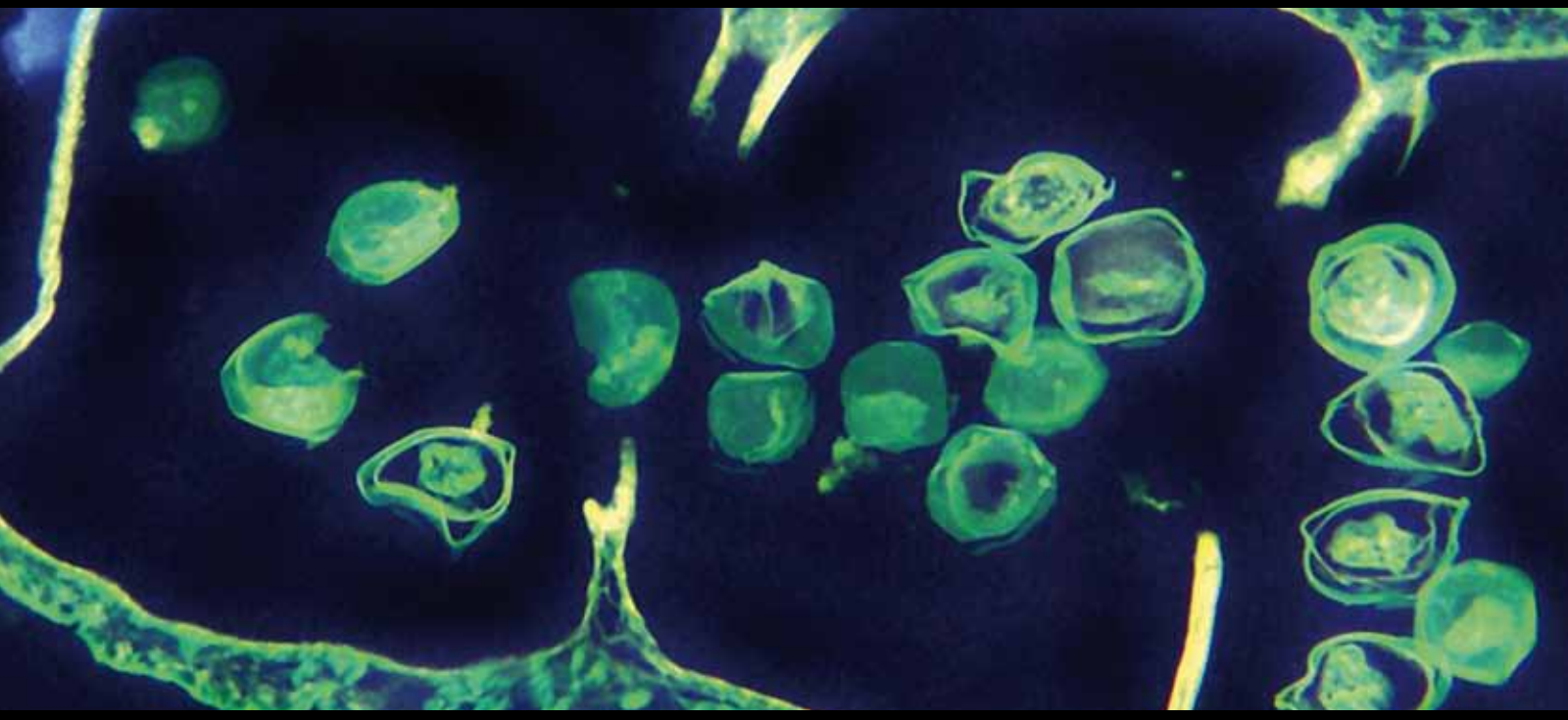
Journal of Parasitology Research

Host Parasite Interaction



Host Parasite Interaction

Host Parasite Interaction

Editorial Board

Contents

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 926706, 10 pages
doi:10.1155/2011/926706

Review Article

A Sequential Model of Host Cell Killing and Phagocytosis by *Entamoeba histolytica*

Adam Sateriale[1,2] and Christopher D. Huston[1,3]

[1] *Department of Medicine, College of Medicine, The University of Vermont, Room 320 Stafford Hall, 95 Carrigan Drive, Burlington, VT 05405, USA*
[2] *Cell and Molecular Biology Graduate Program, College of Medicine, The University of Vermont, Burlington, VT 05405, USA*
[3] *Department of Microbiology and Molecular Genetics, College of Medicine, The University of Vermont, Room 320 Stafford Hall, 95 Carrigan Drive, Burlington, VT 05405, USA*

Correspondence should be addressed to Christopher D. Huston, christopher.huston@uvm.edu

Received 24 November 2010; Accepted 19 December 2010

Academic Editor: D. S. Lindsay

The protozoan parasite *Entamoeba histolytica* is responsible for invasive intestinal and extraintestinal amebiasis. The virulence of *Entamoeba histolytica* is strongly correlated with the parasite's capacity to effectively kill and phagocytose host cells. The process by which host cells are killed and phagocytosed follows a sequential model of adherence, cell killing, initiation of phagocytosis, and engulfment. This paper presents recent advances in the cytolytic and phagocytic processes of *Entamoeba histolytica* in context of the sequential model.

1. Introduction

Entamoeba histolytica is an enteric parasite that colonizes the human intestinal lumen and has the capacity to invade the epithelium. Although 90% of amebic infections are asymptomatic and self-limiting, there are an estimated 50 million cases of invasive infection annually [1, 2]. According to the WHO, *Entamoeba histolytica* is ranked third as a cause of death among parasites with 100,000 estimated deaths annually [1]. The morbidity and mortality of this parasite is primarily seen in developing countries. Ingestion of contaminated food or water containing infectious cysts leads to excystation in the intestine. Each cyst produces eight motile trophozoites, which colonize the host's colon. In those cases where the infection is not self limiting, amebic dysentery and liver abscess formation can occur [2].

The process of invasion and hepatic abscess formation has no apparent advantage for *Entamoeba histolytica* [3]. The logical question would then be why did this organism evolve to be a pathogen and not a commensal like its noninvasive cousin, *Entamoeba dispar*? One theory of *Entamoeba histolytica's* origin of virulence is coincidental evolution. Host cells may have recognition patterns similar to those of enteric bacteria that the parasite has evolved to identify. *Entamoeba histolytica* has been shown to preferentially phagocytose cells coated with collectins, C-type lectins involved in recognition of ligands that are common to both bacteria and apoptotic cells [4]. An effective hijacking of the host's own innate immune system to increase phagocytosis may have led to an invasive phenotype. In further support of this theory, Ghosh and Samuelson [3] have shown that several signaling proteins required for *Entamoeba histolytica's* virulence are also utilized to kill and phagocytose bacteria. Another seemingly plausible explanation is that *Entamoeba histolytica*'s invasive phenotype arose in response to host defense mechanisms [5]. Directed apoptosis and subsequent phagocytosis may serve to limit host inflammatory mechanisms by suppressing necrosis and subsequent Th1-type immunity [6]. Cysteine proteases that are known to degrade host extracellular matrix also protect *Entamoeba histolytica* from complement, secretory IgA, and serum IgG [7–9].

While the evolutionary basis behind virulence is uncertain, the mechanism behind virulence is slowly becoming clearer. Invasion by *Entamoeba histolytica* is strongly

correlated with the parasite's capacity to kill and phagocytose host cells [10–13]. The function of this review is to highlight some of the recent advances in understanding the mechanism of cell killing and phagocytosis, and to place these findings in the context of previous knowledge. For the purpose of this review, cell killing and phagocytosis have been organized in a sequential model involving (i) adherence to the host cell surface, (ii) contact-dependent cell killing, (iii) initiation of phagocytosis, and (iv) engulfment (see Figure 1).

2. Adherence

The D-galactose/N-acetyl-D-galactosamine- (GalNAc-) specific lectin is the major amebic surface adhesin responsible for adherence to intestinal mucus and host cells [14]. The GalNAc lectin is composed of a light subunit (Lgl), heavy subunit (Hgl), and a noncovalently bound intermediate subunit (Igl) [15, 16]. The light and heavy subunits are linked via a disulfide bond and exist predominantly at the parasite cell membrane as a 260 kDa heterodimer [15]. The heavy subunit contains a carbohydrate recognition domain (CRD) that recognizes D-galactose and N-acetyl-D-galactosamine [17]. MUC-2, the predominant mucin in the host intestine, is bound by the GalNAc lectin with high affinity (K_d = 8.2×10^{-11} M), allowing for *Entamoeba histolytica* to colonize mucosal surfaces [18, 19]. The CRD also recognizes host cell surface protein glycoconjugates and inhibition of adherence to host cells has been shown using monoclonal antibodies that bind the CRD specifically [20, 21]. Host cell adherence can also be strongly inhibited using μM concentrations of either galactose or N-acetyl-D-galactosamine [14, 22, 23]. Inhibition of adherence through the GalNAc lectin invariably leads to a subsequent decrease in host cell cytotoxicity [23]. Tetracycline-regulated expression of a truncated intracellular domain of the GalNAc lectin heavy subunit has been shown to significantly decrease adherence to host cells *in vitro* [24]. These data suggest that the lectin participates in outside-to-inside signaling, which is likely through the $\beta 2$ integrin homologous intracellular domain of the GalNAc heavy subunit. These functions in adhesion and signaling place the GalNAc lectin firmly at the nexus of virulence, though there are other *Entamoeba histolytica* proteins that have been implicated in adherence.

The EhCPADH complex is a 124 kDa heterodimer formed by a cysteine protease (EhCP112) and an adhesin (EhADH112). Targeted monoclonal antibodies to the C-terminus adhesion epitope of ADH112 results in greater than 50% reduced adherence to host cells, and ensuing decreases in cytotoxicity and phagocytosis [25]. ADH112 has three putative transmembrane domains, a putative Bro1 domain, and an intracellular domain with potential phosphorylation sites [26]. It will be interesting to see whether targeted mutations to the intracellular region or a truncated version of this protein produce a parasite with diminished adherence. The ADH112 intracellular domain is highly divergent from that of the GalNAc lectin heavy subunit [26]. Adhesion signaling mechanisms of these complexes are, therefore, likely to be distinct.

Many of the proteins recently implicated in adherence have arisen from genomic and transcriptomic analyses of *Entamoeba histolytica* and nonvirulent *Entamoeba*. Sequencing of the *Entamoeba histolytica* genome has led to many new discoveries, truly advancing the field of *Entamoeba* research in a manner not seen since Diamond et al. first axenically cultured the parasite [27–29]. One such discovery is STIRP (serine-threonine-isoleucine rich protein), a protein family exclusively expressed in virulent strains of *Entamoeba, in vitro*. shRNA-mediated silencing of the STIRP family led to a 35% decrease in adhesion to host cells and a subsequent reduction in cytotoxicity [30]. ROM1 is a serine protease functionally related to the rhomboid proteases first identified in *Drosophila melanogaster* [31, 32]. Rhomboid proteases are seven-pass transmembrane proteases with the ability to cleave transmembrane proteins at their transmembrane domain [33]. The ROM1 gene appears to be the only rhomboid protease expressed by both *Entamoeba histolytica* and *Entamoeba dispar*. shRNA-mediated silencing of ROM1 reduced adhesion to healthy Chinese hamster ovary (CHO) cells, but not to apoptotic CHO cells, the mechanism of which is still to be determined. It is hypothesized that the ROM1 protease could be involved in cleavage and activation of amebic transmembrane proteins involved in adherence and phagocytosis. ROM1 silenced ameba were shown to have an ordinary amount of GalNAc lectin at their cell surface, but other amebic adhesins may be modulated by ROM1 [31]. There is experimental evidence of at least one additional *Entamoeba histolytica* surface lectin activity involved in phagocytosis [34].

Another recently described potential adhesin is TMKB1-9, a member of a large family of transmembrane kinases (the relevance of which is more thoroughly discussed later) [35]. The expression of TMKB1-9 was shown, quite conclusively, to correlate with decreased adherence to and destruction of CHO cell monolayers. Intriguingly, the expression of TMKB1-9 also correlated to serum content in the culture medium, suggesting a possible mechanism for sensing environmental conditions [36]. As this exciting new research unfolds, we shall hopefully better understand what serum component(s) is regulating TMKB1-9 expression, and how TMKB1-9 modulates cell adherence.

Trophozoites of *Entamoeba histolytica* express GPI-anchored lipoglycoconjugates on their cell surface, referred to as lipopeptidophosphoglycans or EhLPPG [37, 38]. These molecules have been implicated in host-parasite interactions based on the finding that nonvirulent and virulent strains of *Entamoeba histolytica* express different amounts and structures of EhLPPG [39–42]. Recent research has shown that EhLPPG are the primary NKT cell ligands, helping to explain why CD1d−/− mice show significantly larger liver abscesses [43, 44]. Marinets et al. [45] found that passive immunization with antibody to LPPG conferred protection from invasive amebiasis in the severe combined immunodeficient (SCID) mouse model of hepatic abscess. This effect was also seen using a SCID intestinal xenograph model of invasion [46]. LPPG antibody also caused agglutination of ameba *in vitro*, which may have been a confounding factor in an earlier report showing an LPPG antibody-mediated

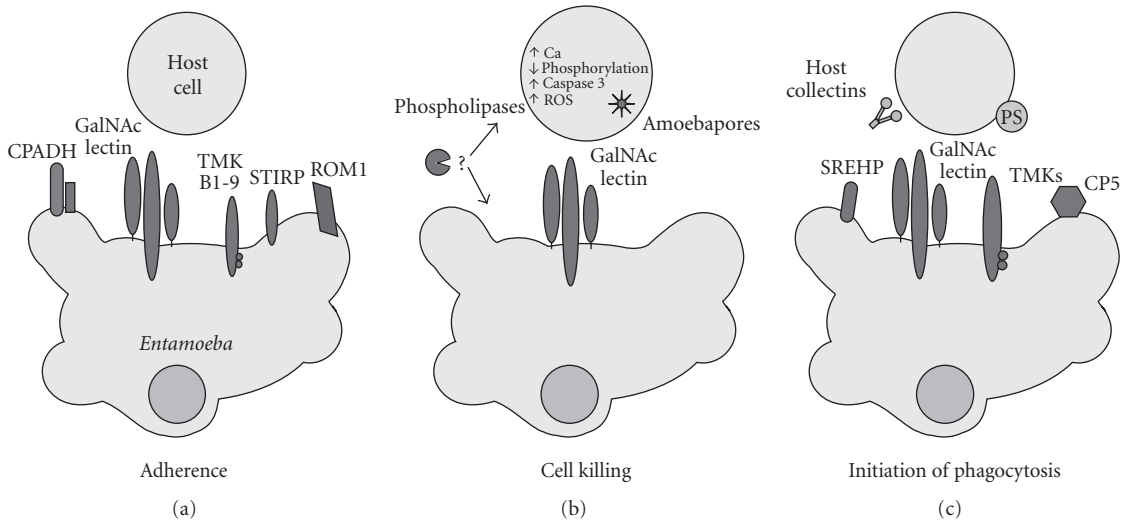


Figure 1: Sequential model of cell killing and phagocytosis by *Entamoeba histolytica*. Adherence, cell killing, and initiation of phagocytosis leading to engulfment of host cells are depicted from left to right. Abbreviations: cysteine protease adhesin (CPADH), transmembrane kinase (TMK), serine-threonine-isoleucine rich protein (STIRP), reactive oxygen species (ROS), serine-rich *Entamoeba histolytica* protein (SREHP), cysteine protease 5 (CP5), and phosphatidylserine (PS).

decrease in adherence [47]. LPPG may be vitally important in immune recognition, but the role it plays in host cell-parasite adherence remains uncertain. Finally, the lysine and glutamic acid-rich protein, KERP1, remains an attractive potential adhesion, as it has been shown to bind epithelial cells and is absent in the *Entamoeba dispar* genome [48]. Its role in adhesion has yet to be formally tested, but KERP1 has recently been evidenced to play a role in liver abscess formation [49].

3. Cell Killing

The GalNAc lectin is a striking example of a crossover function between adherence and cell killing. Antibodies targeting the heavy subunit (Hgl) on a separate domain from the CRD decrease cell killing by approximately 50% [50]. It should be noted that exclusion of any adherence protein from the subsequent processes of cell killing and initiation of phagocytosis does not rule out their involvement, only a lack of evidence to suggest significant involvement in the latter two. It is quite possible that many of the proteins involved in the recognition of healthy host cells are also involved in the cytolysis and/or recognition of apoptotic cells, much like the GalNAc lectin.

The *Entamoeba histolytica* genome encodes three amoebapore proteins that can be secreted upon contact, and the purified proteins cause target host cell membrane permeability at μM concentrations [51, 52]. When inserted into host cell membrane, amoebapore proteins oligomerize through peptide-peptide interactions to produce ion channels [53]. Antisense silencing of amoebapore A expression significantly impairs *Entamoeba histolytica's* ability to kill baby hamster kidney (BHK) cells, assayed by trypan blue exclusion [54]. The G3 strain of *Entamoeba histolytica* has an almost complete transcriptional silencing of the amoebapore A protein [55]. The G3 strain was also shown to be deficient in cell monolayer destruction and incapable of forming liver abscess in the hamster model of hepatic abscess [55]. Conversely, the G3 strain produced abscesses, though of smaller size, in the SCID mouse model [56]. The authors speculate this difference may have been due to the increased susceptibility of the SCID mice, variable timing of liver assessment, or variation in the role that amoebapore plays in different animal models.

While target host cells and bacteria are susceptible to amoebapore, *Entamoeba histolytica* is surprisingly resistant at μM concentrations. Experiments using liposomes with *Entamoeba histolytica* cell membrane composition demonstrated that the phospholipid composition of the parasite plasma membrane, along with its high cholesterol content, prevents binding of fluorescently labeled amoebapore [57]. The plasma membrane of *Entamoeba histolytica* is also resistant to another protein implicated in host cell killing, phospholipase [58]. Pharmacological inhibitors of eukaryotic phospholipase A significantly reduced CHO cell killing, as measured by trypan blue exclusion criteria [58]. The predominant phospholipid found on the *Entamoeba* cell membrane is ceramide aminoethylphosphonate (CAEP), which is a phospholipase resistant species of phospholipid [59, 60]. While phosphonolipids have been found in small amounts in various mammals, such large amounts of CAEP have only been seen in marine bacteria, gastropods, and bivalve mollusks [61]. CAEP was also detected in the plasma membrane of *Entamoeba histolytica*'s reptilian relative, *Entamoeba invadens* [62]. It is possible that CAEP confers resistance to *Entamoeba histolytica*'s resident phospholipases.

Following contact with *Entamoeba histolytica* host cells undergo the morphological and phenotypic changes of apoptosis, including nuclear chromatin condensation, DNA

fragmentation, and membrane blebbing [63]. These cells stain positive by terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick-end labeling (TUNEL) and by annexin V, indicating DNA degradation and phosphatidylserine increases on the outer leaflet of the host cell plasma membrane [64]. Although one study has shown necrotic features of *Entamoeba histolytica*-induced cell death, predominant amount of the literature supports an apoptotic result [65–71]. The mechanism by which this host-cell apoptosis is initiated in a variety of different cell types is still unclear, but there are some common factors. Target cells show a sustained increase in intracellular Ca^{2+} concentration, protein tyrosine dephosphorylation, and caspase 3 activation following contact with *Entamoeba histolytica* [66, 72, 73]. Recent work has shown that pretreatment of Jurkat lymphocytes with the calpain inhibitor calpeptin leads to a decrease in protein tyrosine dephosphorylation. It is hypothesized that the increase in host cell intracellular Ca^{2+} concentration activates calpain, which cleaves and activates host SHP-1 and SHP-2. SHP-1 and SHP-2 then act as protein tyrosine phosphatases. Although calpeptin pretreatment leads to a decrease in protein tyrosine dephosphorylation, it is insufficient to halt ensuing apoptosis [74]. Caspase 8 deficiency and caspase 9 inhibition have likewise been shown to be ineffective in abrogating apoptosis in target Jurkat lymphocytes. Conversely, the caspase 3 inhibitor Ac-DEVD-CHO was found to block Jurkat cell apoptosis, measured by DNA fragmentation and 51Cr release [66]. In a C57BL/6 mouse model of hepatic abscess, *Entamoeba histolytica*-induced apoptosis was also found to be Fas/Fas ligand independent [64]. These findings support a Fas/Fas ligand and caspase 8/9 independent activation of caspase 3.

Recent research using a CBA mouse model of colitis has shown that intraperitoneal injection with the pan-caspase inhibitor ZVAD reduced the mouse parasite burden and, further, that caspase 3 knockout C57BL/6 mice showed an even lower parasite burden [6]. The fact that caspase 3 knockout mice were not fully protected from *Entamoeba* invasion suggests a possible second mechanism of cell death. Sim et al. [70] have shown in neutrophils that intracellular reactive oxygen species (ROS) are induced upon contact from *Entamoeba histolytica*. This induction also coincides with an increasing ERK1/2 activation. Incubation with a MEK1 inhibitor decreased ERK1/2 activation and neutrophil apoptosis. Recent work from this group indicates that apoptosis in neutrophils is also inhibited by host cell preincubation with monoclonal antibodies to CD18 [75]. CD18 is a $\beta 2$ integrin that mediates neutrophil adhesion and is known to promote activation of NADPH oxidase [76]. Treatment with an NADPH oxidase inhibitor also partially decreased neutrophil apoptosis, as measured by annexin-V staining of phosphatidylserine [70]. Previous studies have shown GalNAc lectin deposition on target host cell membranes following parasite contact [77]. It is interesting to speculate that, if integrated into the host cell membrane, the $\beta 2$ integrin domain of the GalNAc lectin heavy subunit may be capable of stimulating NADPH oxidase. Whether the ROS-dependent pathway and the caspase 3-dependent pathway are part of the same mechanism of apoptosis or separate, the end result is membrane blebbing and increased phosphatidylserine exposure on the outer leaflet of the host plasma membrane [13, 67].

4. Initiation of Phagocytosis

Experiments have shown, conclusively, that *Entamoeba histolytica* more readily phagocytoses host cells that have already undergone apoptosis [13, 67]. Apoptotic Jurkat lymphocytes and Ca^{2+} ionophore-treated erythrocytes are both phagocytosed at a higher rate than their viable counterparts. Jurkat lymphocytes made artificially apoptotic by insertion of phosphatidylserine into the outer leaflet are also phagocytosed by *Entamoeba histolytica* at a higher rate [67]. When healthy Jurkat lymphocytes were incubated with *Entamoeba histolytica in vitro*, caspase 3 activity was detected by immunofluorescence using an antiactive caspase 3 antibody in virtually all intact cells ingested [67]. Thus, apoptosis appears to be a requirement for phagocytosis to occur, though it remains possible that viable cells are just engulfed less efficiently.

Galactose inhibition of the GalNAc lectin leads to a 22% reduction in amebic adherence to Ca^{2+} ionophore-treated erythrocytes, in contrast to healthy erythrocytes which show approximately 81% reduction in adherence [13]. Similarly, D-galactose inhibits adherence to apoptotic Jurkat lymphocytes inefficiently [67]. These results clearly implicate other *Entamoeba histolytica* receptors in adhesion to apoptotic host cells and initiation of phagocytosis.

Ideal candidates for apoptotic receptors are members of the *Entamoeba histolytica* transmembrane kinase family of proteins. *Entamoeba histolytica* has over 90 transmembrane kinases (TMKs), categorized into subfamilies (A, B1-3, C, D1-2, E, F) based on signature motifs in their kinase domains [35]. Single-cell microarray analysis of *Entamoeba histolytica* has shown that multiple TMKs are expressed by individual parasites *in vitro* [78]. A small subset of these proteins has been characterized, thus far, with surprising results. Certain members of the TMK family have been implicated in proliferation, possibly due to signaling involving the extracellular milieu [36, 78, 79]. TMKB1-9 levels have been shown to correlate with serum levels in culture media; in fact, many of the TMKs have expression patterns that fluctuate over time [35, 36]. Other TMKs have exhibited a role in the uptake of host cells, specifically in the recognition of apoptotic host cells [78, 80]. Expression of a carboxy-truncated version of TMK39, possessing only extracellular and transmembrane domains, decreased uptake of apoptotic Jurkat lymphocytes by approximately 50% [78]. Similarly, expression of a truncated version of TMKB3-96 (PATMK) decreased uptake of Ca^{2+} ionophore-treated erythrocytes [80]. This decrease was also shown using shRNA-mediated knockdown and using polyclonal antiserum specific for PATMK, which localized to the phagocytic cup during erythrophagocytosis.

Exactly what these TMKs are recognizing on apoptotic cells is unknown. Phosphatidylserine exposure is a hallmark of host cell apoptosis, making it a strong candidate ligand [81, 82]. Annexin V masking of phosphatidylserine on

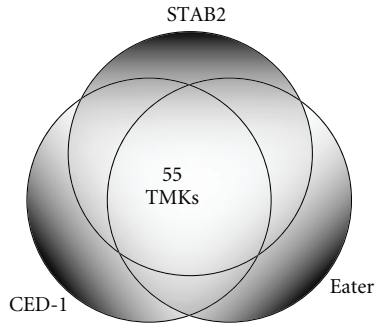


Figure 2: Venn diagram summarizing results of *Entamoeba histolytica* BLAST searches using the extracellular domains of CED-1 (*C. elegans*), eater (*D. melanogaster*), and STAB2 (*H. sapiens*). Fifty-five members of the *E. histolytica* transmembrane kinase gene family share significant homology to these representative scavenger receptors.

Table 1: Members of the *Entamoeba histolytica* transmembrane kinase family found in phagosome preparations at various time points [80, 86].

TMK	Pathema ID
EhTMKA-4	EHI_068720
EhTMKA-85	EHI_128430
EhTMKB1-1	EHI_103240
EhTMKB1-5	EHI_062090
EhTMKB2-14	EHI_068160
EhTMKB2-31	EHI_180320
EhTMKB2-36	EHI_074740
EhTMKB2-41	EHI_064490
EhTMKB2-75	EHI_092260
EhTMKB3-29	EHI_050820
EhTMKB3-96	EHI_167650
EhTMKC-13	EHI_025280
EhTMKC-71	EHI_030420
EhTMKD1-3	EHI_201270
EhTMKD1-40	EHI_064500
EhTMKD1-70	EHI_189290
EhTMKD1-79	EHI_180150
EhTMKD2-19	EHI_081790
EhTMKD2-44	EHI_127000
EhTMKD2-64	EHI_086050
EhTMKE-22	EHI_186990
EhTMKE-54	EHI_188110

apoptotic erythrocytes leads to a decrease in phagocytosis [13]. Annexin V treatment along with galactose inhibition of the GalNAc lectin also leads to an astonishing >95% reduction in erythrophagocytosis. If phosphatidylserine were the only driving force behind apoptotic cell recognition, then annexin V treatment of other apoptotic cell types should also decrease phagocytosis. Interestingly, this effect is not seen. Annexin V treatment of Jurkat lymphocytes does not affect the rate of phagocytosis *in vitro* (C. Huston, unpublished data). These findings lead us to believe that, while phosphatidylserine may be a strong signal for initiation of phagocytosis, other ligands present on nucleated apoptotic host cells must be also capable of stimulating *Entamoeba histolytica* phagocytosis.

Research on macrophage uptake of apoptotic cells has shown that recognition of phosphatidylserine alone involves multiple receptors [83, 84]. As previous studies have noted, the extracellular domain of TMKs contain many epidermal growth factor- (EGF-) like repeats characteristic of scavenger receptors conserved in eukaryotes [78, 85]. A Boolean exploration of BLAST searches involving the extracellular domains of the representative scavenger receptors CED-1 (*C. elegans*), eater (*D. melanogaster*), and STAB2 (*H. sapiens*) returns 55 members of the *Entamoeba histolytica* TMK family (Figure 2). This number is remarkable considering that many of the transmembrane kinase genes encode truncated forms, lacking substantial extracellular domains [35, 79]. Proteomic analysis of the *Entamoeba histolytica* phagosome using carboxylated paramagnetic beads as bait identified 22 TMKs over various time points (Table 1) [80]. It is an attractive hypothesis that TMKs are acting as scavenger receptors, yet more research is needed to characterize TMK ligands and the downstream signaling induced. Buss et al. [78] observed heterodimerization of wild type and truncated TMKs in transfected parasites. It will be interesting to see whether TMK homodimerization alone is sufficient to initiate phagocytosis, and whether TMKs are able to dimerize with other family members.

Another large family of genes in *Entamoeba histolytica* is the cysteine proteases, of which there are 50 known members [87]. EhCP1, EhCP2, and EhCP5 appear to make up nearly 90% of the cysteine protease transcripts in cultured parasites [88, 89]. At different time points of infection, the expression of cysteine proteases can shift greatly, leading to the increase of EhCP4 and others [90]. In cultured parasites, antisense knockdown of EhCP5 resulted in a 90% decrease in cysteine protease activity compared to wild type [91]. Strangely, this strain of *Entamoeba histolytica* had a decrease in phagocytosis, while having no apparent defect in hemolytic activity or monolayer destruction. This is in stark contrast to the known roles of cysteine proteases that include degradation of extracellular matrix, mucin, complement proteins, immunoglobulins, and cytokines [7–9, 92]. EhCP5-attenuated parasites were also unable to penetrate the colonic lamina propria in an *ex vivo* human colonic model of invasion [93]. Targeted inhibitors to EhCP1 and EhCP4 have also been shown to be protective in the SCID mouse-human intestinal xenograph model and in the SCID mouse hepatic abscess model, respectively [94, 95]. The connection between cysteine proteases and phagocytosis has not been determined, but their importance for host invasion has been proven *ex vivo* and *in vivo*. The availability of pharmacologic inhibitors for cysteine proteases makes them attractive targets for drug design, and the inhibitors are potential tools to dissect the roles of individual cysteine proteases in phagocytosis.

The serine rich *Entamoeba histolytica* protein (SREHP) was first identified based on its strong immunogenic properties, and characterized as a potential parasite chemoattractant [96]. These results are perplexing considering that the SREHP does not appear to be secreted, but does show localization to the plasma membrane of *Entamoeba histolytica*. An *in vitro* screen of 43 monoclonal antibodies raised against *Entamoeba histolytica* membrane preparations identified a single antibody that inhibited phagocytosis, which was found to be specific for SREHP [97]. This antibody blocked uptake of apoptotic Jurkat lymphocytes by over 90%, and the reduction was shown to be GalNAc lectin-independent via saturating amounts of galactose. Adherence and induction of apoptosis were also reduced to a much lesser degree. The SREHP has a putative transmembrane domain but no appreciable cytoplasmic domain, implicating a possible coreceptor that is still to be identified.

The host collectins C1q, SP-A, and MBL have all been shown to be ligands that stimulate *Entamoeba histolytica* phagocytosis [4]. Structurally, the collectin family all have a collagenous N-terminal tail and a globular C-terminal head generally involved in opsonization [98]. Collectins are found throughout the host mucosal lining, including those of the intestine [99–101]. Collectin-mediated opsonization of bacteria and apoptotic host cells is stimulatory for *Entamoeba histolytica* as well as macrophages [4, 102] (A. Sateriale, unpublished data). Pretreatment with C1q increased amebic uptake of apoptotic Jurkat lymphocytes *in vitro*, but not of viable Jurkats, even though C1q was detectable on the surface of both. The localization of C1q to apoptotic Jurkat membrane blebs in these experiments indicates possible concentration dependence. C1q and MBL were also found to be chemoattractants for *Entamoeba histolytica,* via a transwell migration assay [4]. As the host collectins have been shown to be structurally similar, a single receptor may show cross-reactivity. However, a putative *Entamoeba histolytica* collectin receptor has yet to be identified.

5. Engulfment

The process of host-cell engulfment following initiation of phagocytosis has been shown to be actin and myosin dependent [103]. Rhodamine-labeled phalloidin localizes to the phagocytic cup during target cell ingestion, and cytochalasin D blocking of actin polymerization has been shown to inhibit phagocytosis [104–106]. An *Entamoeba histolytica* strain with a threefold overexpression of myosin 1B exhibited marked deficiency in erythrophagocytosis [107]. Recent research has also posited that *Entamoeba* lipid rafts are involved in the organization of host-cell adhesion and endocytosis [108]. In a cholesterol-rich organism such as *Entamoeba histolytica,* it is not difficult to imagine the large role lipid rafts could play in organizing pathogenic events [59]. *Entamoeba histolytica* signaling proteins that have been shown to regulate host-cell engulfment include p21 activated kinase (PAK), protein kinase C (PKC), RacA, and phosphatidylinositol 3-kinase (PI3 kinase) [3, 109, 110]. Recent proteomic research involving purified phagosomes has given supporting evidence to these observations and offers a more complete picture of the various proteins involved in amebic endocytosis [80, 111–113]. Okada and Nozaki [114] and Marion and Guillén [85] offer very concise and comprehensive reviews of the endocytosis mechanism.

6. Future Directions

Some of the original mysteries surrounding *Entamoeba histolytica* pathogenicity still plague researchers today. The Zulu word for *Entamoeba histolytica*-derived liver abscess is *isigwebedhla*, which translates to disease of the strong young men [115]. The cause behind the gender bias still remains unknown. This is not particularly surprising, considering that the mechanism by which *Entamoeba histolytica* causes host cell apoptosis is largely uncertain. Models for assaying parasite invasion such as the SCID mouse-human xenograph model and the recent *ex vivo* human intestinal model may allow for a better understanding of host-parasite interactions [93, 116]. While animal models are invaluable, discrepancies between species and even between strains highlight the variability of the host-parasite interface. Models better representing the parasite's natural human host may allow for a better understanding of the invasive phenotype. Many of the proteins described in this sequential model of invasion also happen to be the most immunogenic [117]. The characterization of novel proteins involved in adherence, cell killing, and phagocytosis still holds the promise of identifying future vaccine candidates.

Acknowledgments

The authors thank their fellow laboratory members for their thoughtful comments on this paper. C. D. Huston is supported by NIAID R01 AI072021-03.

References

[1] "WHO/PAHO/UNESCO report. A consultation with experts on amoebiasis. Mexico City, Mexico 28-29 January, 1997," *Epidemiological Bulletin*, vol. 18, no. 1, pp. 13–14, 1997.

[2] R. Haque, C. D. Huston, M. Hughes, E. Houpt, and W. A. Petri Jr., "Amebiasis," *New England Journal of Medicine*, vol. 348, no. 16, pp. 1565–1573, 2003.

[3] S. K. Ghosh and J. Samuelson, "Involvement of p21(racA), phosphoinositide 3-kinase, and vacuolar ATPase in phagocytosis of bacteria and erythrocytes by *Entamoeba histolytica*: suggestive evidence for coincidental evolution of amebic invasiveness," *Infection and Immunity*, vol. 65, no. 10, pp. 4243–4249, 1997.

[4] J. E. Teixeira, B. T. Heron, and C. D. Huston, "C1q- and collectin-dependent phagocytosis of apoptotic host cells by the intestinal protozoan *Entamoeba histolytica*," *Journal of Infectious Diseases*, vol. 198, no. 7, pp. 1062–1070, 2008.

[5] R. Campos-Rodríguezp and A. Jarillo-Luna, "The pathogenicity of *Entamoeba histolytica* is related to the capacity of evading innate immunity," *Parasite Immunology*, vol. 27, no. 1-2, pp. 1–8, 2005.

[6] S. M. Becker, K. N. Cho, X. Guo et al., "Epithelial cell apoptosis facilitates *Entamoeba histolytica* infection in the gut,"

American Journal of Pathology, vol. 176, no. 3, pp. 1316–1322, 2010.

[7] S. L. Reed, J. A. Ember, D. S. Herdman, R. G. DiScipio, T. E. Hugli, and I. Gigli, "The extracellular neutral cysteine proteinase of *Entamoeba histolytica* degrades anaphylatoxins C3a and C5a," *Journal of Immunology*, vol. 155, no. 1, pp. 266–274, 1995.

[8] B. L. Kelsall and J. I. Ravdin, "Degradation of human IgA by *Entamoeba histolytica*," *Journal of Infectious Diseases*, vol. 168, no. 5, pp. 1319–1322, 1993.

[9] V. Q. Tran, D. S. Herdman, B. E. Torian, and S. L. Reed, "The neutral cysteine proteinase of *Entamoeba histolytica* degrades IgG and prevents its binding," *Journal of Infectious Diseases*, vol. 177, no. 2, pp. 508–511, 1998.

[10] A. Martinez-Palomo, A. Gonzalez-Robles, and B. Chavez, "Structural bases of the cytolytic mechanisms of *Entamoeba histolytica*," *Journal of Protozoology*, vol. 32, no. 1, pp. 166–175, 1985.

[11] E. Orozco, G. Guarneros, A. martinez Palomo, and T. Sanchez, "*Entamoeba histolytica*. Phagocytosis as a virulence factor," *Journal of Experimental Medicine*, vol. 158, no. 5, pp. 1511–1521, 1983.

[12] M. A. Rodriguez and E. Orozco, "Isolation and characterization of phagocytosis- and virulence-deficient mutants of *Entamoeba histolytica*," *Journal of Infectious Diseases*, vol. 154, no. 1, pp. 27–32, 1986.

[13] D. R. Boettner, C. D. Huston, J. A. Sullivan, and W. A. Petri Jr., "*Entamoeba histolytica* and Entamoeba dispar utilize externalized phosphatidylserine for recognition and phagocytosis of erythrocytes," *Infection and Immunity*, vol. 73, no. 6, pp. 3422–3430, 2005.

[14] J. I. Ravdin and R. L. Guerrant, "Role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*. Study with mammalian tissue culture cells and human erythrocytes," *Journal of Clinical Investigation*, vol. 68, no. 5, pp. 1305–1313, 1981.

[15] W. A. Petri Jr., M. D. Chapman, T. Snodgrass, B. J. Mann, J. Broman, and J. I. Ravdin, "Subunit structure of the galactose and N-acetyl-D-galactosamine-inhibitable adherence lectin in *Entamoeba histolytica*," *Journal of Biological Chemistry*, vol. 264, no. 5, pp. 3007–3012, 1989.

[16] X. J. Cheng, M. A. Hughes, C. D. Huston et al., "Intermediate subunit of the Gal/GalNAc lectin of *Entamoeba histolytica* is a member of a gene family containing multiple CXXC sequence motifs," *Infection and Immunity*, vol. 69, no. 9, pp. 5892–5898, 2001.

[17] J. M. Dodson, P. W. Lenkowski, A. C. Eubanks et al., "Infection and immunity mediated by the carbohydrate recognition domain of the *Entamoeba histolytica* Gal/GalNAc lectin," *Journal of Infectious Diseases*, vol. 179, no. 2, pp. 460–466, 1999.

[18] K. Chadee, M. L. Johnson, E. Orozco, W. A. Petri, and J. I. Ravdin, "Binding and internalization of rat colonic mucins by the galactose/N-acetyl-D-galactosamine adherence lectin of *Entamoeba histolytica*," *Journal of Infectious Diseases*, vol. 158, no. 2, pp. 398–406, 1988.

[19] K. Chadee, W. A. Petri, D. J. Innes, and J. I. Ravdin, "Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*," *Journal of Clinical Investigation*, vol. 80, no. 5, pp. 1245–1254, 1987.

[20] B. J. Mann, C. Y. Chung, J. M. Dodson, L. S. Ashley, L. L. Braga, and T. L. Snodgrass, "Neutralizing monoclonal antibody epitopes of the *Entamoeba histolytica* galactose adhesin map to the cysteine-rich extracellular domain of the 170- kilodalton subunit," *Infection and Immunity*, vol. 61, no. 5, pp. 1772–1778, 1993.

[21] W. A. Petri Jr., R. Haque, and B. J. Mann, "The bittersweet interface of parasite and host: lectin-carbohydrate interactions during human invasion by the parasite *Entamoeba histolytica*," *Annual Review of Microbiology*, vol. 56, pp. 39–64, 2002.

[22] D. Kobiler and D. Mirelman, "Adhesion of *Entamoeba histolytica* trophozoites to monolayers of human cells," *Journal of Infectious Diseases*, vol. 144, no. 6, pp. 539–546, 1981.

[23] J. I. Ravdin, C. F. Murphy, R. A. Salata, R. L. Guerrant, and E. L. Hewlett, "N-acetyl-d-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica*. I. Partial purification and relation to amoebic virulence in vitro," *Journal of Infectious Diseases*, vol. 151, no. 5, pp. 804–815, 1985.

[24] R. R. Vines, G. Ramakrishnan, J. B. Rogers, L. A. Lockhart, B. J. Mann, and W. A. Petri Jr., "Regulation of adherence and virulence by the *Entamoeba histolytica* lectin cytoplasmic domain, which contains a $\beta 2$ integrin motif," *Molecular Biology of the Cell*, vol. 9, no. 8, pp. 2069–2079, 1998.

[25] G. García-Rivera, M. A. Rodríguez, R. Ocádiz et al., "*Entamoeba histolytica*: a novel cysteine protease and an adhesin form the 112 kDa surface protein," *Molecular Microbiology*, vol. 33, no. 3, pp. 556–568, 1999.

[26] C. Bañuelos, G. García-Rivera, I. López-Reyes, and E. Orozco, "Functional characterization of EhADH112: an *Entamoeba histolytica* Bro1 domain-containing protein," *Experimental Parasitology*, vol. 110, no. 3, pp. 292–297, 2005.

[27] L. S. Diamond, "Axenic cultivation of *Entamoeba histolytica*," *Science*, vol. 134, no. 3475, pp. 336–337, 1961.

[28] B. Loftus, I. Anderson, R. Davies et al., "The genome of the protist parasite *Entamoeba histolytica*," *Nature*, vol. 433, no. 7028, pp. 865–868, 2005.

[29] L. A. Baxt and U. Singh, "New insights into *Entamoeba histolytica* pathogenesis," *Current Opinion in Infectious Diseases*, vol. 21, no. 5, pp. 489–494, 2008.

[30] R. C. MacFarlane and U. Singh, "Identification of an *Entamoeba histolytica* serine-, threonine-, and isoleucine-rich protein with roles in adhesion and cytotoxicity," *Eukaryotic Cell*, vol. 6, no. 11, pp. 2139–2146, 2007.

[31] L. A. Baxt, E. Rastew, R. Bracha, D. Mirelman, and U. Singh, "Downregulation of an *Entamoeba histolytica* rhomboid protease reveals roles in regulating parasite adhesion and phagocytosis," *Eukaryotic Cell*, vol. 9, no. 8, pp. 1283–1293, 2010.

[32] E. Bier, L. Y. Jan, and Y. N. Jan, "rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in Drosophila melanogaster," *Genes and Development*, vol. 4, no. 2, pp. 190–203, 1990.

[33] S. Urban and M. Freeman, "Substrate specificity of rhomboid intramembrane proteases is governed by helix-breaking residues in the substrate transmembrane domain," *Molecular Cell*, vol. 11, no. 6, pp. 1425–1434, 2003.

[34] B. T. Heron, A. Sateriale, J. E. Teixeira, and C. D. Huston, "Evidence for a novel Entamoeba histolytica lectin activity that recognises carbohydrates present on ovalbumin," *International Journal for Parasitology*. In press.

[35] D. L. Beck, D. R. Boettner, B. Dragulev, K. Ready, T. Nozaki, and W. A. Petri Jr., "Identification and gene expression analysis of a large family of transmembrane kinases related to the Gal/GalNAc lectin in *Entamoeba histolytica*," *Eukaryotic Cell*, vol. 4, no. 4, pp. 722–732, 2005.

[36] S. Shrimal, S. Bhattacharya, and A. Bhattacharya, "Serum-dependent selective expression of EhTMKB1-9, a member of

Entamoeba histolytica B1 family of transmembrane kinases," *PLoS Pathogens*, vol. 6, no. 6, Article ID e1000929, 2010.

[37] S. Moody, S. Becker, Y. Nuchamowitz, M. J. McConville, and D. Mirelman, "The lipophosphoglycan-like molecules of virulent and avirulent *E. histolytica* as well as of *E. dispar* differ in both composition and abundance," *Archives of Medical Research*, vol. 28, pp. 98–102, 1997.

[38] S. Moody-Haupt, J. H. Patterson, D. Mirelman, and M. J. McConville, "The major surface antigens of *Entamoeba histolytica* trophozoites are GPI-anchored proteophosphoglycans," *Journal of Molecular Biology*, vol. 297, no. 2, pp. 409–420, 2000.

[39] A. Bhattacharya, R. Prasad, and D. L. Sacks, "Identification and partial characterization of a lipophosphoglycan from a pathogenic strain of *Entamoeba histolytica*," *Molecular and Biochemical Parasitology*, vol. 56, no. 1, pp. 161–168, 1992.

[40] G. Srivastava, M. T. Anand, S. Bhattacharya, and A. Bhattacharya, "Lipophosphoglycan is present in distinctly different form in different *Entamoeba histolytica* strains and absent in Entamoeba moshkovskii and Entamoeba invadens," *Journal of eukaryotic microbiology*, vol. 42, no. 5, pp. 617–622, 1995.

[41] S. Moody, S. Becker, Y. Nuchamowitz, and D. Mirelman, "Virulent and avirulent *Entamoeba histolytica* and *E. dispar* differ in their cell surface phosphorylated glycolipids," *Parasitology*, vol. 114, no. 2, pp. 95–104, 1997.

[42] S. Moody, S. Becker, Y. Nuchamowitz, and D. Mirelman, "Identification of significant variation in the composition of lipophosphoglycan-like molecules of *E. histolytica* and *E. dispar*," *Journal of Eukaryotic Microbiology*, vol. 45, no. 2, pp. 9S–12S, 1998.

[43] H. Lotter, T. Jacobs, I. Gaworski, and E. Tannich, "Sexual dimorphism in the control of amebic liver abscess in a mouse model of disease," *Infection and Immunity*, vol. 74, no. 1, pp. 118–124, 2006.

[44] H. Lotter, N. González-Roldán, B. Lindner et al., "Natural killer T cells activated by a lipopeptidophosphoglycan from *Entamoeba histolytica* are critically important to control amebic liver abscess," *PLoS Pathogens*, vol. 5, no. 5, Article ID e1000434, 2009.

[45] A. Marinets, T. Zhang, N. Guillén et al., "Protection against invasive amebiasis by a single monoclonal antibody directed against a lipophosphoglycan antigen localized on the surface of *Entamoeba histolytica*," *Journal of Experimental Medicine*, vol. 186, no. 9, pp. 1557–1565, 1997.

[46] Z. Zhang, M. Duchêne, and S. L. Stanley Jr., "A monoclonal antibody to the amebic lipophosphoglycan-proteophosphoglycan antigens can prevent disease in human intestinal xenografts infected with *Entamoeba histolytica*," *Infection and Immunity*, vol. 70, no. 10, pp. 5873–5876, 2002.

[47] S. L. Stanley Jr., H. Huizenga, and E. Li, "Isolation and partial characterization of a surface glycoconjugate of *Entamoeba histolytica*," *Molecular and Biochemical Parasitology*, vol. 50, no. 1, pp. 127–138, 1992.

[48] M. Seigneur, J. Mounier, M. C. Prevost, and N. Guillén, "A lysine- and glutamic acid-rich protein, KERP1, from *Entamoeba histolytica* binds to human enterocytes," *Cellular Microbiology*, vol. 7, no. 4, pp. 569–579, 2005.

[49] J. Santi-Rocca, C. Weber, G. Guigon, O. Sismeiro, J. Y. Coppée, and N. Guillén, "The lysine- and glutamic acid-rich protein KERP1 plays a role in *Entamoeba histolytica* liver abscess pathogenesis," *Cellular Microbiology*, vol. 10, no. 1, pp. 202–217, 2008.

[50] L. D. Saffer and W. A. Petri Jr., "Role of the galactose lectin of *Entamoeba histolytica* in adherence-dependent killing of mammalian cells," *Infection and Immunity*, vol. 59, no. 12, pp. 4681–4683, 1991.

[51] M. Leippe, S. Ebel, O. L. Schoenberger, R. D. Horstmann, and H. J. Muller-Eberhard, "Pore-forming peptide of pathogenic *Entamoeba histolytica*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 17, pp. 7659–7663, 1991.

[52] M. Leippe, J. Andrä, R. Nickel, E. Tannich, and H. J. Müller-Eberhard, "Amoebapores, a family of membranolytic peptides from cytoplasmic granules of *Entamoeba histolytica*: isolation, primary structure, and pore formation in bacterial cytoplasmic membranes," *Molecular Microbiology*, vol. 14, no. 5, pp. 895–904, 1994.

[53] M. Leippe, "Ancient weapons: NK-lysin, is a mammalian homolog to pore-forming peptides of a protozoan parasite," *Cell*, vol. 83, no. 1, pp. 17–18, 1995.

[54] R. Bracha, Y. Nuchamowitz, M. Leippe, and D. Mirelman, "Antisense inhibition of amoebapore expression in *Entamoeba histolytica* causes a decrease in amoebic virulence," *Molecular Microbiology*, vol. 34, no. 3, pp. 463–472, 1999.

[55] R. Bracha, Y. Nuchamowitz, and D. Mirelman, "Transcriptional silencing of an amoebapore gene in *Entamoeba histolytica*: molecular analysis and effect on pathogenicity," *Eukaryotic Cell*, vol. 2, no. 2, pp. 295–305, 2003.

[56] X. Zhang, Z. Zhang, D. Alexander, R. Bracha, D. Mirelman, and S. L. Stanley Jr., "Expression of amoebapores is required for full expression of *Entamoeba histolytica* virulence in amebic liver abscess but is not necessary for the induction of inflammation or tissue damage in amebic colitis," *Infection and Immunity*, vol. 72, no. 2, pp. 678–683, 2004.

[57] J. Andrä, O. Berninghausen, and M. Leippe, "Membrane lipid composition protects *Entamoeba histolytica* from self-destruction by its pore-forming toxins," *FEBS Letters*, vol. 564, no. 1-2, pp. 109–115, 2004.

[58] J. I. Ravdin, C. F. Murphy, R. L. Guerrant, and S. A. Long-Krug, "Effect of antagonists of calcium and phospholipase A on the cytopathogenicity of *Entamoeba histolytica*," *Journal of Infectious Diseases*, vol. 152, no. 3, pp. 542–549, 1985.

[59] S. B. Aley, W. A. Scott, and Z. A. Cohn, "Plasma membrane of *Entamoeba histolytica*," *Journal of Experimental Medicine*, vol. 152, no. 2, pp. 391–404, 1980.

[60] G. Simon and G. Rouser, "Species variations in phospholipid class distribution of organs: II. Heart and skeletal muscle," *Lipids*, vol. 4, no. 6, pp. 607–614, 1969.

[61] K. S. Mukhamedova and A. I. Glushenkova, "Natural phosphonolipids," *Chemistry of Natural Compounds*, vol. 36, no. 4, pp. 329–341, 2000.

[62] J. Cerbón and J. Flores, "Phospholipid composition and turnover of pathogenic amebas," *Comparative Biochemistry and Physiology B*, vol. 69, no. 3, pp. 487–492, 1981.

[63] B. D. Ragland, L. S. Ashley, D. L. Vaux, and W. A. Petri Jr., "*Entamoeba histolytica*: target cells killed by trophozoites undergo DNA fragmentation which is not blocked by Bcl-2," *Experimental Parasitology*, vol. 79, no. 3, pp. 460–467, 1994.

[64] K. B. Seydel and S. L. Stanley Jr., "*Entamoeba histolytica* induces host cell death in amebic liver abscess by a non-fas-dependent, non-tumor necrosis factor alpha-dependent pathway of apoptosis," *Infection and Immunity*, vol. 66, no. 6, pp. 2980–2983, 1998.

[65] O. Berninghausen and M. Leippe, "Necrosis versus apoptosis as the mechanism of target cell death induced by *Entamoeba*

histolytica," *Infection and Immunity*, vol. 65, no. 9, pp. 3615–3621, 1997.

[66] C. D. Huston, E. R. Houpt, B. J. Mann, C. S. Hahn, and W. A. Petri Jr., "Caspase 3-dependent killing of host cells by the parasite *Entamoeba histolytica*," *Cellular Microbiology*, vol. 2, no. 6, pp. 617–625, 2000.

[67] C. D. Huston, D. R. Boettner, V. Miller-Sims, and W. A. Petri Jr., "Apoptotic killing and phagocytosis of host cells by the parasite *Entamoeba histolytica*," *Infection and Immunity*, vol. 71, no. 2, pp. 964–972, 2003.

[68] L. Yan and S. L. Stanley Jr., "Blockade of caspases inhibits amebic liver abscess formation in a mouse model of disease," *Infection and Immunity*, vol. 69, no. 12, pp. 7911–7914, 2001.

[69] K. B. Seydel and S. L. Stanley Jr., "*Entamoeba histolytica* induces host cell death in amebic liver abscess by a non-fas-dependent, non-tumor necrosis factor alpha-dependent pathway of apoptosis," *Infection and Immunity*, vol. 66, no. 6, pp. 2980–2983, 1998.

[70] S. Sim, T. S. Yong, S. J. Park et al., "NADPH oxidase-derived reactive oxygen species-mediated activation of ERK1/2 is required for apoptosis of human neutrophils induced by *Entamoeba histolytica*," *Journal of Immunology*, vol. 174, no. 7, pp. 4279–4288, 2005.

[71] K. A. Kim, Y. A. Lee, and M. H. Shin, "Calpain-dependent calpastatin cleavage regulates caspase-3 activation during apoptosis of Jurkat T cells induced by *Entamoeba histolytica*," *International Journal for Parasitology*, vol. 37, no. 11, pp. 1209–1219, 2007.

[72] J. I. Ravdin, N. Sperelakis, and R. L. Guerrant, "Effect of ion channel inhibitors on the cytopathogenicity of *Entamoeba histolytica*," *Journal of Infectious Diseases*, vol. 146, no. 3, pp. 335–340, 1982.

[73] J. E. Teixeira and B. J. Mann, "*Entamoeba histolytica*-induced dephosphorylation in host cells," *Infection and Immunity*, vol. 70, no. 4, pp. 1816–1823, 2002.

[74] K. A. Kim, Y. A. Lee, and M. H. Shin, "Calpain-dependent cleavage of SHP-1 and SHP-2 is involved in the dephosphorylation of Jurkat T cells induced by *Entamoeba histolytica*," *Parasite Immunology*, vol. 32, no. 3, pp. 176–183, 2010.

[75] S. Sim, S. J. Park, T. S. Yong, K. I. Im, and M. H. Shin, "Involvement of β-integrin in ROS-mediated neutrophil apoptosis induced by *Entamoeba histolytica*," *Microbes and Infection*, vol. 9, no. 11, pp. 1368–1375, 2007.

[76] T. N. Mayadas and X. Cullere, "Neutrophil β integrins: moderators of life or death decisions," *Trends in Immunology*, vol. 26, no. 7, pp. 388–395, 2005.

[77] J. Pacheco, M. Shibayama, R. Campos et al., "*In vitro* and *in vivo* interaction of *Entamoeba histolytica* Gal/GalNAc lectin with various target cells: an immunocytochemical analysis," *Parasitology International*, vol. 53, no. 1, pp. 35–47, 2004.

[78] S. N. Buss, S. Hamano, A. Vidrich et al., "Members of the *Entamoeba histolytica* transmembrane kinase family play non-redundant roles in growth and phagocytosis," *International Journal for Parasitology*, vol. 40, no. 7, pp. 833–843, 2010.

[79] A. Mehra, J. Fredrick, W. A. Petri Jr., S. Bhattacharya, and A. Bhattacharya, "Expression and function of a family of transmembrane kinases from the protozoan parasite *Entamoeba histolytica*," *Infection and Immunity*, vol. 74, no. 9, pp. 5341–5351, 2006.

[80] D. R. Boettner, C. D. Huston, A. S. Linford et al., "*Entamoeba histolytica* phagocytosis of human erythrocytes involves PATMK, a member of the transmembrane kinase family," *PLoS Pathogens*, vol. 4, no. 1, pp. 122–133, 2008.

[81] S. J. Martin, C. P. M. Reutelingsperger, A. J. McGahon et al., "Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl," *Journal of Experimental Medicine*, vol. 182, no. 5, pp. 1545–1556, 1995.

[82] V. A. Fadok, D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson, "Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages," *Journal of Immunology*, vol. 148, no. 7, pp. 2207–2216, 1992.

[83] D. L. Bratton and P. Henson, "Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up?" *Current Biology*, vol. 18, no. 2, pp. R76–R79, 2008.

[84] K. S. Ravichandran, "Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums," *Journal of Experimental Medicine*, vol. 207, no. 9, pp. 1807–1817, 2010.

[85] S. Marion and N. Guillén, "Genomic and proteomic approaches highlight phagocytosis of living and apoptotic human cells by the parasite *Entamoeba histolytica*," *International Journal for Parasitology*, vol. 36, no. 2, pp. 131–139, 2006.

[86] C. Aurrecoechea, J. Brestelli, B. P. Brunk et al., "EuPathDB: a portal to eukaryotic pathogen databases," *Nucleic Acids Research*, vol. 38, database issue, pp. D415–D419, 2010.

[87] C. G. Clark, U. C. M. Alsmark, M. Tazreiter et al., "Structure and content of the *Entamoeba histolytica* genome," *Advances in Parasitology*, vol. 65, pp. 51–190, 2007.

[88] I. Bruchhaus, T. Jacobs, M. Leippe, and E. Tannich, "*Entamoeba histolytica* and Entamoeba dispar: differences in numbers and expression of cysteine proteinase genes," *Molecular Microbiology*, vol. 22, no. 2, pp. 255–263, 1996.

[89] I. Bruchhaus, B. J. Loftus, N. Hall, and E. Tannich, "The intestinal protozoan parasite *Entamoeba histolytica* contains 20 cysteine protease genes, of which only a small subset is expressed during in vitro cultivation," *Eukaryotic Cell*, vol. 2, no. 3, pp. 501–509, 2003.

[90] C. A. Gilchrist, E. Houpt, N. Trapaidze et al., "Impact of intestinal colonization and invasion on the *Entamoeba histolytica* transcriptome," *Molecular and Biochemical Parasitology*, vol. 147, no. 2, pp. 163–176, 2006.

[91] S. Ankri, T. Stolarsky, and D. Mirelman, "Antisense inhibition of expression of cysteine proteinases does not affect *Entamoeba histolytica* cytopathic or haemolytic activity but inhibits phagocytosis," *Molecular Microbiology*, vol. 28, no. 4, pp. 777–785, 1998.

[92] X. Que, S. H. Kim, M. Sajid et al., "A surface amebic cysteine proteinase inactivates interleukin-18," *Infection and Immunity*, vol. 71, no. 3, pp. 1274–1280, 2003.

[93] D. Bansal, P. Ave, S. Kerneis et al., "An ex-vivo human intestinal model to study *Entamoeba histolytica* pathogenesis," *PLoS Neglected Tropical Diseases*, vol. 3, no. 11, article e551, 2009.

[94] S. G. Meléndez-López, S. Herdman, K. Hirata et al., "Use of recombinant *Entamoeba histolytica* cysteine proteinase 1 to identify a potent inhibitor of amebic invasion in a human colonic model," *Eukaryotic Cell*, vol. 6, no. 7, pp. 1130–1136, 2007.

[95] C. He, G. P. Nora, E. L. Schneider et al., "A novel *Entamoeba histolytica* cysteine proteinase, EhCP4, is key for invasive amebiasis and a therapeutic target," *Journal of Biological Chemistry*, vol. 285, no. 24, pp. 18516–18527, 2010.

[96] S. L. Stanley Jr., A. Becker, C. Kunz-Jenkins, L. Foster, and E. Li, "Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats,"

Proceedings of the National Academy of Sciences of the United States of America, vol. 87, no. 13, pp. 4976–4980, 1990.

[97] J. E. Teixeira and C. D. Huston, "Participation of the serine-rich *Entamoeba histolytica* protein in amebic phagocytosis of apoptotic host cells," *Infection and Immunity*, vol. 76, no. 3, pp. 959–966, 2008.

[98] J. K. van de Wetering, L. M. G. van Golde, and J. J. Batenburg, "Collectins: players of the innate immune system," *European Journal of Biochemistry*, vol. 271, no. 7, pp. 1229–1249, 2004.

[99] S. Rubio, T. Lacaze-Masmonteil, B. Chailley-Heu, A. Kahn, J. R. Bourbon, and R. Ducroc, "Pulmonary surfactant protein A (SP-A) is expressed by epithelial cells of small and large intestine," *Journal of Biological Chemistry*, vol. 270, no. 20, pp. 12162–12169, 1995.

[100] J. Akiyama, A. Hoffman, C. Brown et al., "Tissue distribution of surfactant proteins A and D in the mouse," *Journal of Histochemistry and Cytochemistry*, vol. 50, no. 7, pp. 993–996, 2002.

[101] K. Uemura, M. Saka, T. Nakagawa et al., "L-MBP is expressed in epithelial cells of mouse small intestine," *Journal of Immunology*, vol. 169, no. 12, pp. 6945–6950, 2002.

[102] C. A. Ogden, A. DeCathelineau, P. R. Hoffmann et al., "C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells," *Journal of Experimental Medicine*, vol. 194, no. 6, pp. 781–795, 2001.

[103] I. Meza, P. Talamás-Rohana, and M. A. Vargas, "The cytoskeleton of *Entamoeba histolytica*: structure, function, and regulation by signaling pathways," *Archives of Medical Research*, vol. 37, no. 2, pp. 234–243, 2006.

[104] G. B. Bailey, D. B. Day, and J. W. Gasque, "Rapid polymerization of *Entamoeba histolytica* actin induced by interaction with target cells," *Journal of Experimental Medicine*, vol. 162, no. 2, pp. 546–558, 1985.

[105] R. L. Guerrant, J. Brush, and J. I. Ravdin, "Interaction between *Entamoeba histolytica* and human polymorphonuclear neutrophils," *Journal of Infectious Diseases*, vol. 143, no. 1, pp. 83–93, 1981.

[106] G. B. Bailey, D. B. Day, C. Nokkaew, and C. C. Harper, "Stimulation by target cell membrane lipid of actin polymerization and phagocytosis by *Entamoeba histolytica*," *Infection and Immunity*, vol. 55, no. 8, pp. 1848–1853, 1987.

[107] H. Voigt, J. C. Olivo, P. Sansonetti, and N. Guillén, "Myosin IB from *Entamoeba histolytica* is involved in phagocytosis of human erythrocytes," *Journal of Cell Science*, vol. 112, no. 8, pp. 1191–1201, 1999.

[108] K. Mittal, B. H. Welter, and L. A. Temesvari, "*Entamoeba histolytica*: lipid rafts are involved in adhesion of trophozoites to host extracellular matrix components," *Experimental Parasitology*, vol. 120, no. 2, pp. 127–134, 2008.

[109] E. Labruyère, C. Zimmer, V. Galy, J. C. Olivo-Marin, and N. Guillén, "EhPAK, a member of the p21-activated kinase family, is involved in the control of *Entamoeba histolytica* migration and phagocytosis," *Journal of Cell Science*, vol. 116, no. 1, pp. 61–71, 2003.

[110] E. D. J. O. Batista and W. De Souza, "Involvement of protein kinases on the process of erythrophagocytis by *Entamoeba histolytica*," *Cell Biology International*, vol. 28, no. 4, pp. 243–248, 2004.

[111] M. Okada, C. D. Huston, B. J. Mann, W. A. Petri Jr., K. Kita, and T. Nozaki, "Proteomic analysis of phagocytosis in the enteric protozoan parasite *Entamoeba histolytica*," *Eukaryotic Cell*, vol. 4, no. 4, pp. 827–831, 2005.

[112] S. Marion, C. Laurent, and N. Guillén, "Signalization and cytoskeleton activity through myosin IB during the early steps of phagocytosis in *Entamoeba histolytica*: a proteomic approach," *Cellular Microbiology*, vol. 7, no. 10, pp. 1504–1518, 2005.

[113] M. Okada, C. D. Huston, M. Oue et al., "Kinetics and strain variation of phagosome proteins of *Entamoeba histolytica* by proteomic analysis," *Molecular and Biochemical Parasitology*, vol. 145, no. 2, pp. 171–183, 2006.

[114] M. Okada and T. Nozaki, "New insights into molecular mechanisms of phagocytosis in *Entamoeba histolytica* by proteomic analysis," *Archives of Medical Research*, vol. 37, no. 2, pp. 244–252, 2006.

[115] S. L. Stanley Jr., "Amoebiasis," *Lancet*, vol. 361, no. 9362, pp. 1025–1034, 2003.

[116] K. B. Seydel, E. Li, P. E. Swanson, and S. L. Stanley Jr., "Human intestinal epithelial cells produce proinflammatory cytokines in response to infection in a SCID mouse-human intestinal xenograft model of amebiasis," *Infection and Immunity*, vol. 65, no. 5, pp. 1631–1639, 1997.

[117] L. Mortimer and K. Chadee, "The immunopathogenesis of *Entamoeba histolytica*," *Experimental Parasitology*, vol. 126, no. 3, pp. 366–380, 2010.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 965369, 10 pages
doi:10.1155/2011/965369

Review Article

Malaria Vaccine Development: Are Bacterial Flagellin Fusion Proteins the Bridge between Mouse and Humans?

Daniel Y. Bargieri,[1,2,3] Irene S. Soares,[4] Fabio T. M. Costa,[5] Catarina J. Braga,[6] Luis C. S. Ferreira,[6] and Mauricio M. Rodrigues[1,2]

[1] *Centro de Terapia Celular e Molecular (CTCMol), Universidade Federal de São Paulo, Escola Paulista de Medicina, Rua Mirassol 207, São Paulo 04044-010, SP, Brazil*
[2] *Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, Escola Paulista de Medicina, Rua Mirassol, 207, São Paulo 04044-010, SP, Brazil*
[3] *Unité de Biologie et Génétique du Paludisme, Institut Pasteur, 75724 Paris Cedex 15, France*
[4] *Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas, Avenida Prof. Lineu Prestes 580, São Paulo 05508-900, SP, Brazil*
[5] *Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas, Rua Monteiro Lobato, 255, Campinas 13083-970, SP, Brazil*
[6] *Departamento de Microbiologia do Instituto de Ciências Biomédicas Universidade de São Paulo, Avenida Prof. Lineu Prestes, 1374, São Paulo 05508-900, SP, Brazil*

Correspondence should be addressed to Mauricio M. Rodrigues, mrodrigues@unifesp.br

Received 1 December 2010; Accepted 18 January 2011

Academic Editor: Michael Lanzer

In the past 25 years, the development of an effective malaria vaccine has become one of the biggest riddles in the biomedical sciences. Experimental data using animal infection models demonstrated that it is possible to induce protective immunity against different stages of malaria parasites. Nonetheless, the vast body of knowledge has generated disappointments when submitted to clinical conditions and presently a single antigen formulation has progressed to the point where it may be translated into a human vaccine. In parallel, new means to increase the protective effects of antigens in general have been pursued and depicted, such as the use of bacterial flagellins as carriers/adjuvants. Flagellins activate pathways in the innate immune system of both mice and humans. The recent report of the first Phase I clinical trial of a vaccine containing a *Salmonella* flagellin as carrier/adjuvant may fuel the use of these proteins in vaccine formulations. Herein, we review the studies on the use of recombinant flagellins as vaccine adjuvants with malarial antigens in the light of the current state of the art of malaria vaccine development. The available information indicates that bacterial flagellins should be seriously considered for malaria vaccine formulations to the development of effective human vaccines.

1. Whole Parasite Vaccines

1.1. Preerythrocytic Stages. The seminal work using radiation-attenuated sporozoites has been reproduced in multiple experimental systems, demonstrating that attenuated infective forms of *Plasmodium sp.* administered by the endovenous route can provide solid vaccination status against any symptoms of malaria [1]. However, the use of radiation-attenuated, viable live sporozoites imposes a number of restrictions because if the radiation fails, then these parasites would cause the disease, thereby nullifying any protective effects. To definitively solve this problem, a number of genetically attenuated lines of parasites have been recently generated. These genetically attenuated parasites are now being pursued as possible antigenic sources for vaccine development for humans, and Phase I and II studies are about to begin [2–5].

1.2. Erythrocytic Stages. Likewise, genetically attenuated blood-stage forms of rodent malaria parasites have been successfully generated in the past few years and have been

proposed as an antigen source for human vaccination trials [5–7]. To our knowledge, none of these parasite lines are being tested in Phase I or II trials. Although it is technically feasible to produce large amounts of genetically attenuated sporozoites or blood-stage forms, it would be difficult to commercialise these vaccines because the parasites would have to be injected live (cryopreserved) by the endovenous route to confer protective immunity. There are also safety issues related to intravenous injections of these formulations because vaccines are not conventionally administered by this route. These two issues will certainly become the main obstacles that must be overcome for successful whole parasite vaccine development.

2. Recombinant Subunit Vaccines

The difficulties associated with large-scale generation of whole parasite antigens for mass vaccination led to the search for recombinant subunit vaccines based on immunodominant malarial antigens. Again, the mouse malaria model was very instructive for narrowing the search for the most promising antigens and testing different recombinant formulations, which provided the proof of principle for these subunit recombinant vaccines. Based on many years of detailed studies, some leading candidate antigens and formulations for vaccines were selected and exhaustively tested, but these tests have mainly occurred in the mouse model of infection as well as nonhuman primates.

2.1. Preerythrocytic Stages. Immunity generated by radiation-attenuated sporozoites is targeted to a dominant protective antigen, the circumsporozoite (CS) protein [8]. Nevertheless, other protective antigens yet to be characterized do exist [8, 9]. This protein is recognized by antibodies, $CD4^+$ and $CD8^+$ T cells induced by immunisation with radiation-attenuated sporozoites that can eliminate the pre-erythrocytic stages of the parasite [10–14]. These results led to the development of vaccine formulations that elicit high antibody titres against sporozoites and/or increased numbers of $CD4^+$ and $CD8^+$ T cells specific for malaria liver stages. Although other proteins such as Liver Stage 1 or 3 and Thrombospondin-Related Anonymous Protein (TRAP) are also being explored as vaccine candidates, the CS protein can be used as a prototypical example of a pre-erythrocytic stage antigen, and the results can be extrapolated to the other surface antigens.

High antibody titres can be achieved, for example, using multiple synthetic peptides (MAPs) formulated in the presence of strong adjuvants [15–17]. Because of the success in rodent models, human safety and efficacy trials are warranted.

The most reliable and reproducible results of vaccination against pre-erythrocytic stages in rodent malaria models were obtained by vaccination with two recombinant viral vectors. Using attenuated viruses containing the entire CS protein or its immunodominant epitopes, a solid and long-lasting protective immunity against sporozoite challenge was elicited [18–20]. Although some protocols managed to use only a single vector for vaccination [21], heterologous prime-boost vaccination using two different vectors for priming and boosting may be essential for eliciting protective immunity [22–25].

These exciting results obtained in the mouse model led to a number of clinical trials. Unfortunately, these trials provided only limited protective immunity in Phase II trials performed in the laboratory, and none in the field [26–28]. The reasons for such failures are difficult to explain precisely. The failures may be related to low antibody levels and/or fewer specific $CD4^+$ and $CD8^+$ T cells generated by these recombinant viruses in humans compared to mice. Based on the mouse model, relatively large amounts of $CD4^+$ and $CD8^+$ T cells are required for complete protective immunity because high numbers of these cells are already required to patrol the entire liver [13, 18, 19, 29, 30]. More powerful viral vectors and strategies of vaccination are being developed every day, and future approaches may increase the number of these protective T cells. Nevertheless, it is unclear whether it will ever be possible to reproduce in humans the levels of immunity induced by viral vectors that are found in the mouse models.

Despite the great progress in malaria vaccine research against pre-erythrocytic stages in the past 20 years, a single type of formulation has shown promising results when tested in humans. This vaccine formulation consists of a large C-terminal fragment of the CS protein sequence fused to the Hepatitis B antigen S (conventional hepatitis B vaccine, Engerix B) and expressed as a recombinant protein in *Saccharomyces cerevisiae*. The fusion protein, named RTS, when expressed together with antigen S, naturally assembles into virus-like particles called RTS,S. The efficacy of the RTS,S formulation is dependent on the use of adjuvant systems (AS), which consist of two different formulations that include monophosphoryl lipid A (MPL, a detoxified form of LPS) and QS21 (saponin purified from *Quillaja saponaria*) in an oil-in-water emulsion (AS02) or in a liposomal suspension (AS01) [31].

In recent Phase II trials performed in naïve human volunteers challenged with *P. falciparum* sporozoites, efficacies ranging from 32% to 50% were observed. Immunological studies performed on these vaccinated individuals indicated that protection correlated with the concentration of specific antibodies and the frequency of IFN-γ producing cells as detected by ELISPOT [32]. A number of Phase IIb trials have been carried out, and they continue to be carried out in the field. These results are more difficult to interpret, but in trials performed in children in the endemic areas, 49.5% and 62% efficacy were reported during the 6-month period that was studied retarding the first malaria episode [33, 34]. Although partial, the protective immunity afforded by this vaccination protocol can be considered the most hopeful path for vaccine development to date. Because protective immunity was highly dependent on the adjuvants used in the formulation, these studies highlighted the importance of adjuvant development for a reliable malaria vaccine (see below). Currently, this formulation is being tested in Phase III trials in malaria-endemic areas, and the results are anxiously expected for the years 2011-12 [31].

2.2. Erythrocytic Stages. The erythrocytic forms of malaria grow and multiply within the host erythrocytes, and the rupture of the host cells is responsible for the clinical symptoms, including fever, of the noncomplicated forms of the disease. Nevertheless, infected erythrocytes (iEs) can also cytoadhere by means of variant surface antigens (VSA) to endothelial receptors on the microvasculature of certain organs or to the placental trophoblast, thereby disappearing from peripheral blood circulation. This phenomenon, named sequestration, is responsible for the majority of the deaths caused by malaria because it leads to the severe forms of malaria, including cerebral malaria, severe anaemia, acute respiratory distress symptoms or pregnancy-associated malaria (PAM) [35]. Because blood-stage forms of the parasite are responsible for the clinical manifestations of the disease, a vaccine against them would prevent or reduce the morbidity and mortality of malaria through the elimination or reduction of the parasitic burden (reviewed in [36, 37]).

In the case of PAM, the scenario is complex because cytoadherence is mediated by VSA. Given that *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) is involved on parasite cytoadhesion and several studies indicate that PfEMP-1 is the major target during natural acquired immunity, this protein family is the only VSA under consideration for vaccine development based on the induction of adhesion-blocking antibodies (reviewed in [38]). Furthermore, because PAM is associated with a selection of antigenically distinct iEs that express a particular PfEMP-1 variant named VAR2CSA, a specific vaccine against this syndrome, based on this variant, should also be developed [35]. Several bodies of evidence point the VAR2CSA protein as a leading vaccine candidate against PAM and strategies based on induction of antibodies capable of inhibiting the adhesion of iEs to chondroitin sulphate A (CSA) in the placenta have recently been pursued [38, 39]. In fact, disruption of *var2csa* gene abrogates iEs CSA binding ability, plasma antibody levels against VAR2CSA are gender-specific, and naturally acquisition of high levels of VAR2CSA specific antibodies is parity-dependent and correlates to protection against PAM. However, large-scale development of a full-length VAR2CSA recombinant protein is hampered by its size (350 kDa). An alternative is the expression of all or some of its six Duffy binding-like (DBL) domains, although full-length VAR2CSA displays higher specificity and affinity to human CSA and induced antibodies abrogate adhesion of iEs to CSA [40]. Nevertheless, detailed characterization regarding VAR2CSA DBL domains interaction with CSA backbone, the resolution of their molecular structure (individually or as large fragments), and their involvement in PAM are still puzzling [38, 39].

Concerning blood-stage antigens of *Plasmodium* not directly related to PAM, in the last 20 years significant progress has been made in their molecular characterisation as well as in the identification of the immunologic mechanisms capable of eliminating these forms of the parasite (reviewed in [41–43]). Among the immunodominant antigens already characterised from blood-stage parasites, one of the main candidates for the development of a vaccine against *Plasmodium* is the Merozoite Surface Protein 1 (MSP-1) (revised in [44]). Although other proteins, such as Apical Membrane Antigen 1 (AMA-1) and Duffy-Binding Protein (DBP), are also being explored as vaccine candidates (revised in [45–47]), MSP-1 can be used as a prototypical example of a blood-stage antigen, and the results obtained from a vaccine developed around this antigen can be extrapolated to other surface antigens.

In different species of *Plasmodium,* the structure of the C-terminal region of MSP-1 ($MSP1_{19}$) consists of two domains. These domains contain 10–12 cysteine residues. Domain 1 or 2 contain 4–6 or 6 cysteine residues and form 2-3 or 3 disulphide bonds, respectively. Both domains are configured as epidermal growth factor-(EGF-) like domains. This type of conformation was initially suggested by the primary amino acid structure and was later confirmed by the three-dimensional analysis of recombinant proteins representing the $MSP1_{19}$ regions of *P. cynomolgi* and *P. falciparum*. The structure of the $MSP1_{19}$ of *P. vivax* ($PvMSP1_{19}$) was recently elucidated through NMR, and it corroborated the $MSP1_{19}$ structures previously described in other species [48].

During invasion of the host erythrocytes, MSP-1 is a target of proteolytic processes. Various studies have demonstrated that $MSP1_{42}$ is found as a dimer, while $MSP1_{19}$ remains monomeric, suggesting a secondary process that would cause the dissociation of the protein before the erythrocytes are invaded (reviewed in [44]).

The precise biological role of MSP-1 is still unknown. However, several studies have demonstrated that antibodies specific for the $MSP1_{19}$ of *P. falciparum* ($PfMSP1_{19}$) can inhibit the invasion of merozoites, suggesting that this protein is involved in the process of erythrocyte invasion. In this context, attempts to knock down the coding genes of six merozoite proteins of *P. falciparum* associated with the membrane through the GPI anchor (MSP-1, MSP-2, MSP-4, MSP-5, MSP-10, RAMA, and Pf92) demonstrated that successful genetic deletion could not be obtained, with the exception of the *msp-5* gene. These observations highlight the importance of these proteins in the blood cycle of the malaria parasite [49, 50]. Finally, developmental knockout proved the importance of MSP-1 in the structure of *Plasmodium* merozoites [51].

Based on these positive attributes, the C-terminal region of MSP-1 ($MSP1_{19}$ or $MSP1_{42}$) has been heavily pursued as a vaccine candidate. Initial studies in mouse models demonstrated solid levels of protective immunity after vaccination with strong adjuvants, such as Complete Freund's Adjuvant [52, 53]. Other adjuvants did not elicit a similar effect, which clearly demonstrates the importance of the adjuvant in vaccine formulations. Although protective immunity is mostly mediated by antibodies, a recent study showed that the presence of $CD4^+$ T helper epitopes improves the protective immunity induced in mouse models [54]. Subsequent studies performed in nonhuman primates duplicated these results by demonstrating a strict requirement for a strong adjuvant (e.g., Complete Freund's Adjuvant) to stimulate protective immunity against experimental infection with *P. cynomolgi* or *P. falciparum* [55, 56].

Based on the results obtained from the experimental models of malaria infection, a recent Phase IIb vaccine trial was performed in Africa. In this clinical trial, children were vaccinated with a formulation containing a recombinant His-tagged fusion protein encompassing the $MSP1_{42}$ of the *P. falciparum* 3D7 strain (FMP1) that was formulated with the adjuvant AS02 [57]. This vaccine formulation was shown to be safe and immunogenic, as demonstrated by detection of specific antibody titres by ELISA. Unfortunately, the trial failed, and no significant reduction in the incidence of malaria infection could be observed in children receiving the FMP1/AS02 formulation [57]. The precise reason why the vaccination failed should be investigated. It might be attributed to a polymorphism of the MSP-1 protein. This fact may be relevant to the interpretation of the results because protective immunity to the C-terminal region of *P. falciparum* MSP-1 can be strain-specific, and antibodies targeting this antigen may not show parasite inhibitory activity [56]. Furthermore, some of the MSP-1-specific antibodies are endowed with the ability to block the activity of inhibitory antibodies [44]. Although negative, these results do not completely refute the hypothesis that the C-terminal region of *P. falciparum* MSP-1 could be part of a subunit malaria vaccine in a new recombinant form or formulation. Currently, the immunogenic properties of distinct recombinant proteins are being compared in experimental animal models to select possible candidates for human trials [58].

3. Bacterial Flagellin Fusion Proteins: Bridging the Gap between Mice and Humans?

As thoroughly discussed above, vaccination of mice with recombinant proteins or viral vectors may confer significant protection against an infectious challenge with malaria parasites. The main problem is that some of these formulations, such as the ones containing strong adjuvants, simply cannot be used in humans. Others, such as the recombinant viruses, failed to reproduce in humans the immunogenicity observed in mice. These successive failures have led to the search for new alternatives that could eventually bridge the gap between mice and humans.

3.1. Bacterial Flagellins. Flagella represent a complex locomotion device expressed by different bacterial species. They encompass at least 50 different proteins. Flagella may also behave as virulence-associated factors by helping pathogenic bacteria to bind to epithelial cells and to overcome non-immune defence mechanisms such as liquid flow in the urinary tract. Flagellin, the structural protein of the flagellar filament, is the most abundant protein of the flagellar apparatus, which can assemble by the polymerisation of thousands of flagellin monomers. The molecular masses of flagellins can vary from 28 kDa to 80 kDa. The N- and C-terminal regions of these molecules are highly conserved among different bacterial species and are folded together in parallel as a packed α-helix structure that is recognised by innate receptors of the immune system (see below). On the other hand, the central region shows a striking degree of sequence heterogeneity, even among flagellins from a single bacterial species. This heterogeneity has been observed in *Salmonella enterica* serovars, an immune escape mechanism reflecting the fact that most B cell epitopes are exposed to this region of the molecule [59].

Flagellin is a Pathogen-Associated Molecular Pattern (PAMP) recognised by Pattern Recognition Receptors (PRRs) in the innate immune system that triggers inflammatory responses and activates the adaptative immune system. At least two classes of PRRs, Toll-like Receptors (TLRs) and NOD-like Receptors (NLRs), respond to flagellin.

TLR5 is a transmembrane receptor with a conserved cytoplasmic signalling domain—the Toll/interleukin (IL)-1 receptor homology domain (TIR), a common signature for TLRs—that specifically recognises a conserved site in the N- and C-terminal regions of flagellin that is formed after the correct assembly of the molecule; in fact, this site is essential for the correct flagella assembly [60, 61]. The receptor is expressed by monocytes, dendritic cells (DCs), and epithelial cells that sense extracellular flagellin. When activated, TLR5 signals through the adaptor protein MyD88 to trigger NF-κB activation and the subsequent expression of proinflammatory cytokines. Flagellin also induces the expression of a number of activation markers in antigen-presenting cells (APCs), such as CD80, CD86, CD40, and MHC class II, all of which augment antigen presentation to T cells [62–67]. Interestingly, flagellin is one of the two proteic agonist of TLRs.

In the case of the NLRs, flagellin that is expressed by intracellularly replicating pathogens or specifically injected by a secretory apparatus displayed by some bacterial pathogens, such as *S. enterica*, activates a multiprotein cytosolic complex, the inflammasome, which is involved in the regulation of inflammation and cell death responses in a manner dependent on ICE-protease-activating factor (Ipaf) and neuronal apoptosis inhibitory protein 5 (Naip5) [68, 69]. Activation of the inflammasome by cytosolic flagellin is related to the control of bacterial growth [70, 71] and triggers Caspase-1 activation followed by proinflammatory cell death (pyroptosis) with secretion of IL-1β and IL-18 and activation of inducible nitric-oxide synthase, iNOS [68, 72, 73]. A short C-terminal region of flagellin, which is distinct from the sequences required for TLR5 activation, is responsible for Naip5/Ipaf-containing inflammasome activation [69]. Nevertheless, it remains elusive whether flagellin binds directly to Naip5 or Ipaf or if it acts through an undefined upstream receptor.

Clearly, mammalian cells evolved the ability to very efficiently sense the presence of extra- and intracellular flagellins and respond with strong inflammatory signals that may ultimately enhance the induction of adaptive immune defences. Based on these natural properties, bacterial flagellins have been intensively investigated as vaccine adjuvants that can either be co-administered with purified recombinant antigens or as a carrier/adjuvant molecule genetically fused to the target antigen.

3.2. Bacterial Flagellins As Vaccine Adjuvants. In the late 80s, a seminal work envisaged the use of bacterial flagellins as carriers/adjuvants for vaccine antigens by cloning synthetic oligonucleotides encoding specific B or T-cell epitopes at the central hypervariable region of the *S.* Muenchen FliC*d* flagellin expressed in an attenuated *S*. Dublin vaccine strain that was delivered via mucosal or parenteral routes to mice [74]. Purified recombinant forms of flagellin genetically fused to short synthetic oligonucleotides were also successfully used as subunit vaccines by targeting either influenza virus haemagglutinin or an adhesin expressed by enterotoxigenic *Escherichia coli* strains even before the discovery that flagellin activates innate immune responses [75, 76].

More recently, the description of innate immune receptors that recognise flagellin and their capacity to activate innate and adaptive immune responses intensified the studies testing bacterial flagellins as vaccine adjuvants for the induction of both antibody and cellular immune responses. Different systems that used flagellins as carriers/adjuvants for recombinant fusion proteins or with coadministered recombinant antigens were tested in the last few years; for a detailed and updated compilation of these data, we recommend a very recently published paper [77].

For brevity and the purpose of our review, we selected the vaccine field that is highly advanced: the development of an anti-influenza vaccine. Two formulations of fusion proteins of flagellin and influenza antigen were generated. First, four tandem copies of the ectodomain of the conserved matrix protein M2 (M2e) of human influenza A virus were fused to the C-terminal domain of the flagellin of *Salmonella typhimurium* fljB (Type 2). A second formulation was generated by the fusion of the viral haemagglutinin globular head domain to the C-terminus of flagellin or in place of the D3 domain (VAX125). All proteins were expressed as soluble fusion proteins in the *E. coli* system. The preclinical studies testing these vaccines reported induction of strong humoral-specific immune responses against the influenza antigens after immunisation of mice and rabbits with the recombinant fusion proteins alone. In both cases, the antibody response was dependent on the physical fusion of the antigen to the flagellin. Most relevant for vaccination studies was the fact that immunisation with either construct protected mice from a lethal challenge with influenza A virus and significantly reduced weight loss and clinical symptoms compared to control animals [78, 79].

Based on these successful pre-clinical studies, the influenza vaccine candidate VAX125 underwent a complete Phase I clinical trial that tested safety, reactogenicity, immunogenicity, tolerogenicity, and escalating dose-range. The results from this trial showed that recombinant flagellin was generally well tolerated by vaccinated individuals. Importantly, 91% of the individuals who received any dose of recombinant protein developed titres of neutralising antibodies compatible with protective status against influenza infection (http://clinicaltrials.gov/NCT00921947/) [80].

3.3. Bacterial Flagellin As a Carrier/Adjuvant for Malaria Antigens. Based on the great capacity of flagellin to induce humoral and cellular immune responses against foreign antigens, we explored the use of this molecule for the generation of fusion proteins containing malarial antigens. We selected for our initial studies the 19-kDa C-terminal region of MSP-1 from *P. vivax*. This region of MSP-1, termed $PvMSP1_{19}$, was selected because it is arguably the most immunogenic region of *P. vivax* [81]. In immuno-epidemiological studies performed all around the world, this molecule has been shown to be highly conserved [82] and recognised by more than 95% of individuals after their first contact with the *P. vivax* [83]. Most pertinent for vaccine development, immunisation of nonhuman primates with a recombinant protein based on $PvMSP1_{19}$ resulted in protection against an experimental challenge [84].

$PvMSP1_{19}$ was fused to the C-terminal end of the FliC flagellin from *S. typhimurium*. This fusion protein was able to bind and activate TLR5 that was expressed by *in vitro* cultured transfected cells, and it was recognised by serum from *P. vivax* malaria patients. Moreover, the fusion protein was recognised by monoclonal antibodies directed against the three-dimensional structural epitopes, indicating that it was correctly folded when expressed in the *E. coli* system [85].

We immunised mice and rabbits with the recombinant fusion protein in the absence of any other adjuvant. Immunised animals developed strong, specific, and long-lasting antibody-mediated responses. The antibody titres after the second dose were similar to the ones obtained following immunisation with a recombinant protein containing only the $PvMSP1_{19}$ emulsified in Complete/Incomplete Freund's Adjuvant. Additionally, the antibodies raised after these immunisations recognised *P. vivax* merozoites by immunofluorescence [85].

In the absence of any other adjuvant, the pattern of the immune response was biased toward a type 2 response, with a high IgG1/2 ratio and limited amounts of IFN-γ secreted by the splenic immune cells. Nevertheless, the addition of other adjuvants to the fusion protein, such as TLR-9 agonist, modulated the immune response towards a type 1 response, with a lower IgG1/2 ratio and secretion of significantly more IFN-γ [85].

Because long-term *in vitro* culture of *P. vivax* is not feasible, and it was difficult to find a reliable biological model to test our vaccine formulation, we reproduced entirely the study using the 19-kDa C-terminal region of MSP-1 from *P. falciparum*. This region of MSP-1, termed $PfMSP1_{19}$, was selected because it has been extensively studied as a vaccine candidate, as described above. Rabbits injected with $PfMSP1_{19}$ fused to flagellin raised high antibody titres that dramatically inhibited the *in vitro* growth of the parasite lines 3D7, S20, and FCR3 [86]. A similar approach is now being used to generate recombinant fusion proteins containing immuno-dominant epitopes of the sporozoite stage CS protein.

Using another approach, we inserted the CS protein $CD8^+$ T-cell epitope $CS_{280–288}$) from the murine malaria parasite *P. yoelii* into the central hypervariable (D3) domain of the *S.* Muenchen FliC flagellin [74]. The flagellin adjuvant effects were determined with two vaccine formulations: (i) attenuated *S.* Dublin strains administered orally expressing

hybrid flagella composed of the flagellin-CS fusion protein and (ii) purified flagellins administered s.c. to mice either as hybrid flagellin or native flagellin mixed with synthetic $CS_{280–288}$ peptide. Both formulations induced CS-specific $CD8^+$ T-cell responses in the absence of any conventional adjuvant, as measured by ELISPOT [87]. These results suggest that *Salmonella* flagellins are promising soluble adjuvants for synthetic peptides in regard to the activation of specific cytotoxic T cell responses.

To our knowledge, these were the first reports of an induction of specific humoral and cellular immune responses in experimental immunisations with recombinant proteins based on malaria vaccine antigens without the addition of any conventional adjuvant. As mentioned above, the magnitude of an immune response induced against recombinant proteins is highly dependent on the potency of the adjuvant formulation.

4. Adjuvanticity Mechanism of Flagellin

The mechanisms by which flagellin acts as an adjuvant are not fully understood, but it is likely to be dependent on direct APC activation, especially DC activation, to enhance antigen presentation. Flagellin up-regulates the expression of costimulatory molecules such as B7-1, B7-2, and CD40 in murine bone marrow-derived DCs [88, 89] and B7-1, B7-2, CD83, and CCR7 in human primary blood DCs [62]. Moreover, flagellin-maturated human DCs down-regulate endocytic activity, a hallmark of DC activation, and they have enhanced T-cell stimulatory activity [62]. Another remarkable capacity of flagellin is to convert tolerogenic DCs into activated antigen-presenting cells, which is an unusual ability [90]. The effects of flagellin on DCs are at least in part dependent on TLR5 activation, as evidenced by the fact that OVA-specific $CD4^+$ T cell proliferation in response to immunisation with flagellin-OVA is impaired by depletion of $TLR5^{+/+}CD11c^{+/+}$ cells or in $TLR5^{-/-}$) mice [67]. However, $TLR5^{-/-}$) mice still develop strong antiflagellin antibody responses after injection of flagellin; in addition, $MyD88^{-/-}$) mice, which have impaired TLR- and NOD-dependent signalling, respond to flagellin injection with less robust antibody production [91]. In fact, recent results have shown that both TLR5 and NOD-like receptors operate in a complementary manner to support the flagellin adjuvant effects, at least in relation to the induction of humoral responses [92].

The requirement for the physical linking of flagellin to the antigen is still a matter of debate. The fusion requirement may be dependent on the amount of antigen injected. One possibility is that when flagellin is bound to the antigen, there is concomitant APC activation and antigen uptake, as opposed to coadministered soluble antigen and flagellin when an activated APC may not uptake the antigen at the same time of activation. The fact that activated DCs down-regulate endocytic activity supports this hypothesis, indicating that if activation does not happen in the presence of the antigen, uptake may be impaired, and more antigen would be required to increase the chance of concomitant antigen uptake and APC activation. In fact, when we tested equimolar amounts of flagellin and antigen that were fused or co-administered, we found that the responses are comparable in the range of 10 μg; however, the fusion protein was more efficient in the induction of antigen-specific antibody responses at the levels of 1 μg or 0.1 μg (Bargieri DY and Rodrigues MM, unpublished data).

5. Conclusions

RTS,S is arguably the most advanced vaccine against *P. falciparum* malaria. Its development provided great lessons; for example, we learned that the delivery system/adjuvant is integral for the induction of any degree of immunity against malaria. Despite the on-going RTS,S Phase III clinical trials, we should not stop looking for new methods to improve the immunogenicity of recombinant antigens in humans. One possibility would be to add other recombinant vectors, such as adenoviral vectors, in combination with RTS,S to boost its potency. In this paper, we proposed an alternative approach that involved the structural modification of malarial antigens to increase their intrinsic immunogenicity. The use of recombinant fusion flagellins containing malarial epitopes may be a simple and inexpensive way to enhance protein antigenicity. The fact that recombinant flagellins have reached Phase I/II clinical trials should accelerate further studies in this direction.

Acknowledgments

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (Proc. no. 09/15132-4), The National Institute for Vaccine Technology (INCTV-CNPq), and The Millennium Institute for Vaccine Development and Technology (CNPq-420067/2005-1). Daniel Y. Bargieri is supported by a Manlio Cantarini fellowship. Catarina J. Braga is supported by a fellowship from FAPESP. Irene S. Soares, Fabio T. M. Costa, Luis C. S. Ferreira and Mauricio M. Rodrigues are recipients of fellowships from CNPq. The authors thank Dr. Bruna C. de Alencar for the suggestions.

References

[1] R. S. Nussenzweig, J. Vanderberg, H. Most, and C. Orton, "Protective immunity produced by the injection of X-irradiated sporozoites of plasmodium berghei," *Nature*, vol. 216, no. 5111, pp. 160–162, 1967.

[2] A. K. Mueller, M. Labaied, S. Kappe, and K. Matuschewski, "Genetically modified Plasmodium parasites as a protective experimental malaria vaccine," *Nature*, vol. 13, no. 7022, pp. 164–167, 2005.

[3] M. R. Van Dijk, B. Douradinha, B. Franke-Fayard et al., "Genetically attenuated P36p-deficient malarial sporozouites induce protective immunity and apoptosis of infected liver cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 34, pp. 12194–12199, 2005.

[4] K. M. VanBuskirk, M. T. O'Neill, P. De La Vega et al., "Preerythrocytic, live-attenuated Plasmodium falciparum vaccine

candidates by design," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 31, pp. 13004–13009, 2009.

[5] L. M. Ting, M. Gissot, A. Coppi, P. Sinnis, and K. Kim, "Attenuated Plasmodium yoelii lacking purine nucleoside phosphorylase confer protective immunity," *Nature Medicine*, vol. 14, no. 9, pp. 954–958, 2008.

[6] A. S. I. Aly, M. J. Downie, C. B. Mamoun, and S. H. I. Kappe, "Subpatent infection with nucleoside transporter 1-deficient Plasmodium blood stage parasites confers sterile protection against lethal malaria in mice," *Cellular Microbiology*, vol. 12, no. 7, pp. 930–938, 2010.

[7] R. Spaccapelo, C. J. Janse, S. Caterbi et al., "Plasmepsin 4-deficient Plasmodium berghei are virulence attenuated and induce protective immunity against experimental malaria," *American Journal of Pathology*, vol. 176, no. 1, pp. 205–217, 2010.

[8] K. A. Kumar, G. I. Sano, S. Boscardin et al., "The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites," *Nature*, vol. 444, no. 7121, pp. 937–940, 2006.

[9] M. Mauduit, A. C. Grüner, R. Tewari et al., "A role for immune responses against non-CS components in the cross-species protection induced by immunization with irradiated malaria sporozoites," *PLoS ONE*, vol. 4, no. 11, article e7717, 2009.

[10] P. Romero, J. L. Maryanski, G. Corradin, R. S. Nussenzweig, V. Nussenzweig, and F. Zavala, "Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria," *Nature*, vol. 341, no. 6240, pp. 323–326, 1989.

[11] E. H. Nardin, D. A. Herrington, J. Davis et al., "Conserved repetitive epitope recognized by CD4 clones from a malaria-immunized volunteer," *Science*, vol. 246, no. 4937, pp. 1603–1606, 1989.

[12] L. Renia, M. S. Marussig, D. Grillot et al., "In vitro activity of CD4+ and CD8+ T lymphocytes from mice immunized with a synthetic malaria peptide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 18, pp. 7963–7967, 1991.

[13] M. M. Rodrigues, A. S. Cordey, G. Arreaza et al., "CD8+ cytolytic T cell clones derived against the Plasmodium yoelii circumsporozoite protein protect against malaria," *International Immunology*, vol. 3, no. 6, pp. 579–585, 1991.

[14] A. Malik, J. E. Egan, R. A. Houghten, J. C. Sadoff, and S. L. Hoffman, "Human cytotoxic T lymphocytes against the Plasmodium falciparum circumsporozoite protein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 8, pp. 3300–3304, 1991.

[15] J. P. Tam, P. Clavijo, Y. A. Lu, V. Nussenzweig, R. Nussenzweig, and F. Zavala, "Incorporation of T and B epitopes of the circumsporozoite protein in a chemically defined synthetic vaccine against malaria," *Journal of Experimental Medicine*, vol. 171, no. 1, pp. 299–306, 1990.

[16] E. H. Nardin, G. A. Oliveira, J. M. Calvo-Calle, and R. S. Nussenzweig, "The use of multiple antigen peptides in the analysis and induction of protective immune responses against infectious diseases," *Advances in Immunology*, vol. 60, pp. 105–149, 1995.

[17] J. M. Calvo-Calle, G. A. Oliveira, C. O. Watta, J. Soverow, C. Parra-Lopez, and E. H. Nardin, "A linear peptide containing minimal T- and B-cell epitopes of Plasmodium falciparum circumsporozoite protein elicits protection against transgenic sporozoite challenge," *Infection and Immunity*, vol. 74, no. 12, pp. 6929–6939, 2006.

[18] O. Bruña-Romero, G. González-Aseguinolaza, J. C. R. Hafalla, M. Tsuji, and R. S. Nussenzweig, "Complete, long-lasting protection against malaria of mice primed and boosted with two distinct viral vectors expressing the same plasmodial antigen," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 20, pp. 11491–11496, 2001.

[19] A. Reyes-Sandoval, T. Berthoud, N. Alder et al., "Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses," *Infection and Immunity*, vol. 78, no. 1, pp. 145–153, 2010.

[20] O. J. Ophorst, K. Radošević, M. J. E. Havenga et al., "Immunogenicity and protection of a recombinant human adenovirus serotype 35-based malaria vaccine against Plasmodium yoelii in mice," *Infection and Immunity*, vol. 74, no. 1, pp. 313–320, 2006.

[21] T. Shiratsuchi, U. Rai, A. Krause, S. Worgall, and M. Tsuji, "Replacing adenoviral vector HVR1 with a malaria B cell epitope improves immunogenicity and circumvents preexisting immunity to adenovirus in mice," *Journal of Clinical Investigation*, vol. 120, no. 10, pp. 3688–3701, 2010.

[22] S. Li, M. Rodrigues, D. Rodriguez et al., "Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 11, pp. 5214–5218, 1993.

[23] M. Rodrigues, S. Li, K. Murata et al., "Influenza and vaccinia viruses expressing malaria CD8+ T and B cell epitopes: comparison of their immunogenicity and capacity to induce protective immunity," *Journal of Immunology*, vol. 153, no. 10, pp. 4636–4648, 1994.

[24] M. Sedegah, T. R. Jones, M. Kaur et al., "Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 13, pp. 7648–7653, 1998.

[25] J. Schneider, S. C. Gilbert, T. J. Blanchard et al., "Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara," *Nature Medicine*, vol. 4, no. 4, pp. 397–402, 1998.

[26] D. P. Webster, S. Dunachie, J. M. Vuola et al., "Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 13, pp. 4836–4841, 2005.

[27] S. J. Dunachie, M. Walther, J. E. Epstein et al., "A DNA prime-modified vaccinia virus Ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naive adults against Plasmodium falciparum sporozoite challenge," *Infection and Immunity*, vol. 74, no. 10, pp. 5933–5942, 2006.

[28] P. Bejon, E. Ogada, T. Mwangi et al., "Extended follow-up following a phase 2b randomized trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya," *PLoS ONE*, vol. 2, no. 8, article e707, 2007.

[29] N. W. Schmidt, R. L. Podyminogin, N. S. Butler et al., "Memory CD8+ T cell responses exceeding a large but definable threshold provide long-term immunity to malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 37, pp. 14017–14022, 2008.

[30] M. Rodrigues, R. S. Nussenzweig, P. Romero, and F. Zavala, "The in vivo cytotoxic activity of CD8+ T cell clones correlates with their levels of expression of adhesion molecules," *Journal of Experimental Medicine*, vol. 175, no. 4, pp. 895–905, 1992.

[31] J. Cohen, V. Nussenzweig, R. Nussenzweig, J. Vekemans, and A. Leach, "From the circumsporozoite protein to the RTS,S/AS candidate vaccine," *Human Vaccines*, vol. 6, no. 1, pp. 90–96, 2010.

[32] K. E. Kester, J. F. Cummings, O. Ofori-Anyinam et al., "Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection," *Journal of Infectious Diseases*, vol. 200, no. 3, pp. 337–346, 2009.

[33] P. Bejon, J. Lusingu, A. Olotu et al., "Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age," *New England Journal of Medicine*, vol. 359, no. 24, pp. 2521–2532, 2008.

[34] S. Abdulla, R. Oberholzer, O. Juma et al., "Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants," *New England Journal of Medicine*, vol. 359, no. 24, pp. 2533–2544, 2008.

[35] B. Gamain, J. D. Smith, N. K. Viebig, J. Gysin, and A. Scherf, "Pregnancy-associated malaria: parasite binding, natural immunity and vaccine development," *International Journal for Parasitology*, vol. 37, no. 3-4, pp. 273–283, 2007.

[36] J. Wipasa and E. M. Riley, "The immunological challenges of malaria vaccine development," *Expert Opinion on Biological Therapy*, vol. 7, no. 12, pp. 1841–1852, 2007.

[37] B. Genton and Z. H. Reed, "Asexual blood-stage malaria vaccine development: facing the challenges," *Current Opinion in Infectious Diseases*, vol. 20, no. 5, pp. 467–475, 2007.

[38] L. Hviid, "The role of Plasmodium falciparum variant surface antigens in protective immunity and vaccine development," *Human Vaccines*, vol. 6, no. 1, pp. 84–89, 2010.

[39] M. Dahlbäck, M. A. Nielsen, and A. Salanti, "Can any lessons be learned from the ambiguous glycan binding of PfEMP1 domains?" *Trends in Parasitology*, vol. 26, no. 5, pp. 230–235, 2010.

[40] P. Khunrae, M. Dahlbäck, M. A. Nielsen et al., "Full-length recombinant Plasmodium falciparum VAR2CSA binds specifically to CSPG and induces potent parasite adhesion-blocking antibodies," *Journal of Molecular Biology*, vol. 397, no. 3, pp. 826–834, 2010.

[41] Z. H. Reed, M. Friede, and M. P. Kieny, "Malaria vaccine development: progress and challenges," *Current Molecular Medicine*, vol. 6, no. 2, pp. 231–245, 2006.

[42] S. S. Yazdani, P. Mukherjee, V. S. Chauhan, and C. E. Chitnis, "Immune responses to asexual blood-stages of malaria parasites," *Current Molecular Medicine*, vol. 6, no. 2, pp. 187–203, 2006.

[43] J. Langhorne, F. M. Ndungu, A. M. Sponaas, and K. Marsh, "Immunity to malaria: more questions than answers," *Nature Immunology*, vol. 9, no. 7, pp. 725–732, 2008.

[44] M. Kadekoppala and A. A. Holder, "Merozoite surface proteins of the malaria parasite: the MSP1 complex and the MSP7 family," *International Journal for Parasitology*, vol. 40, no. 10, pp. 1155–1161, 2010.

[45] E. J. Remarque, B. W. Faber, C. H. M. Kocken, and A. W. Thomas, "Apical membrane antigen 1: a malaria vaccine candidate in review," *Trends in Parasitology*, vol. 24, no. 2, pp. 74–84, 2008.

[46] C. E. Chitnis and A. Sharma, "Targeting the Plasmodium vivax Duffy-binding protein," *Trends in Parasitology*, vol. 24, no. 1, pp. 29–34, 2008.

[47] A. Brown and M. K. Higgins, "Carbohydrate binding molecules in malaria pathology," *Current Opinion in Structural Biology*, vol. 20, no. 5, pp. 560–566, 2010.

[48] J. J. Babon, W. D. Morgan, G. Kelly, J. F. Eccleston, J. Feeney, and A. A. Holder, "Structural studies on Plasmodium vivax merozoite surface protein-1," *Molecular and Biochemical Parasitology*, vol. 153, no. 1, pp. 31–40, 2007.

[49] R. A. O'Donnell, A. Saul, A. F. Cowman, and B. S. Crabb, "Functional conservation of the malaria vaccine antigen MSP-1 across distantly related Plasmodium species," *Nature Medicine*, vol. 6, no. 1, pp. 91–95, 2000.

[50] P. R. Sanders, L. M. Kats, D. R. Drew et al., "A set of glycosylphosphatidyl inositol-anchored membrane proteins of Plasmodium falciparum is refractory to genetic deletion," *Infection and Immunity*, vol. 74, no. 7, pp. 4330–4338, 2006.

[51] A. Combe, D. Giovannini, T. G. Carvalho et al., "Clonal conditional mutagenesis in malaria parasites," *Cell Host and Microbe*, vol. 5, no. 4, pp. 386–396, 2009.

[52] T. M. Daly and C. A. Long, "A recombinant 15-kilodalton carboxyl-terminal fragment of Plasmodium yoelii yoelii 17XL merozoite surface protein 1 induces a protective immune response in mice," *Infection and Immunity*, vol. 61, no. 6, pp. 2462–2467, 1993.

[53] T. M. Daly and C. A. Long, "Influence of adjuvants on protection induced by a recombinant fusion protein against malarial infection," *Infection and Immunity*, vol. 64, no. 7, pp. 2602–2608, 1996.

[54] B. Singh, M. Cabrera-Mora, J. Jiang, M. Galinski, and A. Moreno, "Genetic linkage of autologous T cell epitopes in a chimeric recombinant construct improves anti-parasite and anti-disease protective effect of a malaria vaccine candidate," *Vaccine*, vol. 28, no. 14, pp. 2580–2592, 2010.

[55] K. L. R. L. Perera, S. M. Handunnetti, I. Holm, S. Longacre, and K. Mendis, "Baculovirus merozoite surface protein 1 C-terminal recombinant antigens are highly protective in a natural primate model for human plasmodium vivax malaria," *Infection and Immunity*, vol. 66, no. 4, pp. 1500–1506, 1998.

[56] J. A. Lyon, E. Angov, M. P. Fay et al., "Protection induced by plasmodium falciparum MSP1 is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses," *PLoS ONE*, vol. 3, no. 7, article e2830, 2008.

[57] B. R. Ogutu, O. J. Apollo, D. McKinney et al., "Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya," *PLoS ONE*, vol. 4, no. 3, article e4708, 2009.

[58] Z. H. Reed, M. P. Kieny, H. Engers et al., "Comparison of immunogenicity of five MSP1-based malaria vaccine candidate antigens in rabbits," *Vaccine*, vol. 27, no. 10, pp. 1651–1660, 2009.

[59] H. C. Ramos, M. Rumbo, and J. C. Sirard, "Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa," *Trends in Microbiology*, vol. 12, no. 11, pp. 509–517, 2004.

[60] F. Hayashi, K. D. Smith, A. Ozinsky et al., "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5," *Nature*, vol. 410, no. 6832, pp. 1099–1103, 2001.

[61] K. D. Smith, E. Andersen-Nissen, F. Hayashi et al., "Toll-like receptor 5 recognizes a conserved site on flagellin required

for protofilament formation and bacterial motility," *Nature Immunology*, vol. 4, no. 12, pp. 1247–1253, 2003.

[62] T. K. Means, F. Hayashi, K. D. Smith, A. Aderem, and A. D. Luster, "The toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells," *Journal of Immunology*, vol. 170, no. 10, pp. 5165–5175, 2003.

[63] S. Arimilli, J. B. Johnson, K. M. Clark et al., "Engineered expression of the TLR5 ligand flagellin enhances paramyxovirus activation of human dendritic cell function," *Journal of Virology*, vol. 82, no. 22, pp. 10975–10985, 2008.

[64] A. Merlo, C. Calcaterra, S. Ménard, and A. Balsari, "Cross-talk between Toll-like receptors 5 and 9 on activation of human immune responses," *Journal of Leukocyte Biology*, vol. 82, no. 3, pp. 509–518, 2007.

[65] S. Agrawal, A. Agrawal, B. Doughty et al., "Cutting Edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos," *Journal of Immunology*, vol. 171, no. 10, pp. 4984–4989, 2003.

[66] A. Didierlaurent, I. Ferrero, L. A. Otten et al., "Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response," *Journal of Immunology*, vol. 172, no. 11, pp. 6922–6930, 2004.

[67] J. T. Bates, S. Uematsu, S. Akira, and S. B. Mizel, "Direct stimulation of tlr5+/+ CD11c+ cells is necessary for the adjuvant activity of flagellin," *Journal of Immunology*, vol. 182, no. 12, pp. 7539–7547, 2009.

[68] E. A. Miao, C. M. Alpuche-Aranda, M. Dors et al., "Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1β via Ipaf," *Nature Immunology*, vol. 7, no. 6, pp. 569–575, 2006.

[69] K. L. Lightfield, J. Persson, S. W. Brubaker et al., "Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin," *Nature Immunology*, vol. 9, no. 10, pp. 1171–1178, 2008.

[70] D. S. Zamboni, K. S. Kobayashi, T. Kohlsdorf et al., "The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of Legionella pneumophila infection," *Nature Immunology*, vol. 7, no. 3, pp. 318–325, 2006.

[71] M. Vinzing, J. Eitel, J. Lippmann et al., "NAIP and Ipaf control Legionella pneumophila replication in human cells," *Journal of Immunology*, vol. 180, no. 10, pp. 6808–6815, 2008.

[72] C. L. Buzzo, J. C. Campopiano, L. M. Massis et al., "A novel pathway for inducible nitric-oxide synthase activation through inflammasomes," *Journal of Biological Chemistry*, vol. 285, no. 42, pp. 32087–32095, 2010.

[73] K. R. Bortoluci and R. Medzhitov, "Control of infection by pyroptosis and autophagy: role of TLR and NLR," *Cellular and Molecular Life Sciences*, vol. 67, no. 10, pp. 1643–1651, 2010.

[74] S. M. C. Newton, C. O. Jacob, and B. A. D. Stocker, "Immune response to cholera toxin epitope inserted in Salmonella flagellin," *Science*, vol. 244, no. 4900, pp. 70–72, 1989.

[75] J. McEwen, R. Levi, R. J. Horwitz, and R. Arnon, "Synthetic recombinant vaccine expressing influenza haemagluttinin epitope in Salmonella flagellin leads to partial protection in mice," *Vaccine*, vol. 10, no. 6, pp. 405–411, 1992.

[76] M. G. Luna, M. M. Martins, S. M. C. Newton, S. O. P. Costa, D. F. Almeida, and L. C. S. Ferreira, "Cloning and expression of colonization factor antigen I (CFA/I) epitopes of enterotogenic *Escherichia coli* (ETEC) in Salmonella flagellin," *Research in Microbiology*, vol. 148, no. 3, pp. 217–228, 1997.

[77] S. B. Mizel and J. T. Bates, "Flagellin as an adjuvant: cellular mechanisms and potential," *Journal of Immunology*, vol. 185, no. 10, pp. 5677–5682, 2010.

[78] J. W. Huleatt, V. Nakaar, P. Desai et al., "Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin," *Vaccine*, vol. 26, no. 2, pp. 201–214, 2008.

[79] L. Song, V. Nakaar, U. Kavita et al., "Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs," *PLoS ONE*, vol. 3, no. 5, article e2257, 2008.

[80] J. J. Treanor, D. N. Taylor, L. Tussey et al., "Safety and immunogenicity of a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125) in healthy young adults," *Vaccine*, vol. 28, no. 52, pp. 8268–8274, 2010.

[81] M. B. Barbedo, R. Ricci, M. C. S. Jimenez et al., "Comparative recognition by human IgG antibodies of recombinant proteins representing three asexual erythrocytic stage vaccine candidates of Plasmodium vivax," *Memorias do Instituto Oswaldo Cruz*, vol. 102, no. 3, pp. 335–339, 2007.

[82] I. S. Soares, J. W. Barnwell, M. U. Ferreira et al., "A Plasmodium vivax vaccine candidate displays limited allele polymorphism, which does not restrict recognition by antibodies," *Molecular Medicine*, vol. 5, no. 7, pp. 459–470, 1999.

[83] M. H. C. Rodrigues, M. G. Cunha, R. L. D. Machado, O. C. Ferreira, M. M. Rodrigues, and I. S. Soares, "Serological detection of Plasmodium vivax malaria using recombinant proteins corresponding to the 19-kDa C-terminal region of the merozoite surface protein-1," *Malaria Journal*, vol. 2, no. 1, pp. 1–7, 2003.

[84] S. Dutta, D. C. Kaushal, L. A. Ware et al., "Merozoite surface protein 1 of Plasmodium vivax induces a protective response against Plasmodium cynomolgi challenge in rhesus monkeys," *Infection and Immunity*, vol. 73, no. 9, pp. 5936–5944, 2005.

[85] D. Y. Bargieri, D. S. Rosa, C. J. M. Braga et al., "New malaria vaccine candidates based on the Plasmodium vivax Merozoite Surface Protein-1 and the TLR-5 agonist Salmonella Typhimurium FliC flagellin," *Vaccine*, vol. 26, no. 48, pp. 6132–6142, 2008.

[86] D. Y. Bargieri, J. A. Leite, S. C. P. Lopes et al., "Immunogenic properties of a recombinant fusion protein containing the C-terminal 19 kDa of Plasmodium falciparum merozoite surface protein-1 and the innate immunity agonist FliC flagellin of Salmonella Typhimurium," *Vaccine*, vol. 28, no. 16, pp. 2818–2826, 2010.

[87] C. J. M. Braga, L. M. Massis, M. E. Sbrogio-Almeida et al., "CD8+ T cell adjuvant effects of Salmonella FliCd flagellin in live vaccine vectors or as purified protein," *Vaccine*, vol. 28, no. 5, pp. 1373–1382, 2010.

[88] O. Pino, M. Martin, and S. M. Michalek, "Cellular mechanisms of the adjuvant activity of the flagellin component FljB of Salmonella enterica serovar typhimurium to potentiate mucosal and systemic responses," *Infection and Immunity*, vol. 73, no. 10, pp. 6763–6770, 2005.

[89] S. K. Datta, V. Redecke, K. R. Prilliman et al., "A subset of toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells," *Journal of Immunology*, vol. 170, no. 8, pp. 4102–4110, 2003.

[90] I. Vicente-Suarez, J. Brayer, A. Villagra, F. Cheng, and E. M. Sotomayor, "TLR5 ligation by flagellin converts tolerogenic dendritic cells into activating antigen-presenting cells that preferentially induce T-helper 1 responses," *Immunology Letters*, vol. 125, no. 2, pp. 114–118, 2009.

[91] C. J. Sanders, L. Franchi, F. Yarovinsky et al., "Induction of adaptive immunity by flagellin does not require robust activation of innate immunity," *European Journal of Immunology*, vol. 39, no. 2, pp. 359–371, 2009.

[92] M. Vijay-Kumar, F. A. Carvalho, J. D. Aitken, N. H. Fifadara, and A. T. Gewirtz, "TLR5 or NLRC4 is necessary and sufficient for promotion of humoral immunity by flagellin," *European Journal of Immunology*, vol. 40, no. 12, pp. 3528–3534, 2010.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 101848, 11 pages
doi:10.1155/2011/101848

Review Article

The Influence of MHC and Immunoglobulins A and E on Host Resistance to Gastrointestinal Nematodes in Sheep

C. Y. Lee,[1] K. A. Munyard,[1] K. Gregg,[1] J. D. Wetherall,[1] M. J. Stear,[2] and D. M. Groth[1]

[1] *Curtin Health Innovation Research Institute and Western Australian Biomedical Research Institute, Curtin University, Perth, WA 6845, Australia*
[2] *Department of Animal Production and Public Health, School of Veterinary Medicine, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK*

Correspondence should be addressed to D. M. Groth, d.groth@curtin.edu.au

Received 29 November 2010; Revised 17 February 2011; Accepted 18 February 2011

Academic Editor: Alvin A. Gajadhar

Gastrointestinal nematode parasites in farmed animals are of particular importance due to their effects on production. In Australia, it is estimated that the direct and indirect effects of parasite infestation cost the animal production industries hundreds of millions of dollars each year. The main factors considered by immunologists when studying gastrointestinal nematode infections are the effects the host's response has on the parasite, which immunological components are responsible for these effects, genetic factors involved in controlling immunological responses, and the interactions between these forming an interconnecting multilevel relationship. In this paper, we describe the roles of immunoglobulins, in particular IgA and IgE, and the major histocompatibility complex in resistance to gastrointestinal parasites in sheep. We also draw evidence from other animal models to support the involvement of these immune components. Finally, we examine how IgA and IgE exert their influence and how methods may be developed to manage susceptible animals.

1. Introduction

Gastrointestinal worm infestation is one of the major causes of reduced productivity in domestic sheep in tropical and temperate regions of the world. In common with other parasitic infections, there is a complex interaction between the host's innate and adaptive defence mechanisms and consequent adaptations by the parasite. An understanding of these interactions is essential for the development of sustainable strategies to minimise the impact of the parasite burden on the host. Analysis of the problem is made more difficult by the diversity of nematode species and strains that commonly infect sheep and the apparently variable manner in which sheep respond to these organisms.

Inherited factors play an important role in determining susceptibility to nematode infections. For example, over the past two decades, the Rylington Merino Project has selected sheep for resistance to nematodes on the basis of annual worm egg counts [1, 2]. Relative to a control flock, the selected flock now has sufficient inherited resistance to nematodes that anthelminthic chemicals are not required during the lambing season. Selective breeding has been successful in other research flocks [1, 3, 4] and many commercial farms. Resistant animals can be identified by measuring faecal egg counts (FECs) over the first year of life. Selection for nematode resistance is widely practised in Australia and New Zealand but less common in the rest of the world.

In Australia and New Zealand, the correlations between FEC and growth rate have been weak [5–7]. In contrast, in Europe, the correlations are strong [8–10] but have been shown to change over time. The differences may reflect the breed of sheep in the different regions, that is, Australian Merino, New Zealand Romney, Scottish Blackface, and Polish long wool sheep. Alternatively, the differences may be a consequence of the nematode community. In the two European FEC studies, egg counts were predominantly *Teladorsagia circumcincta* but in the Australian and New Zealand studies, *Haemonchus contortus* or *Trichostrongylus colubriformis* made a much greater contribution to egg

counts. Alternatively, the differences between Europe and Australasia could reflect the different husbandry conditions; European sheep generally reach sale weights at an earlier age. IgA and IgE responses have been associated with reduced egg counts, but IgE responses have been shown to develop more slowly and are associated with pathology [11].

Many studies have implicated variation within the major histocompatibility complex (MHC) as a determinant of host resistance and/or sensitivity to gastrointestinal parasitism in several species [12]. In addition, mucosal humoral responses to parasites have been implicated in mechanisms that restrict parasite growth and mediate the expulsion of worms [13]. In this paper, the roles of the MHC and immunoglobulin synthesis, especially IgA and IgE, are discussed with particular emphasis on nematode infections in sheep.

2. Role of Adaptive Immunity in Gastrointestinal Parasitic Infestation

Parasitic gastroenteritis is caused by nematodes that include species from the genera *Trichostrongylus*, *Teladorsagia*, *Haemonchus, Nematodirusi,* and *Cooperia* [14]. Infections usually arise from ingestion of parasite larvae or eggs from pasture, and it is well established that the presence of parasite antigens in the host's gastrointestinal system triggers innate immune responses, in addition to humoral and cell-mediated adaptive responses, with recruitment of T cells along the gastrointestinal mucosa [15, 16]. During an initial infection, dendritic cells take up and process parasite molecules. The dendritic cells then migrate to the draining lymph nodes and activate T cells, although additional interactions between antigen presenting cells and T cells may occur close to the site of uptake. In the small intestine, soluble antigens (metabolic or excretory-secretory components) are absorbed by specialised microfold cells in the follicle-associated epithelium overlying the Peyer's patches either through phagocytosis or pinocytosis [17]. Antigens are transported from the intestinal lumen to the subepithelial dome, where the antigen-presenting cells interact with T cells.

The importance of T lymphocytes, which regulate the host adaptive response against gastrointestinal parasites, has been demonstrated in several laboratory animal models, including *Trichinella spiralis*, *Heligmosomoides bakeri*, and *Strongyloides stercoralis* [12, 37, 38] and also in sheep infected with *Haemonchus contortus* [39]. However, it is also clear that adaptive immune responses to nematode parasites do not completely prevent subsequent infection, at least in most animals within a flock.

The three major manifestations of resistance to nematodes are reduced numbers of adult nematodes, decreased size of adult nematodes, and increased numbers of inhibited larvae, compared to susceptible contemporaries. However, not all resistant animals manifest all the three primary indicators, and the three indicators do not develop at the same rate [40, 41]. Large worms tend to lay more eggs [42] and are generally more pathogenic [11]. Reduced egg counts, increased expulsion of parasites, altered growth rates in resistant hosts, increased numbers of eosinophils, mast cells, plasma cells, and lymphocytes as well as increased concentrations of antibody are common secondary indicators in most nematode infections of sheep.

Much of the current knowledge concerning the mammalian immune response to parasites comes from studies on laboratory animals, particularly rodents. Experimental infections in rodents have provided valuable information for the analysis of immunological and genetic mechanisms that determine resistance to gastrointestinal nematode parasites [32, 43]. The demonstration that genetic factors influence resistance and susceptibility in mice allows the identification of genetic markers or genes that confer resistance [43]. Although the genes controlling resistance in different species are unlikely to be identical, many of the pathways are likely to be similar.

3. The Role of IgA in Nematode Resistance

In several host-parasite systems, parasite-specific IgA has been associated with resistance [44–48]. However, careful experimental design and interpretation are needed because IgA responses to nematode infection are correlated with IgE production, together with infiltration of eosinophils and mast cells and the subsequent degranulation of mast cells [49]. The mutual correlations could be a consequence of cytokines from Th2 cells, which recruit the relevant cells. Therefore, it is possible that increased IgA activity may be a marker of an increased mucosal immune response. IgA is not complement fixing and recently has been implicated in anti-inflammatory mechanisms [50]. Evidence for an active role is discussed below.

In mice, the humoral immune response has been reported to exert a direct effector role against gastrointestinal nematode parasites. Immunity against murine *Trichuris muris* has been achieved through monoclonal IgA antibody infusion that resulted in the expulsion of the parasites from the gastrointestinal tract [51]. The immune mechanism was thought to be through antibody binding directly to parasite excretion/secretion antigens [51].

Smith et al. [52] were the first to report a relationship between IgA response and reduced worm length following infection with *T. circumcincta.* They examined the length of all nematodes, including larval stages, to identify inhibited larvae. They found an increase in lymphatic IgA and IgA-positive cells in the gastric lymph. Pooling data across age classes produced an extremely strong correlation between the increased IgA response and increased numbers of inhibited larvae. A large study in naturally infected sheep supported this finding by showing that lambs with higher peripheral IgA activity against fourth-stage larvae showed inhibition of a higher proportion of larvae [53].

More recent data have cast doubt on the role of IgA in nematode inhibition [54]. Sheep were trickle-infected, then, challenged with 50,000 *T. circumcincta*. Parasite development ceased approximately five days after challenge and preceded the peak of IgA activity in the gastric lymph on day 9. The IgA response was apparently too slow to play a direct role in the inhibition of larval development. However, more research is necessary before firm conclusions can be made.

The relationship between IgA levels in the gastric lymph and IgA levels at the site of infection in the abomasal mucosa is unknown. In addition, there is density-dependent inhibition of larval development [55]. The mechanism of density-dependent inhibition may differ from that of immune-mediated inhibition, and the inhibition observed in this experiment may not have been immune mediated.

In contrast to the uncertain relationship between IgA level and numbers of inhibited larvae, the parasite-specific IgA response is consistently correlated with a reduction in adult worm length in infected animals. In Scottish Blackface sheep matched for age, sex, breed, farm of origin, and parasite exposure history, Stear et al. [49] observed considerable variation in the number of IgA-positive plasma cells and the activity of parasite-specific IgA in the abomasal mucosa. There was a negative correlation between IgA and worm length, which was stronger for mucosal IgA than for serum IgA. The correlations observed were also stronger against fourth-stage larvae (L4) than against third-stage larvae (L3). Recently, Henderson and Stear [56] showed a direct correlation between mucosal IgA and plasma IgA levels of 0.66. The negative correlation observed between parasite-specific IgA levels and worm length was likely to have been a direct effect of IgA on the parasite, rather than a change in the quantity of antibody produced in response to changes in worm number [49]. Similar correlations have been observed in Santa Ines, Suffolk, and Ile de France lambs infected by *H. contortus*, Scottish Blackface lambs infected by *H. contortus*, and Churra lambs infected with *T. circumcincta* [57–59]. In addition, Scottish Blackface lambs that were naturally infected with *T. circumcincta* have shown a similar relationship [53, 60].

Stear et al. [49] estimated that approximately 38% of nematode parasite worm length variation could be accounted for by mucosal IgA activity directed against L4 worms, a value considerably less than the over 90% estimated by Smith et al. [52]. However, the high value reported by Smith et al. may have been an artefact created by pooling data from sheep of different ages. The level of variation in nematode parasite worm length due to L4 parasite-specific IgA activity has been independently estimated as ~38% in Churra sheep [59], with similar estimates reported by Sinski et al. [61], Strain and Stear [57], Strain et al. [60], Stear et al. [53], Amarante et al. [58], and Henderson and Stear [56].

In addition to the effects of IgA, two other factors influence the size of adult nematodes: IgA specificity and worm density dependence. Variance analysis in sheep intentionally infected with *T. circumcincta* [53] indicated that these three components accounted for most of the variation in adult female worm length. This conclusion is consistent with the hypothesis that, in this host-parasite system, IgA is the major host mechanism influencing parasite growth and fecundity. In *Strongyloides ratti*, the density-dependent response is abolished in immunosuppressed rats [62], which suggests that density dependence is mediated through the immune system in at least some host-parasite systems.

There are several methods by which IgA could influence nematode growth. Parasitic nematodes release a variety of proteases that partially predigest proteins and may also break down antibodies and other mediators of host resistance. Antibodies against these enzymes or other molecules could inhibit enzyme activity and feeding by the parasite [63–67]. This appears to be a mechanism underlying the success of vaccination against H-Gal-GP (a galactose-containing glycoprotein complex purified from intestinal membranes of adult *H. contortus* worms) from *H. contortus* [68, 69]. Alternatively, IgA could interact with eosinophils to control nematode growth and fecundity (see below).

There does not appear to be a consistent association between IgA activity and the number of adult *T. circumcincta* [49]. There is also no consistent association with the number of *H. contortus* [70–72]. The absence of a relationship suggests that IgA activity does not determine worm numbers.

Hertzberg et al. [73] trickle infected White Alpine lambs with *Ostertagia leptospicularis* and showed that there was a gradual increase in serum IgA levels during infection. As expected from other species, IgA has a short half-life and IgA activity declined rapidly after anthelminthic treatment. When subsequently challenged with 100,000 infective L3 parasites, the serum IgA level rose rapidly but was observed to decrease earlier than either IgG1 or IgG2.

4. IgA and Eosinophilia

Variation in the number of mast cells, globule leucocytes, eosinophils, and IgA plasma cells has been observed in sheep that were infected with nematodes [49, 58]. Globule leucocytes are derived from subepithelial mast cells [74, 75]. Stear et al. [49] found that sheep with more mast cells had higher abomasal concentrations of globule leucocytes, eosinophils, IgA plasma cells, and more larval antigen-specific IgA antibody. Henderson and Stear [56] measured the level of IgA and eosinophil numbers in Scottish Blackface lambs over a period of 60 days after challenge and observed that both variables had similar response kinetics. IgA and eosinophil activity peaked at 8–10 days after infection and declined subsequently. Stear et al. [49] measured eosinophil numbers at the end of the experiment during necropsy of the animals while Henderson and Stear [56] measured mucosal eosinophilia over a 60-day period. A similar study using Caribbean hair sheep and wool sheep [19] found that the hair breed had higher serum levels of IgA and IgE in uninfected sheep, and that there were significant differences in IgA, IgE, and tissue eosinophils levels between the two sheep breeds which was negatively correlated with worm counts. IgA levels accounted for 38% and eosinophil numbers 40% of the variation in worm length, respectively. In correlation studies that analysed the two variables together, the combination accounted for 53% of worm length variation. Therefore, it appears that IgA and eosinophilia have a combined or synergistic effect on worm length [56]. Eosinophils have been shown to express receptors for IgA [76, 77], which can be activated by binding of parasite antigen/IgA to IgA cell surface receptors [78]. Therefore, IgA could help target eosinophils to nematodes. Interestingly, eosinophils in mice lack receptors for IgA [76], and this could explain the relative ineffectiveness of eosinophils in some murine models [79, 80].

5. The Role of IgE in Nematode Resistance

Increased numbers of mast cells is a hallmark of many nematode infections, and they have been implicated in the control of worm numbers in some but not all infections. For example, mast cells appear crucial for the control of *Trichinella spiralis* but not for *Trichuris muris* or *Nippostrongylus brasiliensis* [81]. Sheep that are resistant to *T. circumcincta* have increased numbers of mast cells or globule leucocytes compared to more susceptible contemporaries [49]. Similarly, mast cells are important for resistance to *H. contortus* [82, 83].

As binding of parasite molecules by cell-surface IgE is the major trigger for mast cell degranulation, IgE is implicated by default in resistance to nematode infection. An association between high plasma IgE activity against a high-molecular-weight allergen and low egg counts was reported in 20 lambs selected from a group of 72 naturally infected crossbred sheep [84]. A study using lymphatic cannulation to allow continuous assessment of the migrating immune cells from the intestinal mucosa and mesenteric lymph nodes showed differential changes in the expression of IL-5 in the afferent intestinal lymph in two lines of sheep selected for susceptibility or resistance to *T. colubriformis* [85]. Furthermore, in a parallel study by the same group, the resistant line had higher IgE in lymph than the susceptible line [86]. Naturally infected Texel lambs with high IgE activity against recombinant tropomyosin from *T. circumcincta* also had lower egg counts than lambs with lower IgE responses [87]. An independent study from New Zealand also showed an association between increased IgE activity against an aspartyl protease inhibitor from *T. colubriformis* and reduced egg counts [88].

6. Genetic Factors in Gastrointestinal Parasite Immunity

Quantitative genetic analysis in sheep and cattle has clearly shown that resistance to nematode infection is under genetic control [2, 89–93]. The heritability of a single egg count varies among populations but is usually between 0.2 and 0.4 in animals that have been previously exposed to infection [94]. This is similar to the heritability of milk production in dairy cattle or growth rate in beef cattle and indicates the feasibility of selective breeding [95]. Quantitative trait loci (QTLs) for resistance to the intestinal nematode *Heligmosomoides polygyrus* were located on mouse chromosomes 1, 2, 8, 13, 17, and 19 by Iraqi et al. [32]. Interestingly, one chromosomal region identified by these researchers was the MHC located on mouse chromosome 17. Their observations were confirmed independently by Behnke et al. [33] who found associations between eight immunological traits (FEC at weeks 2, 4, and 6, mucosal mast cell protease 1, granuloma score, IgG1 against L5, and IgG1, and IgE to L4) and QTLs on chromosome 1 and 17 associated with resistance to the *H. polygyrus* infection. More specifically, the MHC genes, most notably, the class II and TNF regions were significantly associated with gastrointestinal parasite infection.

Davies et al. [29] provided evidence of QTLs located on sheep chromosomes 2, 3, 14, and 20 conferring resistance to infection with *T. circumcincta* in Scottish Blackface sheep. Analysis of chromosome 20 showed that the MHC region had a statistically significant association with gastrointestinal nematode parasite resistance. QTLs associated with specific IgA activity against nematode parasites were also located on chromosomes 3 and 20. Alleles of the *DRB1* in the MHC class II region have been associated with nematode resistance in several different breeds of sheep [23–25, 96] and cattle [90, 97, 98]. However, in contrast, Beh et al. [99] found no significant linkage of the MHC in sheep resistance to *Trichostrongylus colubriformis*. Unfortunately, their study used only a single marker to represent the MHC region and chromosome 20 in their whole-genome linkage analysis. Beh et al. [99] also applied an additional two markers to a single-point ANOVA and confirmed no linkage to the MHC region. In another linkage study, no significant QTL was found on chromosome 20, for resistance to parasitic nematode infection in sheep [100]. In this study, only four markers were used to represent chromosome 20, of which only two mapped to the MHC region [100]. Recently, a more extensive whole-genome QTL analysis for resistance to *H. contortus* showed, in one family, weak linkage between egg counts and the *Ovar-DYA* region in the MHC class IIb region [101], consistent with a previous report that associated this region with resistance to *T. circumcincta* [26].

7. The Influence of the MHC on Antibody Production

The role of MHC in controlling IgA concentrations is supported by several human studies, especially on IgA and combined variable immunodeficiency (CVID). One of the first studies that identified an association between IgA deficiency and the MHC region was by Wilton et al. [102], who found an association between MHC class III genes and IgA deficiency. An increase in frequency of certain HLA haplotypes was observed in deficient patients [102, 103]. A number of studies have since focused on the *HLA-A1-B8-DR3* haplotype to locate the IgA deficiency locus [104, 105]. An investigation of the *HLA-DR3*-extended haplotype showed that in the Sardinian population, where a lower prevalence of IgA deficiency exists, the *HLA-DR3-B18* haplotype is more common than the *HLA-DR3-B8* haplotype, suggesting that the IgA deficiency susceptibility gene is located in the more common Northern European DR3-B8 haplotypes [106]. The investigation of features common to the different haplotypes was used to establish the region associated with IgA deficiency, and thus far several different studies have placed the susceptibility locus between the class III region [103, 105, 107, 108] and the class II region [109–111].

Polymorphisms in *MSH5* have also been shown to be associated with CVID and IgA deficiency in a mouse model and through statistical analysis of human populations [112]. This gene, located within the MHC class III region, is involved in DNA mismatch repair as well as in resolving Holliday junctions that form between homologous DNA strands during meiosis [113, 114]. However, Guikema et al. [115] observed a large variety of splice variants of MSH5 mRNA (all of which are unlikely to be stable) and suggested

that *MSH5* was nonfunctional and therefore probably does not participate in Ig class switching. Recently, it has been shown that haplotypes of *MSH5* are associated with IgA deficiency [116, 117] but are not likely to be the causative mutations [117].

8. Mechanisms Underlying the MHC Association with Nematode Resistance

Genetic variation in the mouse MHC has long been associated with resistance to nematode infection [118] and with the specificity of antibody responses [119]. It has been reported that the helminth *Nippostrongylus brasiliensis* may possibly be able to suppress MHC class II molecule expression as an evasive mechanism [120]. Likewise, for sheep, it has been shown that the parasite *T. colubriformis* seems to be capable of downregulating several immune genes, particularly *DRB1* and *DRA*, in afferent lymph migratory cells [121]. In the mouse model infected with *Strongyloides venezuelensis*, class II $^{-/-}$ animals were more susceptible to infection (based on increase in FEC and elimination of worms) than wild-type and class I $^{-/-}$ mice [31]. In addition, parasite-specific IgM, IgA, and IgG were also significantly reduced in class II $^{-/-}$ mice. This study concluded that class II MHC expression was essential to induce a Th2 response against *S. venezuelensis* infection and class I expression was not [31]. Interestingly and somewhat contradictory to the findings discussed above [121], it has been shown that mice strains that lack *I-E*, a homologue of *DRB1*, in their MHC class II region are more resistant [122].

In a comparative study using bovine cDNA microarray analysis of duodenum tissue from an outbred population of resistant and susceptible lambs (which had been subjected to two natural challenges with a range of gastrointestinal parasites), increased expression was observed in a range of genes [18]. Upregulated genes included *DQB1*, *DRA*, and *DQA1* from the MHC class II region [18]. This observation highlights key differences between resistant and susceptible animals in the early immune response to gastrointestinal nematodes. In a separate microarray study, differences were observed in gene expression profiles of hair and wool sheep that had been infected with *H. contortus* [19]. Elevated expression of the MHC class II DM β-chain precursor gene was observed in lymph node tissue of the wool breed. However, no significant change in the expression of this or any other MHC-related gene was observed in abomasal tissue [19]. In another study, using transcriptional profiling of duodenum tissue samples from resistant and susceptible sheep [20], up-regulation of MHC class II genes *Ovar DQA1*, *Ovar DQB1*, and *Ovar DRA* was observed in resistant animals. Subsequent RT-PCR analysis of *Ovar DQA1* showed an average 8.4-fold greater expression in resistant animals than in susceptible animals. Further analysis using GO terms highlighted the significant association between genes highly expressed in resistant animals with terms such as MHC class II activity and exogenous antigen processing and presentation [20]. Furthermore, the frequency of *Ovar DQA1* haplotypes differed between animals from the resistant and susceptible selection lines, with an increase in *Ovar DQA1*Null* in susceptible animals from both Perendale and Romney sheep lines. In Perendale sheep, the frequency of *Ovar DQA1*0101* and *DQA1*0402* alleles was increased in resistant animals and *Ovar DQA1*0103* increased in the susceptible line. However, these observations seemingly contradict earlier findings by the same group, in which no increase was observed in the expression of either MHC class II genes nor any association was found with antigen presentation or processing [123]. Interestingly, a significant increase in expression of a MHC class I gene (*HLA-A* orthologue) in resistant animals was also observed, indicating possible crosstalk between the different responses. Recently, Forrest and colleagues [21] conversely demonstrated no evidence of an interbreed effect of the *Ovar-DQA1*Null* allele on total faecal egg counts. However, the *Ovar-DQA1*Null* appeared to have a significant effect when the analysis was performed within breeds [21].

In a statistical examination of the relationship between MHC polymorphism and parasitological traits in Scottish Blackface sheep, the resistant allele G2 at the DRB1 locus was significantly associated with decreased egg counts and decreased numbers of adult *T. circumcincta* [96]. However, no apparent correlation was observed with adult female parasite length. Hence, the mechanism by which the MHC influences egg counts may operate through the control of worm number and not by controlling nematode fecundity. There are several possible mechanisms but possibly specific class II molecules direct responses to specific peptides, and these responses may play a direct role in protection.

Another possibility is that the observed associations in livestock are a consequence of heterozygote advantage [96]. Heterozygote advantage has complex effects on the power of statistical analyses to detect specific allele effects [27]. As the frequency of an allele increases in a population, an increasing proportion of homozygous sheep will be present and thus the average effect of the specific allele will decline. Also, an allele that is very rare in a population will be present in too few animals to show a significant effect. Conversely, when the allele is very common in the population, its average effect is quite small making its contribution to reduced egg counts difficult to detect. Consequently, only alleles within a narrow frequency range will show effects on parasite resistance. Interestingly, the allele most strongly associated with resistance in Scottish Blackface sheep fell within the narrow detection window, and the most common allele was also associated with the most susceptible animals as predicted by heterozygote advantage. There was also more direct evidence: Heterozygous sheep had lower egg counts following natural *T. circumcincta* infection [96].

Heterozygote advantage is a particularly appealing mechanism for explaining the IgE response to parasites. The specificity of IgE responses is relatively unimportant for mast cell degranulation if the target molecule is soluble and large enough to promote cross-linking of IgE receptors. Therefore, a heterozygote advantage that leads to increased IgE concentrations is more supported than a model of determinant selection (i.e., a direct role of the allele in determining levels of IgE).

Table 1: Summary of studies that have implicated the MHC in resistance to gastointestinal parasites.

Species	Parasite species	Method	MHC association	Reference
Sheep (*Ovis aries*)	mixed	Microarray	*DQB1, DRA, DQA1*	[18]
	H. contortus	Microarray	*DMB*	[19]
	mixed	Microarray	*DQA1*Null, DQB1, DRA*	[20]
	mixed	PCR analysis	*DQA1*Null*	[21]
	mixed	PCR/sequencing	*DQA1*0101, DQA1*0402*	[20]
	mixed	PCR/sequencing	*DRB1*	[22, 23]
	mixed	PCR	*DRB microsatellite*	[23]
	Teladorsagia circumcincta	PCR/sequencing	*DRB1*	[24–27]
	H. contortus	PCR/sequencing	*DRB1, OMHC1*	[28]
	Teladorsagia circumcincta	Linkage	Class IIb region	[29]
Sheep (*Ovis canadensis*)	*n/a*	Population analysis PCR/sequencing	*DRB1*	[30]
Mouse (*Mus musculus*)	*S. venezuelensis*	Knock out	Class II	[31]
	H. polygyrus	Linkage	Class II region	[32, 33]
Striped mouse (*Rhabdomys pumilio*)	mixed	PCR/sequencing	*DRB*	[34]
Yellow necked mouse (*Apodemus flavicollis*)	mixed	PCR/sequencing	*DRB*	[35]
Gray mouse lemur (*Microcebusmurinus*)	mixed	PCR/sequencing	*DRB*	[36]

Charbonnel and Pemberton [124] examined both MHC and neutral loci in free-living Soay sheep that were infected by *T. circumcincta* in St Kilda (Scotland). Over eight years, lower levels of temporal genetic differentiation were observed at MHC loci compared with neutral loci, consistent with balancing selection activity at the MHC loci [124]. These observations confirmed earlier work by Paterson [125] but have not been supported by subsequent research [126]. Significant studies showing positive associations between genes within the MHC and gastrointestinal parasites are summarised in Table 1.

9. Conclusions

There is no single mechanism of nematode resistance in sheep. Resistance to gastrointestinal nematodes involves the control of worm growth as well as worm numbers. The negative correlation between parasite-specific IgA levels and worm length has been well established by many research groups in different breeds of sheep infected by different gastrointestinal parasites. The control of worm numbers involves mast cells in some but not all host-nematode systems. There is a genetic component to nematode resistance, and the MHC is one of the most important components of genetic resistance. QTL analyses have shown a link between the MHC region and FEC in mouse models, as well as in sheep and cattle. The influence of the class II region on parasite resistance has been shown in experimental models as well as by microarray analysis.

Despite the large number of studies that confirmed these relationships, there are other studies in which contradictory results reject these hypotheses. However, correlation studies may generate a complex heterogeneity of results because of the large variety of gastrointestinal nematode parasites and differences in environmental conditions, nutritional status of animals, and geographical locations. Another complication is that the relationship between gene expression from the MHC region, IgA activity, and their effects on parasites is often considered individually rather than as interconnecting multilevel interactions.

Acknowledgment

M. J. Stear thanks the BBSRC, QMS, and Genesis/Faraday for support.

References

[1] L. J. E. Karlsson and J. C. Greeff, "Selection response in fecal worm egg counts in the Rylington Merino parasite resistant flock," *Australian Journal of Experimental Agriculture*, vol. 46, no. 6-7, pp. 809–811, 2006.

[2] K. E. Kemper, D. G. Palmer, S. M. Liu, J. C. Greeff, S. C. Bishop, and L. J. E. Karlsson, "Reduction of faecal worm egg count, worm numbers and worm fecundity in sheep selected for worm resistance following artificial infection with *Teladorsagia circumcincta* and *Trichostrongylus colubriformis*," *Veterinary Parasitology*, vol. 171, pp. 238–246, 2010.

[3] C. A. Morris, T. G. Watson, S. A. Bisset, A. Vlassoff, and P. G. C. Douch, "Breeding sheep in New Zealand for resistance or resilience to nematode parasites," in *Breeding for Resistance to Infectious Diseases of Small Ruminants*, G. D. Gray, R. R. Woolaston, and B. D. Eaton, Eds., pp. 77–98, ACIAR Monograph, Canberra, Australia, 2000.

[4] R. R. Woolaston and R. G. Windon, "Selection of sheep for response to *Trichostrongylus colubriformis* colubriformis larvae: genetic parameters," *Animal Science*, vol. 73, no. 1, pp. 41–48, 2001.

[5] J. C. McEwan, K. G. Dodds, G. J. Greer et al., "Genetic estimates for parasite resistance traits in sheep and their correlations with production traits," *New Zealand Journal of Zoology*, vol. 22, p. 177, 1995.

[6] S. A. Bisset, A. Vlassoff, C. A. Morris, B. R. Southey, R. L. Baker, and A. G. H. Parker, "Heritability of and genetic correlations among faecal egg counts and productivity traits in Romney sheep," *New Zealand Journal of Agricultural Research*, vol. 35, pp. 51–58, 1992.

[7] S. J. Eady, R. R. Woolaston, R. P. Lewer, H. W. Raadsma, A. A. Swan, and R. W. Ponzoni, "Resistance to nematode parasites in Merino sheep: correlation with production traits," *Australian Journal of Agricultural Research*, vol. 49, no. 8, pp. 1201–1211, 1998.

[8] S. C. Bishop, K. Bairden, Q. A. McKellar, M. Park, and M. J. Stear, "Genetic parameters for faecal egg count following mixed, natural, predominantly *Ostertagia circumcincta* infection and relationships with live weight in young lambs," *Animal Science*, vol. 63, no. 3, pp. 423–428, 1996.

[9] M. J. Stear, K. Bairden, J. L. Duncan et al., "How hosts control worms," *Nature*, vol. 389, no. 6646, p. 27, 1997.

[10] J. Bouix, J. Krupinski, R. Rzepecki et al., "Genetic resistance to gastrointestinal nematode parasites in polish long-wool sheep," *International Journal for Parasitology*, vol. 28, no. 11, pp. 1797–1804, 1998.

[11] M. J. Stear, S. C. Bishop, N. G. Henderson, and I. Scott, "A key mechanism of pathogenesis in sheep infected with the nematode *Teladorsagia circumcincta*," *Animal Health Research Reviews*, vol. 4, no. 1, pp. 45–52, 2003.

[12] J. M. Behnke, F. Iraqi, D. Menge, R. L. Baker, J. Gibson, and D. Wakelin, "Chasing the genes that control resistance to gastrointestinal nematodes," *Journal of Helminthology*, vol. 77, no. 2, pp. 99–109, 2003.

[13] W. I. Khan, "Physiological changes in the gastrointestinal tract and host protective immunity: learning from the mouse-*Trichinella spiralis* model," *Parasitology*, vol. 135, no. 6, pp. 671–682, 2008.

[14] G. Mulcahy, S. O'Neill, S. Donnelly, and J. P. Dalton, "Helminths at mucosal barriers—interaction with the immune system," *Advanced Drug Delivery Reviews*, vol. 56, no. 6, pp. 853–868, 2004.

[15] D. W. K. Acheson and S. Luccioli, "Microbial-gut interactions in health and disease. Mucosal immune responses," *Best Practice and Research Clinical Gastroenterology*, vol. 18, pp. 387–404, 2004.

[16] R. M. Maizels and M. Yazdanbakhsh, "Immune regulation by helminth parasites: cellular and molecular mechanisms," *Nature Reviews Immunology*, vol. 3, no. 9, pp. 733–744, 2003.

[17] C. A. Janeway, P. Travers, M. Walport, and M. Schlomchik, *Immunobiology: The Immune System in Health and Disease*, Churchill Levingstone, Edinburgh, UK, 2001.

[18] C. Diez-Tascón, O. M. Keane, T. Wilson et al., "Microarray analysis of selection lines from outbred populations to identify genes involved with nematode parasite resistance in sheep," *Physiological Genomics*, vol. 21, pp. 59–69, 2005.

[19] K. M. MacKinnon, J. L. Burton, A. M. Zajac, and D. R. Notter, "Microarray analysis reveals difference in gene expression profiles of hair and wool sheep infected with *Haemonchus contortus*," *Veterinary Immunology and Immunopathology*, vol. 130, no. 3-4, pp. 210–220, 2009.

[20] O. M. Keane, K. G. Dodds, A. M. Crawford, and J. C. McEwan, "Transcriptional profiling of *Ovis aries* identifies *Ovar-DQA1* allele frequency differences between nematode-resistant and susceptible selection lines," *Physiological Genomics*, vol. 30, no. 3, pp. 253–261, 2007.

[21] R. H.J. Forrest, H. Zhou, Q. Fang, A. Smyth, C. M. Frampton, and J. G.H. Hickford, "No evidence for a universal association between variation in faecal egg count for a mixed field-challenge of gastrointestinal parasites and the presence of the *Ovar-DQA1* null haplotype in sheep," *Veterinary Immunology and Immunopathology*, vol. 135, no. 3-4, pp. 303–305, 2010.

[22] S. Paterson, K. Wilson, and J. M. Pemberton, "Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (*Ovis aries* L.)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 7, pp. 3714–3719, 1998.

[23] P. M. Outteridge, L. Andersson, P. G. C. Douch et al., "The PCR typing of MHC-DRB genes in the sheep using primers for an intronic microsatellite: application to nematode parasite resistance," *Immunology and Cell Biology*, vol. 74, no. 4, pp. 330–336, 1996.

[24] F. W. Schwaiger, D. Gostomski, M. J. Stear et al., "An ovine major histocompatibility complex DRB1 allele is associated with low faecal egg counts following natural, predominantly *Ostertagia circumcincta* infection," *International Journal for Parasitology*, vol. 25, no. 7, pp. 815–822, 1995.

[25] G. Sayers, B. Good, J. P. Hanrahan, M. Ryan, J. M. Angles, and T. Sweeney, "Major Histocompatibility Complex DRB1 gene: its role in nematode resistance in Suffolk and Texel sheep breeds," *Parasitology*, vol. 131, no. 3, pp. 403–409, 2005.

[26] J. Buitkamp, P. Filmether, M. J. Stear, and J. T. Epplen, "Class I and class II major histocompatibility complex alleles are associated with faecal egg counts following natural, predominantly Ostertagia ciucumcincta infection," *Parasitology Research*, vol. 82, no. 8, pp. 693–696, 1996.

[27] M. J. Stear, A. Belch, K. Donskow-Schmelter et al., "Detection of genes with moderate effects on disease resistance using ovine mhc and resistance to nematodes as an example," *Veterinary Immunology and Immunopathology*, vol. 120, no. 1-2, pp. 3–9, 2007.

[28] J. A. Figueroa Castillo, R. D.M. Medina, J. M.B. Villalobos et al., "Association between major histocompatibility complex microsatellites, fecal egg count, blood packed cell volume and blood eosinophilia in Pelibuey sheep infected with *Haemonchus contortus*," *Veterinary Parasitology*. In press.

[29] G. Davies, M. J. Stear, M. Benothman et al., "Quantitative trait loci associated with parasitic infection in Scottish Blackface sheep," *Heredity*, vol. 96, no. 3, pp. 252–258, 2006.

[30] G. A. Gutierrez-Espeleta, P. W. Hedrick, S. T. Kalinowski, D. Garrigan, and W. M. Boyce, "Is the decline of desert bighorn sheep from infectious disease the result of low MHC variation?" *Heredity*, vol. 86, no. 4, pp. 439–450, 2001.

[31] R. M. Rodrigues, N. M. Silva, A. L.R. Gonçalves et al., "Major histocompatibility complex (MHC) class II but not MHC class i molecules are required for efficient control of *Strongyloides venezuelensis* infection in mice," *Immunology*, vol. 128, no. 1, pp. e432–e441, 2009.

[32] F. A. Iraqi, J. M. Behnke, D. M. Menge et al., "Chromosomal regions controlling resistance to gastro-intestinal nematode infections in mice," *Mammalian Genome*, vol. 14, no. 3, pp. 184–191, 2003.

[33] J. M. Behnke, F. A. Iraqi, J. M. Mugambi et al., "High resolution mapping of chromosomal regions controlling resistance to gastrointestinal nematode infections in an advanced intercross line of mice," *Mammalian Genome*, vol. 17, no. 6, pp. 584–597, 2006.

[34] G. Froeschke and S. Sommer, "MHC class II DRB variability and parasite load in the striped mouse (Rhabdomys pumilio) in the Southern Kalahari," *Molecular Biology and Evolution*, vol. 22, no. 5, pp. 1254–1259, 2005.

[35] Y. Meyer-Lucht and S. Sommer, "MHC diversity and the association to nematode parasitism in the yellow-necked mouse (*Apodemus flavicollis*)," *Molecular Ecology*, vol. 14, no. 7, pp. 2233–2243, 2005.

[36] J. Schad, J. U. Ganzhorn, and S. Sommer, "Parasite burden and constitution of major histocompatibility complex in the Malagasy mouse lemur, *Microcebus murinus*," *Evolution*, vol. 59, no. 2, pp. 439–450, 2005.

[37] R. K. Grencis, J. Riedlinger, and D. Wakelin, "L3T4-positive T lymphoblasts are responsible for transfer of immunity to *Trichinella spiralis* in mice," *Immunology*, vol. 56, no. 2, pp. 213–218, 1985.

[38] H. L. Rotman, S. Schnyder-Candrian, P. Scott, T. J. Nolan, G. A. Schad, and D. Abraham, "IL-12 eliminates the Th-2 dependent protective immune response of mice to larval Strongyloides stercoralis," *Parasite Immunology*, vol. 19, no. 1, pp. 29–39, 1997.

[39] H. S. Gill, D. L. Watson, and M. R. Brandon, "Monoclonal antibody to $CD4^+$ T cells abrogates genetic resistance to *Haemonchus contortus* in sheep," *Immunology*, vol. 78, no. 1, pp. 43–49, 1993.

[40] M. J. Stear, S. Strain, and S. C. Bishop, "How lambs control infection with *Ostertagia circumcincta*," *Veterinary Immunology and Immunopathology*, vol. 72, no. 1-2, pp. 213–218, 1999.

[41] M. J. Stear, S. Strain, and S. C. Bishop, "Mechanisms underlying resistance to nematode infection," *International Journal for Parasitology*, vol. 29, no. 1, pp. 51–56, 1999.

[42] M. J. Stear and S. C. Bishop, "The curvilinear relationship between worm length and fecundity of *Teladorsagia circumcincta*," *International Journal for Parasitology*, vol. 29, no. 5, pp. 777–780, 1999.

[43] D. Wakelin, "Rodent models of genetic resistance to parasite infections," in *Breeding for Disease Resistance in Farm Animals*, R. E. F. Axford, S. C. Bishop, F. W. Nicholas, and J. B. Owen, Eds., pp. 107–126, CAB International, Wallingford, Wash, USA, 2nd edition, 2000.

[44] K. Kanobana, H. W. Ploeger, and L. Vervelde, "Immune expulsion of the trichostrongylid *Cooperia oncophora* is associated with increased eosinophilia and mucosal IgA," *International Journal for Parasitology*, vol. 32, no. 11, pp. 1389–1398, 2002.

[45] T. Inaba, H. Sato, and H. Kamiya, "Monoclonal IgA antibody-mediated expulsion of Trichinella from the intestine of mice," *Parasitology*, vol. 126, no. 6, pp. 591–598, 2003.

[46] C. P. Wilkes, C. Bleay, S. Paterson, and M. E. Viney, "The immune response during a *Strongyloides ratti* infection of rats," *Parasite Immunology*, vol. 29, no. 7, pp. 339–346, 2007.

[47] S. Yanming, Y. Ruofeng, C. I. Muleke, Z. Guangwei, X. Lixin, and L. Xiangrui, "Vaccination of goats with recombinant galectin antigen induces partial protection against *Haemonchus contortus* infection," *Parasite Immunology*, vol. 29, no. 6, pp. 319–326, 2007.

[48] K. Matsuzawa, M. Abe, T. Shirakura, W. H. Zhao, and F. Nakamura, "Spontaneous worm expulsion and intestinal IgA response in mice infected by *Vampirolepis nana*," *Parasitology International*, vol. 57, no. 4, pp. 512–514, 2008.

[49] M. J. Stear, S. C. Bishop, M. Doligalska et al., "Regulation of egg production, worm burden, worm length and worm fecundity by host responses in sheep infected with *Ostertagia circumcincta*," *Parasite Immunology*, vol. 17, no. 12, pp. 643–652, 1995.

[50] M. E. Lamm, "Interaction of antigens and antibodies at mucosal surfaces," *Annual Review of Microbiology*, vol. 51, pp. 311–340, 1997.

[51] T. I. A. Roach, K. J. Else, D. Wakelin, D. J. McLaren, and R. K. Grencis, "*Trichuris muris*: antigen recognition and transfer of immunity in mice by IgA monoclonal antibodies," *Parasite Immunology*, vol. 13, no. 1, pp. 1–12, 1991.

[52] W. D. Smith, F. Jackson, E. Jackson, and J. Williams, "Age immunity to *Ostertagia circumcincta*: comparison of the local immune responses of 4 1/2—and 10-month-old lambs," *Journal of Comparative Pathology*, vol. 95, no. 2, pp. 235–245, 1985.

[53] M. J. Stear, K. Bairden, G. T. Innocent, S. Mitchell, S. A. J. Strain, and S. C. Bishop, "The relationship between IgA activity against fourth-stage larvae and density-dependent effects on the number of fourth-stage larvae of *Teladorsagia circumcincta* in naturally infected sheep," *Parasitology*, vol. 129, pp. 363–369, 2004.

[54] A. M. Halliday, C. M. Routledge, S. K. Smith, J. B. Matthews, and W. D. Smith, "Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep," *Parasite Immunology*, vol. 29, no. 8, pp. 425–434, 2007.

[55] J. D. Dunsmore, "Retarded development of *Ostertagia species* in sheep," *Nature*, vol. 186, no. 4729, pp. 986–987, 1960.

[56] N. G. Henderson and M. J. Stear, "Eosinophil and IgA responses in sheep infected with *Teladorsagia circumcincta*," *Veterinary Immunology and Immunopathology*, vol. 112, no. 1-2, pp. 62–66, 2006.

[57] S. A. J. Strain and M. J. Stear, "The influence of protein supplementation on the immune response to *Haemonchus contortus*," *Parasite Immunology*, vol. 23, no. 10, pp. 527–531, 2001.

[58] A. F. T. Amarante, P. A. Bricarello, J. F. Huntley, L. P. Mazzolin, and J. C. Gomes, "Relationship of abomasal histology and parasite-specific immunoglobulin A with the resistance to *Haemonchus contortus* infection in three breeds of sheep," *Veterinary Parasitology*, vol. 128, no. 1-2, pp. 99–107, 2005.

[59] M. Martínez-Valladares, M. P. Vara-Del Río, M. A. Cruz-Rojo, and F. A. Rojo-Vázquez, "Genetic resistance to *Teladorsagia circumcincta*: IgA and parameters at slaughter in Churra sheep," *Parasite Immunology*, vol. 27, no. 6, pp. 213–218, 2005.

[60] S. A. J. Strain, S. C. Bishop, N. G. Henderson et al., "The genetic control of IgA activity against *Teladorsagia circumcincta* and its association with parasite resistance in naturally infected sheep," *Parasitology*, vol. 124, no. 5, pp. 545–552, 2002.

[61] E. Sinski, K. Bairden, J. L. Duncan et al., "Local and plasma antibody responses to the parasitic larval stages of the abomasal nematode *Ostertagia circumcincta*," *Veterinary Parasitology*, vol. 59, no. 2, pp. 107–118, 1995.

[62] S. Paterson and M. E. Viney, "Host immune responses are necessary for density dependence in nematode infections," *Parasitology*, vol. 125, no. 3, pp. 283–292, 2002.

[63] A. C. Chandler, "Experiments on resistance of rats to superinfection with the nematode, *Nippostrongylus muris*," *American Journal of Epidemiology*, vol. 16, no. 3, pp. 750–782, 1932.

[64] A. C. Chandler, "Studies on the nature of immunity to intestinal helminths. I. The local nature of the immunity of white rats to Nippostrongylus infection," *American Journal of Epidemiology*, vol. 22, pp. 157–168, 1935.

[65] A. C. Chandler, "Studies on the nature of immunity to intestinal helminths. II. A study of the correlation between degree of resistance of white rats to Nippostrongylus and interval between infections," *American Journal of Epidemiology*, vol. 22, pp. 243–256, 1935.

[66] A. C. Chandler, "Studies on the nature of immunity to intestinal helminths. III. Renewal of growth and egg production in Nippostrongylus after transfer from immune to non-immune rats," *American Journal of Epidemiology*, vol. 23, pp. 46–54, 1936.

[67] A. C. Chandler, "Studies on the nature of immunity to intestinal helminths. IV. The inter-relationships between parenteral and intestinal immunity in rats infected with Nippostrongylus," *American Journal of Epidemiology*, vol. 24, pp. 129–144, 1936.

[68] D. P. Knox, D. L. Redmond, G. F. Newlands, P. J. Skuce, D. Pettit, and W. D. Smith, "The nature and prospects for gut membrane proteins as vaccine candidates for *Haemonchus contortus* and other ruminant trichostrongyloids," *International Journal for Parasitology*, vol. 33, no. 11, pp. 1129–1137, 2003.

[69] S. e. Ekoja and W. D. Smith, "Antibodies from sheep immunized against *Haemonchus contortus* with H-gal-GP inhibit the haemoglobinase activity of this protéase complex," *Parasite Immunology*, vol. 32, pp. 731–738, 2010.

[70] V. M. Bowles, M. R. Brandon, and E. Meeusen, "Characterization of local antibody responses to the gastrointestinal parasite *Haemonchus contortus*," *Immunology*, vol. 84, no. 4, pp. 669–674, 1995.

[71] H. D. F. H. Schallig, M. A. W. Van Leeuwen, and W. M. L. Hendrikx, "Isotype-specific serum antibody responses of sheep to *Haemonchus contortus* antigens," *Veterinary Parasitology*, vol. 56, no. 1-3, pp. 149–162, 1995.

[72] M. T. Gómez-Muñoz, M. Cuquerella, L. A. Gómez-Iglesias et al., "Serum antibody response of Castellana sheep to *Haemonchus contortus* infection and challenge: relationship to abomasal worm burdens," *Veterinary Parasitology*, vol. 81, no. 4, pp. 281–293, 1999.

[73] H. Hertzberg, H. D. F. H. Schallig, and P. Deplazes, "Development of a protective immunity against *Ostertagia leptospicularis* in trickle-infected sheep and parallel changes of serum gastrin, pepsinogen and antibody levels," *Veterinary Journal*, vol. 157, no. 2, pp. 148–159, 1999.

[74] M. Murray, H. R. P. Miller, and W. F. H. Jarrett, "The globule leukocyte and its derivation from the subepithelial mast cell," *Laboratory Investigation*, vol. 19, pp. 222–234, 1968.

[75] J. F. Huntley, B. McGorum, G. F. J. Newlands, and H. R. P. Miller, "Granulated intraepithelial lymphocytes: their relationship to mucosal mast cells and globule leucocytes in the rat," *Immunology*, vol. 53, no. 3, pp. 525–535, 1984.

[76] M. Van Egmond, C. A. Damen, A. B. Van Spriel, G. Vidarsson, E. Van Garderen, and J. G. J. Van De Winkel, "IgA and the IgA Fc receptor," *Trends in Immunology*, vol. 22, no. 4, pp. 205–211, 2001.

[77] J. M. Woof and M. A. Ken, "The function of immunoglobulin A in immunity," *Journal of Pathology*, vol. 208, no. 2, pp. 270–282, 2006.

[78] C. Prussin and D. D. Metcalfe, "IgE, mast cells, basophils, and eosinophils," *Journal of Allergy and Clinical Immunology*, vol. 111, no. 2, pp. S486–S494, 2003.

[79] B. De Andres, E. Rakasz, M. Hagen et al., "Lack of Fc-ε receptors on murine eosinophils: implications for the functional significance of elevated IgE and eosinophils in parasitic infections," *Blood*, vol. 89, no. 10, pp. 3826–3836, 1997.

[80] C. A. Behm and K. S. Ovington, "The role of eosinophils in parasitic helminth infections: insights from genetically modified mice," *Parasitology Today*, vol. 16, no. 5, pp. 202–209, 2000.

[81] C. E. Lawrence, "Is there a common mechanism of gastrointestinal nematode expulsion?" *Parasite Immunology*, vol. 25, no. 5, pp. 271–281, 2003.

[82] H. D. F. H. Schallig, M. A. W. Van Leeuwen, and A. W. C. A. Cornelissen, "Protective immunity induced by vaccination with two *Haemonchus contortus* excretory secretory proteins in sheep," *Parasite Immunology*, vol. 19, no. 10, pp. 447–453, 1997.

[83] H. D. F. H. Schallig, "Immunological responses of sheep to *Haemonchus contortus*," *Parasitology*, vol. 120, pp. S63–S72, 2000.

[84] J. F. Huntley, J. Redmond, W. Welfare et al., "Studies on the immunoglobulin E responses to *Teladorsagia circumcincta* in sheep: purification of a major high molecular weight allergen," *Parasite Immunology*, vol. 23, no. 5, pp. 227–235, 2001.

[85] A. Pernthaner, S. A. Cole, L. Morrison, and W. R. Hein, "Increased expression of interleukin-5 (IL-5), IL-13, and tumor necrosis factor alpha genes in intestinal lymph cells of sheep selected for enhanced resistance to nematodes during infection with *Trichostrongylus colubriformis*," *Infection and Immunity*, vol. 73, no. 4, pp. 2175–2183, 2005.

[86] A. Pernthaner, R. J. Shaw, M. M. McNeill, L. Morrison, and W. R. Hein, "Total and nematode-specific IgE responses in intestinal lymph of genetically resistant and susceptible sheep during infection with *Trichostrongylus colubriformis*," *Veterinary Immunology and Immunopathology*, vol. 104, no. 1-2, pp. 69–80, 2005.

[87] L. Murphy, P. D. Eckersall, S. C. Bishop et al., "Genetic variation among lambs in peripheral IgE activity against the larval stages of *Teladorsagia circumcincta*," *Parasitology*, pp. 1–12, 2010.

[88] R. J. Shaw, M. M. McNeill, D. R. Maass et al., "Identification and characterisation of an aspartyl protease inhibitor homologue as a major allergen of *Trichostrongylus colubriformis*," *International Journal for Parasitology*, vol. 33, no. 11, pp. 1233–1243, 2003.

[89] S. A. Bisset, A. Vlassoff, C. A. Morris, B. R. Southey, R. L. Baker, and A. G. H. Parker, "Heritability of and genetic correlations among faecal egg counts and productivity traits in Romney sheep," *New Zealand Journal of Agricultural Research*, vol. 35, pp. 51–58, 1992.

[90] M. J. Stear, D. J. S. Hetzel, S. C. Brown, L. J. Gershwin, M. J. Mackinnon, and F. W. Nicholas, "The relationships among ecto- and endoparasite levels, Class I antigens of the bovine major histocompatibility system, immunoglobulin E levels and weight gain," *Veterinary Parasitology*, vol. 34, no. 4, pp. 303–321, 1990.

[91] M. J. Stear and M. Murray, "Genetic resistance to para-sitic disease: particularly of resistance in ruminants to gastrointestinal nematodes," *Veterinary Parasitology*, vol. 54, no. 1–3, pp. 161–176, 1994.

[92] M. J. Stear, L. Fitton, G. T. Innocent, L. Murphy, K. Rennie, and L. Matthews, "The dynamic influence of genetic variation on the susceptibility of sheep to gastrointestinal nematode infection," *Journal of the Royal Society Interface*, vol. 4, no. 16, pp. 767–776, 2007.

[93] S. C. Bishop, F. Jackson, R. L. Coop, and M. J. Stear, "Genetic parameters for resistance to nematode infections in Texel lambs and their utility in breeding programmes," *Animal Science*, vol. 78, no. 2, pp. 185–194, 2004.

[94] M. J. Stear, K. Bairden, S. C. Bishop et al., "The genetic basis of resistance to *Ostertagia circumcincta* in lambs," *Veterinary Journal*, vol. 154, no. 2, pp. 111–119, 1997.

[95] M. J. Stear, S. C. Bishop, B. A. Mallard, and H. Raadsma, "The sustainability, feasibility and desirability of breeding livestock for disease resistance," *Research in Veterinary Science*, vol. 71, no. 1, pp. 1–7, 2001.

[96] M. J. Stear, G. T. Innocent, and J. Buitkamp, "The evolution and maintenance of polymorphism in the major histocompatibility complex," *Veterinary Immunology and Immunopathology*, vol. 108, no. 1-2, pp. 53–57, 2005.

[97] M. J. Stear, T. J. Tierney, F. C. Baldock, S. C. Brown, F. W. Nicholas, and T. H. Rudder, "Class I antigens of the bovine major histocompatibility system are weakly associated with variation in faecal worm egg counts in naturally infected cattle," *Animal Genetics*, vol. 19, no. 2, pp. 115–122, 1988.

[98] L. C. Gasbarre, E. A. Leighton, and C. J. Davies, "Influ-ence of host genetics upon antibody responses against gastrointestinal nematode infections in cattle," *Veterinary Parasitology*, vol. 46, no. 1-4, pp. 81–91, 1993.

[99] K. J. Beh, D. J. Hulme, M. J. Callaghan et al., "A genome scan for quantitative trait loci affecting resistance to *Trichostrongylus colubriformis* in sheep," *Animal Genetics*, vol. 33, no. 2, pp. 97–106, 2002.

[100] A. M. Crawford, K. A. Paterson, K. G. Dodds et al., "Discovery of quantitative trait loci for resistance to parasitic nematode infection in sheep: I. Analysis of outcross pedigrees," *BMC Genomics*, vol. 7, article 178, 2006.

[101] K. Marshall, J. F. Maddox, S. H. Lee et al., "Genetic mapping of quantitative trait loci for resistance to *Haemonchus contortus* in sheep," *Animal Genetics*, vol. 40, no. 3, pp. 262–272, 2009.

[102] A. N. Wilton, T. J. Cobain, and R. L. Dawkins, "Family studies of IgA deficiency," *Immunogenetics*, vol. 21, no. 4, pp. 333–342, 1985.

[103] F. M. Schaffer, J. Palermos, Z. B. Zhu, B. O. Barger, M. D. Cooper, and J. E. Volanakis, "Individuals with IgA deficiency and common variable immunodeficiency share polymorphisms of major histocompatibility complex class III genes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 20, pp. 8015–8019, 1989.

[104] A. Reil, G. Bein, H. K. G. Machulla, B. Sternberg, and M. Seyfarth, "High-resolution DNA typing in immunoglobulin A deficiency confirms a positive association with DRB1*0301, DQB1*02 haplotypes," *Tissue Antigens*, vol. 50, no. 5, pp. 501–506, 1997.

[105] H. W. Schroeder, Z. B. Zhu, R. E. March et al., "Susceptibility locus for IgA deficiency and common variable immunodeficiency in the HLA-DR3, -B8, -A1 haplotypes," *Molecular Medicine*, vol. 4, no. 2, pp. 72–86, 1998.

[106] F. Cucca, Z. B. Zhu, A. Khanna et al., "Evaluation of IgA deficiency in Sardinians indicates a susceptibility gene is encoded within the HLA class III region," *Clinical and Experimental Immunology*, vol. 111, no. 1, pp. 76–80, 1998.

[107] V. B. Matthews, C. S. Witt, M. A. H. French et al., "Central MHC genes affect IgA levels in the human: reciprocal effects in IgA deficiency and IgA nephropathy," *Human Immunology*, vol. 63, no. 5, pp. 424–433, 2002.

[108] J. E. Volanakis, Z. B. Zhu, F. M. Schaffer et al., "Major histocompatibility complex class III genes and susceptibility to immunoglobulin A deficiency and common variable immunodeficiency," *Journal of Clinical Investigation*, vol. 89, no. 6, pp. 1914–1922, 1992.

[109] O. Olerup, C. I. E. Smith, J. Bjorkander, and L. Hammarstrom, "Shared HLA class II-associated genetic susceptibility and resistance, related to the HLA-DQB1 gene, in IgA deficiency and common variable immunodeficiency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 22, pp. 10653–10657, 1992.

[110] I. Vořechovský, M. Cullen, M. Carrington, L. Hammarström, and A. D. B. Webster, "Fine mapping of IGAD1 in IgA deficiency and common variable immunodeficiency: identification and characterization of haplotypes shared by affected members of 101 multiple-case families," *Journal of Immunology*, vol. 164, no. 8, pp. 4408–4416, 2000.

[111] J. Kralovicova, L. Hammarström, A. Plebani, A. D. B. Webster, and I. Vorechovsky, "Fine-scale mapping at IGAD1 and genome-wide genetic linkage analysis implicate HLA-DQ/DR as a major susceptibility locus in selective IgA deficiency and common variable immunodeficiency," *Journal of Immunology*, vol. 170, no. 5, pp. 2765–2775, 2003.

[112] H. Sekine, R. C. Ferreira, Q. Pan-Hammarström et al., "Role for Msh5 in the regulation of Ig class switch recombination," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 17, pp. 7193–7198, 2007.

[113] B. D. Harfe and S. Jinks-Robertson, "DNA mismatch repair and genetic instability," *Annual Review of Genetics*, vol. 34, pp. 359–399, 2000.

[114] J. A. Surtees, J. L. Argueso, and E. Alani, "Mismatch repair proteins: key regulators of genetic recombination," *Cytogenetic and Genome Research*, vol. 107, no. 3-4, pp. 146–159, 2004.

[115] J. E. J. Guikema, C. E. Schrader, N. G. J. Leus et al., "Re-assessment of the role of Mut S homolog 5 in Ig class switch recombination shows lack of involvement in cis- and trans-switching," *Journal of Immunology*, vol. 181, no. 12, pp. 8450–8459, 2008.

[116] S. M. Offer, Q. Pan-Hammarström, L. Hammarström, and R. S. Harris, "Unique DNA repair gene variations and potential associations with the primary antibody deficiency syndromes igAD and CVID," *PLoS ONE*, vol. 5, no. 8, Article ID e12260, 2010.

[117] N. D. Pozo, L. M. Medrano, M. C. Cénit et al., "MSH5 is not a genetic predisposing factor for immunoglobulin A deficiency but marks the HLA-DRB1*0102 subgroup carrying susceptibility," *Human Immunology*, vol. 71, pp. 861–864, 2010.

[118] D. L. Wassom and E. A. B. Kelly, "The role of the major histocompatibility complex in resistance to parasite infections," *Critical Reviews in Immunology*, vol. 10, no. 1, pp. 31–52, 1990.

[119] M. W. Kennedy, "Genetic control of the immune repertoire in nematode infections," *Parasitology Today*, vol. 5, no. 10, pp. 316–324, 1989.

[120] T. Dainichi, Y. Maekawa, K. Ishii et al., "Nippocystatin, a cysteine protease inhibitor from Nippostrongylus brasiliensis, inhibits antigen processing and modulates antigen-specific immune response," *Infection and Immunity*, vol. 69, no. 12, pp. 7380–7386, 2001.

[121] J. S. Knight, D. B. Baird, W. R. Hein, and A. Pernthaner, "The gastrointestinal nématode *Tricostrongylus colubriformis* down-regulates immune gene expression in migratory cells in afferent lymph," *BMC Immunology*, vol. 11, article 51, 2010.

[122] D. L. Wassom, C. J. Krco, and C. S. David, "I-E expression and susceptibility to parasite infection," *Immunology Today*, vol. 8, no. 2, pp. 39–43, 1987.

[123] O. M. Keane, A. Zadissa, T. Wilson et al., "Gene expression profiling of Naïve sheep genetically resistant and susceptible to gastrointestinal nematodes," *BMC Genomics*, vol. 7, article 42, 2006.

[124] N. Charbonnel and J. Pemberton, "A long-term genetic survey of an ungulate population reveals balancing selection acting on MHC through spatial and temporal fluctuations in selection," *Heredity*, vol. 95, no. 5, pp. 377–388, 2005.

[125] S. Paterson, "Evidence for balancing selection at the major histocompatibility complex in a free-living ruminant," *Journal of Heredity*, vol. 89, no. 4, pp. 289–294, 1998.

[126] D. Beraldi, A. F. McRae, J. Gratten et al., "Quantitative trait loci (QTL) mapping of resistance to strongyles and coccidia in the free-living Soay sheep (*Ovis aries*)," *International Journal for Parasitology*, vol. 37, no. 1, pp. 121–129, 2007.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 926812, 10 pages
doi:10.1155/2011/926812

Research Article

Energetic Cost of *Ichthyophonus* Infection in Juvenile Pacific Herring (*Clupea pallasii*)

Johanna J. Vollenweider,[1] Jake L. Gregg,[2] Ron A. Heintz,[1] and Paul K. Hershberger[2]

[1] *Auke Bay Laboratories, Alaska Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, 17109 Point Lena Loop Road, Juneau, AL 99801, USA*
[2] *Marrowstone Marine Field Station, and Western Fisheries Research Center, United States Geological Survey, 616 Marrowstone Point Road, Nordland, WA 98358-9633, USA*

Correspondence should be addressed to Johanna J. Vollenweider, johanna.vollenweider@noaa.gov

Received 2 December 2010; Accepted 14 February 2011

Academic Editor: Bernard Marchand

The energetic costs of fasting and *Ichthyophonus* infection were measured in juvenile Pacific herring (*Clupea pallasii*) in a lab setting at three temperatures. Infected herring incurred significant energetic costs, the magnitude of which depended on fish condition at the time of infection (fat versus lean). Herring that were fed continually and were in relatively good condition at the time of infection (fat) never stored lipid despite *ad libitum* feeding. In feeding herring, the energetic cost of infection was a 30% reduction in total energy content relative to controls 52 days post infection. Following food deprivation (lean condition), infection caused an initial delay in the compensatory response of herring. Thirty-one days after re-feeding, the energetic cost of infection in previously-fasted fish was a 32% reduction in total energy content relative to controls. Body composition of infected herring subsequently recovered to some degree, though infected herring never attained the same energy content as their continuously fed counterparts. Fifty-two days after re-feeding, the energetic cost of infection in previously-fasted fish was a 6% reduction in total energy content relative to controls. The greatest impacts of infection occurred in colder temperatures, suggesting *Ichthyophonus*-induced reductions in body condition may have greater consequences in the northern extent of herring's range, where juveniles use most of their energy reserves to survive their first winter.

1. Introduction

Ichthyophonus is a commonly occurring Mesomycetozoean parasite that has been reported in more than 100 species of fish [1–3]. *Ichthyophonus* is highly pathogenic to Pacific herring (*Clupea pallasii*) [4] and occurs in high prevalence and intensity in herring populations throughout the northeast Pacific Ocean [5–7]. Epizootics of the parasite are known to structure herring populations and populations of other fish species [1, 2, 8, 9].

Infection by *Ichthyophonus* is not 100% fatal (see Gregg et al. this issue). Infection elicits systemic inflammation and tissue destruction resulting in vital organ failure [10]. In survivors, quantification of reduced fitness or identification of chronic problems associated with the parasite is limited to a study which found decreased swimming performance in salmon (*Oncorhynchus*) as a result of tissue damage. Authors surmised that poor swimming ability may lead to fatigue and elevated depletion of lipid reserves that would ultimately interfere with migrations [11]. Differences in swimming stamina between *Ichthyophonus*-infected and uninfected cohorts are more pronounced at higher temperatures [12] where metabolic energy costs are higher. Increased energetic costs and exhaustion of lipid stores could have a host of additional consequences particularly in relation to other energetically demanding processes such as maturation, spawning, or overwinter survival.

At high latitudes, overwinter survival of young-of-the-year fish has been identified as a critical period determining year class strength of many fish species [13–15]. Nearer the poles, growing seasons are short which limits the time in which animals can attain energy. During the brief

summer months, juvenile fish have high energetic demands with conflicting requirements. They must allocate energy to growth to increase foraging opportunities and avoid predation, as well as allocate energy to storage to supply endogenous energy during winter when prey is scarce [16]. Additional energetic demands, such as those imposed by parasite infection, could have serious consequences for fish already contending with energy limitations.

Pacific herring are a northern species adapted to the extreme seasonal conditions of the North Pacific. Herring occupy an intermediate trophic level, transferring energy from zooplankton to higher level consumers such as many fish species, marine mammals, and seabirds [17–19]. As zooplanktivores, they are tightly linked to seasonal fluctuations in productivity [20]. Consequently, their body composition fluctuates significantly in a seasonal manner. Energy content of age-0 herring increases throughout the summer and autumn as herring assimilate energy from foraging and subsequently declines over winter when prey availability is low and endogenous energy stores are utilized. In spring herring reach an energetically sensitive condition when their lipid and energy content are at a minimum [21, 22]. In this low-energy state, juvenile herring may be most susceptible to stress, including disease.

The objectives of the study were to measure the energetic condition of juvenile herring infected with *Ichthyophonus* (relative to control fish) under a variety of energetic states and temperatures to mimic various seasonal environmental scenarios found in the northern Gulf of Alaska. Specifically, we compared the body composition of laboratory-reared herring infected with *Ichthyophonus* to controls that were (1) relatively fat, having fed continuously; representing fish in the late summer and autumn, and (2) relatively lean, having undergone extensive fasting followed by a period of refeeding; representing herring in the spring that had undergone winter fasting and commenced foraging on spring plankton blooms. The latter tests were conducted at three temperatures in order to understand how the cost of infection in the most energetically sensitive fish may vary throughout their range (California to Alaska).

2. Methods

2.1. Study Design. To ensure herring were free of *Ichthyophonus* and immunologically naïve, naturally spawned herring eggs were collected from the southern Strait of Georgia (48°55.85′ N, 122°48.15′ W) on 5 May 2008 and held in filtered, UV-irradiated seawater at the US Geological Survey Marrowstone Marine Field Station, in Nordland, Wash, USA (48°06.08′ N, 122°41.32′ W). Eggs hatched on 18 May 2008 and herring were cultured to age 1 according methods outlined by Gregg et al. (this issue).

Herring were separated into two groups to simulate the impact of *Ichthyophonus* infection on fish in (1) autumn (relatively fat fish), and (2) spring (relatively lean fish) (Figure 1). "Autumn fish" were fed *ad libitum* and maintained in ambient water temperature for the duration of the experiment. On day 0, herring were divided into infected and uninfected (control) treatments. Infection with *Ichthyophonus* occurred via intraperitoneal injection according to procedures outlined by Gregg et al. (this issue). Control fish in the uninfected treatment were injected with sterile phosphate buffered saline to provide equivalent handling conditions.

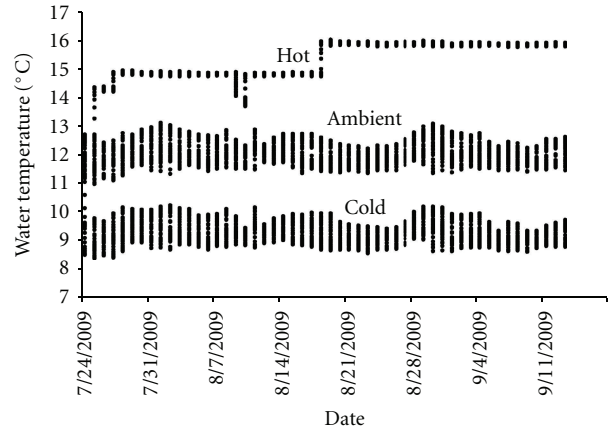


Figure 1: Experimental water temperatures over the course of the study. Ambient water from Puget Sound (mean = 12.0°C) was heated and chilled in head tanks to obtain the "hot" (mean = 15.0°C) and "cold" (mean = 9.5°C) treatments, respectively.

Prior to the study initiation, "spring herring" underwent a 56-day fasting period to simulate limited food availability in winter. On day 0, fish were also divided into infected and uninfected (control) treatments and were maintained in three water temperatures (cold, ambient, hot). At the time of infection, herring were randomly assigned to triplicate tanks for each treatment/temperature condition. After herring were distributed to experimental tanks, ambient seawater pumped from Puget Sound was gradually manipulated over 24 hours to provide cold and hot treatments. The temperature regulating system allowed all temperatures to vary with ambient seawater temperature while maintaining relatively consistent separation between them. Water temperature was recorded every 30 minutes (Figure 2). Mean water temperatures for the three temperature conditions were 9.5, 12.0, and 15.0°C. Subsequent to infection, all fish (spring and autumn conditions) were fed to satiation daily. Subsamples of juvenile herring were collected and frozen for chemical analysis immediately prior to the initiation of the experiment, on day 31 and at the completion of the experiment on day 52. Not all fish in the infected treatments contracted ichthyophoniasis, resulting in disproportionate sample sizes (Table 1). Lengths and weights of all fish were measured at experiment initiation and completion and for fish subsampled on day 31. Mortalities were removed from the experimental tanks daily and their lengths and weights were recorded. All samples and mortalities were assessed for infection via *in vitro* explant culture of the heart according to methods in Gregg et al. (this issue).

Table 1: Sample sizes of juvenile Pacific herring for proximate composition analysis by temperature, where Infect = infected and Control = controls.

Feeding history	Fed-"Autumn"				Fasted-"spring"			
Temperature	Ambient		Cold		Ambient		Hot	
Sampling Day	Infect	Control	Infect	Control	Infect	Control	Infect	Control
0		8			8	8		
31	1	11	5	7	4	8	1	9
52	8	8	8	8	8	8	7	11

2.2. Chemical Analysis. Samples were analyzed for proximate composition including lipid, protein, moisture, and ash content according, standard procedures outlined in Vollenweider et al. [23] (Table 1). Fish selected for chemical analysis represented the entire size range of fish from a given treatment. Briefly, stomach contents were removed and whole fish were dried to a constant mass at 135°C in a LECO Thermogravimetric Analyzer (TGA) 601. Moisture content was calculated from wet and dry masses. Dried fish were homogenized and aliquots were randomly selected for each analysis. Lipid content was measured using a modification of Folch's method outlined by Christie [24] in a Dionex Accelerated Solvent Extractor with 2 : 1 (v : v) chloroform:methanol. Extracts were washed to remove coextractables and percent lipid was measured gravimetrically after solvent evaporated. Protein content was estimated from nitrogen content which was measured using a LECO Nitrogen Analyzer TruSpec following the Dumas method [25]. Protein samples were analyzed in duplicate to ensure accuracy. Ash content was measured gravimetrically after combusting samples at 600°C using the TGA. Quality assurance (QA) samples for each analysis were included with each batch of 17 samples, including blanks, reference material, and replicate sample. If QA samples fell outside prescribed limits, samples were reanalyzed.

Energy content was calculated by summing the energy contributed by total-body lipid and protein as described in Vollenweider et al. [23]. Energy equivalents of 36.43 kJ g^{-1} for lipid and 20.10 kJ g^{-1} for protein were used [26].

2.3. Statistical Analysis. Allometries between energy and length were used to estimate the average energy content of fish in each of the treatment groups. Only a subset of fish from each treatment groups on days 0 and 52 were chemically analyzed. For each of these groups we constructed linear models relating the total energy content of fish to their lengths ($R^2 > 0.50$). The relationships were linearized by transforming the data to their natural logarithms before constructing the models. These resulting models were used to predict the energy content of each fish in a treatment group based on their lengths. Energy densities were calculated for each fish by dividing the predicted energy content by the observed fish weight. Subsequent analyses used the set of predicted values as the response variables for a given treatment. Analyses involving day 31 relied only on observed samples because no measurements of length or weight were recorded for the remaining fish.

Total energy, energy density, and percent lipid were the response variables compared between treatments using a General Linear Model (GLM) for ANOVA and Tukey Test for multiple comparisons. For the first experiment, where fish were continuously fed, the model was

$$\text{Response} = \text{infection} + \text{time} + \text{infection} \times \text{time}. \quad (1)$$

For the second experiment, where fish fasted and then infected upon refeeding, the model was

$$\begin{aligned}\text{Response} &= \text{infection} + \text{time} + \text{temperature} + \text{infection} \times \text{time} \\ &\quad + \text{temperature} \times \text{time} + \text{infection} \times \text{temperature} \times \text{time}.\end{aligned} \quad (2)$$

All factors were considered fixed for this study.

The rate of energy gain over the study period was calculated using a regression slope of natural log-transformed total energy content. Statistical differentiation of slopes was tested using a GLM ANOVA with transformed values of total energy content as the response, day ∗ an indicator variable as the model, and day and indicator variable as covariates following procedures outlined in Minitab 15 statistical package. Herring lengths were compared using 2-Sample t-tests and T statistics are provided. Statistical variation reported in the text and indicated in figures are standard errors.

A brief analysis of mortality was conducted. Nearly half (49%) of the mortalities occurred within the first five days of the experiment. Of these initial mortalities, only 21% were infected fish, suggestive of handling mortality. All subsequent mortality analysis excluded this data.

3. Results

3.1. Mortality Rates from Infection (Ambient Water Temperature). Synergistic interactions of *Ichthyophonus* infection and fasting caused elevated mortality rates in juvenile herring beyond cumulative effects of the two treatments. "Spring herring" that had fasted prior to infection incurred the highest mortality rate over the course of the experiment (53%), followed by "autumn fish" that had been fed continuously prior to infection (37%). Control "spring herring" (uninfected) died at a similar rate as those that had been fed continuously (11% each), indicating that the fasting period was not long enough to cause irreparable nutritional stress

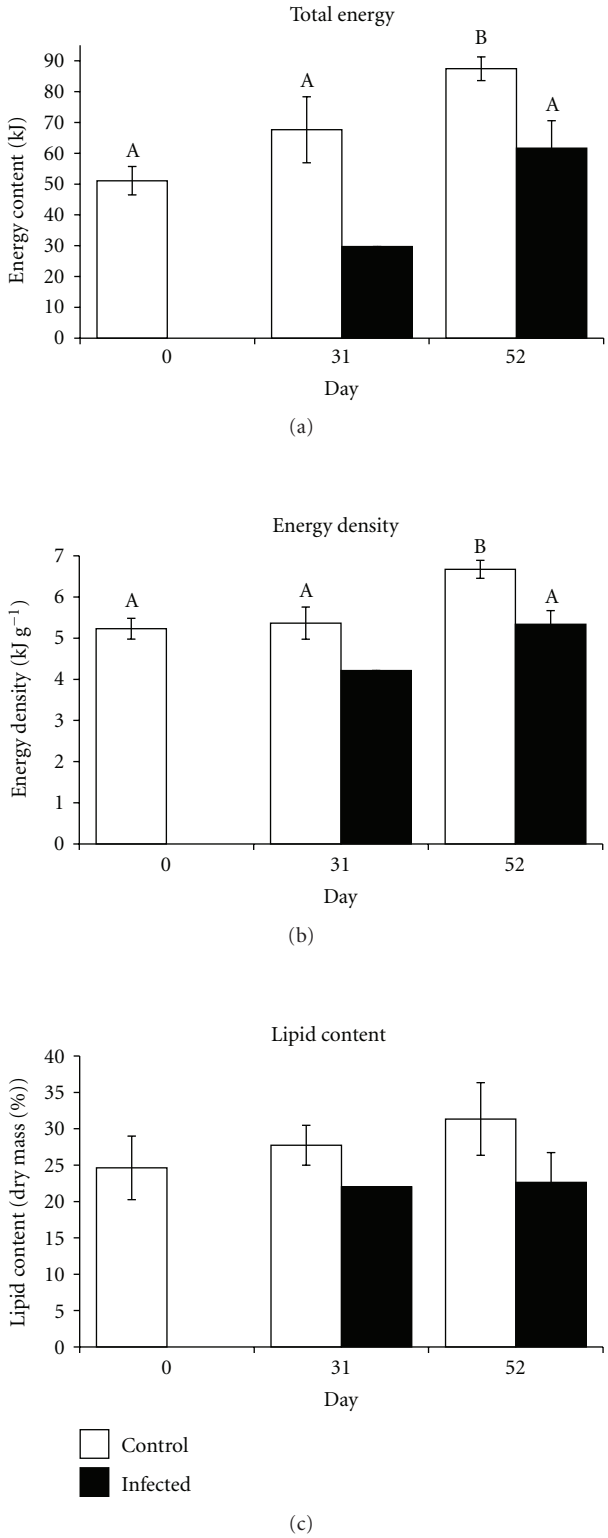


Figure 2: Energetic cost of *Ichthyophonus* infection in "autumn" young-of-the-year Pacific herring depicted by total energy content, energy density, and lipid content (% dry mass). Fish represented in this figure were cultured in ambient water temperature (9.5°C). Different letters represent statistical differentiation. Lack of letters indicates no statistical differentiation. Low sample size of infected fish on day 31 precludes statistical tests.

(See Gregg et al. this issue, for a full discussion of mortality kinetics).

3.2. Energetic Cost of Infection in "Autumn Herring" (Ambient Water Temperature). Compared to the uninfected controls, "autumn herring" with relatively good body condition incurred significant energetic costs from *Ichthyophonus* infection in ambient water temperatures. This was most apparent 31 days after infection, at which time the total energy content of infected juveniles declined by 58% from their initial condition while that of control fish increased by 32% ($F_{(1,38)} = 0.54$, $P = .468$) (Figure 2). Over the ensuing 21 days, infected fish recovered somewhat and stored energy at a rate 61% faster than controls (1520 versus 942 J d^{-1}, $F_{(1,100)} = 0.18$, $P = .677$). Despite elevated energy storage, 52 days of refeeding did not result in infected fish acquiring significant energy since the time of infection ($F_{(1,41)} < 0.01$, $P = .968$). In contrast, controls gained appreciable energy over the same 52-day refeeding period at a rate of 698 J d^{-1}, resulting in a net gain of 42% total energy (kJ) ($F_{(1,105)} = 76.02$, $P < .001$) and 22% energy density (kJ g^{-1}) ($F_{(1,105)} = 13.83$, $P < .001$). Fifty-two days after infection, the energetic cost of infection in feeding juvenile herring relative to controls was a 30% reduction in total energy content (61.6 versus 87.4 kJ; $F_{(1,89)} = 29.74$, $P < .001$) and a 21% reduction in energy density (5.3 versus 6.7 kJ g^{-1}, $F_{(1,89)} = 10.12$, $P = .002$).

Energetic costs of *Ichthyophonus* infection resulted in a depletion of lipid reserves. Despite continuous feeding, lipid content (% dry mass) of infected juvenile herring declined by 11% one month after infection (24.6 versus 22.0%), and remained similarly depleted by day 52 (22.6%; $F_{(1,13)} = 0.71$, $P = .414$) (Figure 2). In contrast, controls that had been fed continuously built up their lipid reserves by the same proportion (11%) after one month (24.6 versus 27.7%; $F_{(1,16)} = 0.01$, $P > .924$), and another 12% over the ensuing 21 days (27.7 versus 31.3%; $F_{(1,16)} = 0.66$, $P = .428$). Consequently, infected juvenile herring had 28% smaller lipid reserves than controls by the end of the 52-day study period (22.6 versus 31.3%; $F_{(1,13)} = 1.84$, $P = .197$).

3.3. Compensatory Response in "Spring Herring" (Ambient Water Temperature). Ichthyophonus infection delayed compensatory growth response following food deprivation in "spring herring". To initiate a compensatory growth response, herring were fasted for 56 days to reduce their body condition to levels representative of wild fish in the spring following food-limited winter months. After the fasting period, total energy content of juvenile herring was 46% less than fed fish (27.4 ± 2.5 versus 51.1 ± 4.6 kJ; $F_{(1,56)} = 12.23$, $P = .001$), and energy densities were 21% less than fed fish (4.1 ± 0.1 versus 5.2 ± 0.3 kJ g^{-1}; $F_{(4,56)} = 4.69$, $P = .035$) (Figure 3). During the first 31 days of refeeding controls demonstrated compensatory growth response, acquiring energy 79% faster than fish that had been fed continuously (954 versus 533 J d^{-1}, $F_{(1,74)} = 1.89$, $P = .173$). Over the same refeeding period, infected fish did not exhibit compensatory response, gaining energy at

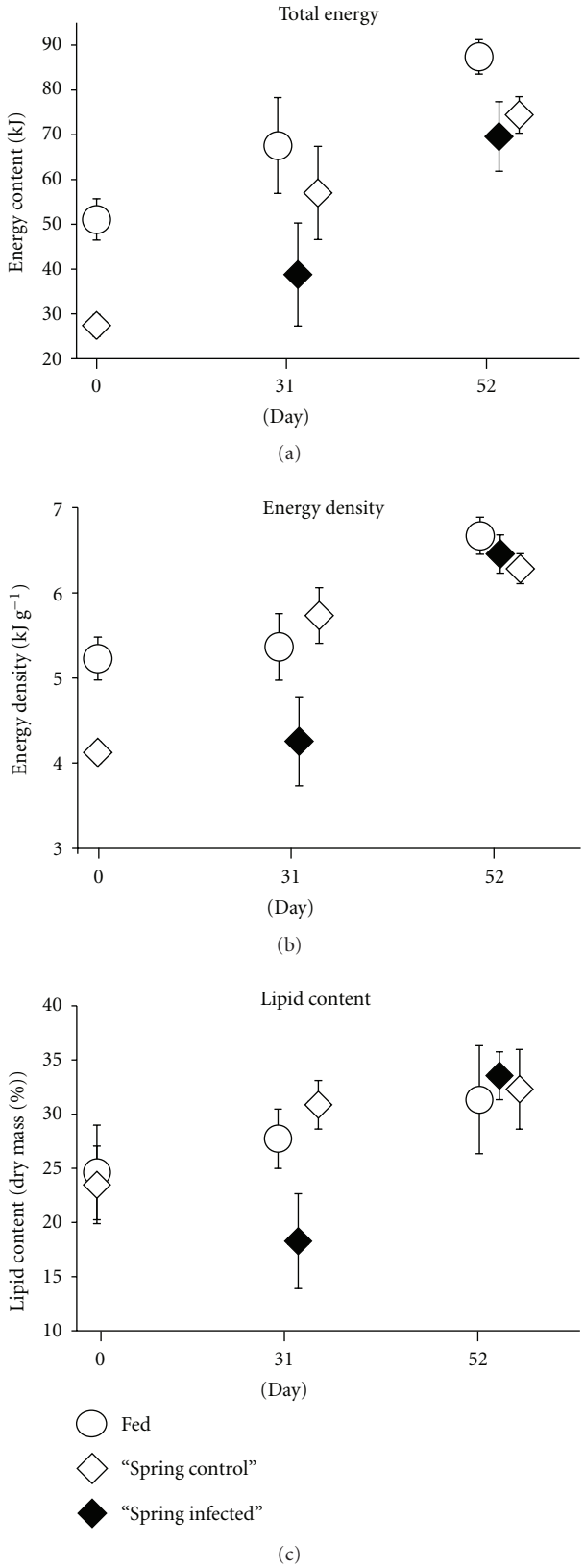


Figure 3: Compensatory response of young-of-the-year Pacific herring infected with *Ichthyophonus* depicted by total energy content, energy density, and lipid content (% dry mass). Fish represented in this figure were cultured in ambient water temperature (9.5°C). Low sample size of infected fish on day 31 precludes statistical tests.

a slower rate than continuously fed fish (367 versus 533 J d^{-1}, $F_{(1,72)} = 0.06$, $P = .813$). As such, infected fish were in relatively poor condition compared to controls by day 31, with a 32% disadvantage in their total energy content (38.8 ± 11.5 versus 57.0 ± 10.4 kJ; $F_{(1,9)} = 13.09$, $P = .006$) and a 26% disadvantage in their energy density (4.3 ± 0.5 versus 5.7 ± 0.3 kJ g^{-1}); $F_{(1,9)} = 15.83$, $P = .003$).

Similar to "autumn herring", the "spring herring" recovered from the initial impacts of infection to some degree during the second half of the experiment. Over the final 21 days of refeeding infected herring increased their rate of energy acquisition substantially which is indicative of compensatory growth response, gaining energy 49% faster than the continually fed fish (1400 versus 942 J d^{-1}, $F_{(1,104)} = 0.64$, $P = .425$). Over the same refeeding period, controls no longer exhibited compensatory growth response, their energy acquisition rates moderating to that of continually fed fish (792 versus 942 J d^{-1}, $F_{(1,163)} = 0.14$, $P = .709$). By the end of the 52-day experiment, infected herring were energetically on par with controls, only incurring a 6% reduction in total energy content relative to controls. (69.6 ± 7.8 versus 74.4 ± 4.1 kJ, resp.; $F_{(1,82)} = 0.28$, $P = .596$ and 6.5 ± 0.2 versus 6.3 ± 0.2 kJ g^{-1}, resp.; $F_{(1,82)} = 2.55$, $P = .114$). Despite elevated energy gains, previously fasted fish never attained the same size as their continuously fed counterparts over the study duration. Hence the total energy of the previously fasted fish was lower than the continuously fed fish (73.6 ± 3.6 versus 87.4 ± 3.8 kJ, resp.; $F_{(1,160)} = 4.57$, $P = .034$) even though their energy densities were the same (6.5 ± 0.2 versus 6.3 ± 0.2 versus 6.7 ± 0.2 kJ g^{-1} for infected "spring herring", control "spring herring," and fed fish, resp.; $F_{(2,159)} = 0.72$, $P = .488$).

Lipid acquisition was also delayed following food deprivation. After the 52-day fasting period, lipid content (% dry mass) was reduced by 4% relative to that of herring that had been continually fed (23.5 ± 3.6 versus 24.6 ± 4.4% lipid; $F_{(1,14)} < 0.01$, $P = .967$) (Figure 3). One month after *Ichthyophonus* infection, lipid content declined significantly by an additional 22% despite refeeding (18.3 ± 4.4 versus 23.5 ± 3.6% lipid; $F_{(1,9)} = 0.49$, $P = .504$). Over the same time period, the compensatory response of control "spring herring" was so strong that their lipid content exceeded the lipid content of continually fed fish (30.9 ± 2.2 versus 25.2 ± 4.6; $F_{(2,14)} = 7.71$, $P = .027$). After 31 days, infected fish experienced a 41% lipid deficit relative to controls (18.3 ± 4.4 versus 30.9 ± 2.2% lipid; $F_{(1,9)} = 17.90$, $P = .002$). Over the following 21 days, infected herring recovered from their initial lipid deficit. Coincidentally, the accelerated rate of lipid storage in controls slowed. Thus by day 52, lipid content of all fish that had undergone previous food restrictions, regardless of infection, were equal to that of continually fed fish (33.6 ± 2.2 versus 32.3 ± 3.7 versus 31.3 ± 5.0% lipid, for infected "spring herring", control "spring herring," and fed fish, resp.; $F_{(2,20)} = 17.90$, $P = .756$).

3.4. Temperature Effects on "Spring Herring". Water temperature influenced the lethality of *Ichthyophonus* infection on juvenile herring. Relative to control mortalities (uninfected

Table 2: Test statistics for comparisons of infected versus uninfected juvenile Pacific herring lengths and energy densities at different water temperatures. Length comparisons were 2-sample *t*-tests (*T* statistic reported), while energy density comparisons were GLM ANOVAs (*F* statistic reported).

	Cold			Ambient			Hot		
	P-value	statistic	df	*P*-value	statistic	df	*P*-value	statistic	df
Length									
Day 31	.863	0.18	8	.695	−0.42	5	.604	0.53	15
Day 52	.245	1.17	99	.605	0.53	18	.604	0.53	15
Energy Density									
Day 31	.552	0.38	1,9	.003	15.83	1,9	.246	1.54	1,9
Day 52	<.001	69.66	1,99	.114	2.55	1,82	.708	0.14	1,107

fish), *Ichthyophonus*-induced mortalities were greatest in juvenile herring reared in ambient water temperatures (42% mortality), followed by hot (12%) and cold (4%) treatments. (See Gregg et al. this issue, for a complete discussion of mortality kinetics).

Warmer temperatures conferred greater rates of energy acquisition in "spring herring" over the 52-day re-feeding period, a pattern which was more pronounced in infected fish. In the cold treatment, controls gained energy at a rate of 1167 J d^{-1} while those in ambient and hot treatments gained energy at rates 7% (1253 J d^{-1}; $F_{(1,198)} = 0.190$, $P = .662$) and 18% (1424 J d^{-1}; $F_{(1,227)} = 1.390$, $P = .239$) more rapidly, respectively (Figure 4). Temperature effects on energy storage rates were more pronounced in infected herring, which gained energy at rates 27% (1095 J d^{-1}; $F_{(1,125)} = 2.81$, $P = .096$) and 37% (1265 J d^{-1}; $F_{(1,121)} = 5.19$, $P = .025$) more rapidly in the ambient and hot treatments than in the cold treatment (797 J d^{-1}). For a given temperature, energy storage rates of infected fish were depressed relative to controls. As temperature increased, the effect of infection on energy storage rates declined, with infected herring having disadvantages of 32%, 13%, and 11% in the cold ($F_{(1,170)} = 6.47$, $P = .012$), ambient ($F_{(1,153)} = 0.55$, $P = .458$) and hot temperatures ($F_{(1,178)} = 0.39$, $P = .535$), respectively.

The influence of temperature and infection on energy storage rates was reflected in the total energy content of "spring herring". After 31 days of refeeding, infected fish failed to gain energy in any water temperature ($F_{(6,107)} = 10.47$, $P > .858$). Only by day 52 had energy content increased significantly in all temperatures ($F_{(6,107)} = 10.47$, $P < .001$). In contrast, all controls had gained appreciable energy after 31 days ($F_{(6,271)} = 86.49$, $P < .002$) and continued to gain energy over the ensuing 21 days in the cold ($F_{(6,271)} = 86.49$, $P < .001$) and hot treatments ($F_{(6,271)} = 86.49$, $P = .028$). Within a given water temperature, infected fish always had reduced energy content relative to controls, though statistical differentiation was intermittent. After 31 days, energetic reductions in infected fish relative to controls were relatively large and increased with water temperature: 9%, 32% and 49% for cold ($F_{(1,9)} = 0.29$, $P = .606$), ambient ($F_{(1,9)} = 13.09$, $P = .006$) and hot ($F_{(1,9)} = 2.94$, $P = 0.121$) treatments, respectively. By day 52, energetic reductions of infected fish remained large, though the trend with increasing water temperature no longer held: 31%, 6%, and 11% for cold ($F_{(1,99)} = 108.56$, $P < .001$), ambient ($F_{(1,82)} = 0.28$, $P = .596$) and hot ($F_{(1,107)} = .01$, $P = .927$) treatments, respectively.

Reduced total energy content of infected herring was partially attributed to decreased growth rates. For all but one day/temperature combination, infected herring were smaller than controls, though never statistically so (Figure 5, Table 2). Growth rates of juvenile herring increased as water temperature increased. Controls grew 0.16, 0.23, and 0.27 mm d^{-1} in cold, ambient, and hot temperatures, respectively, while infected herring grew 0.12, 0.20, 0.23 mm d^{-1} (Figure 5). *Ichthyophonus* infection had relatively little effect on growth rates. Among the controls, "spring herring" reared in the hot treatment grew 40% more rapidly than in the cold treatment and were significantly larger after 52 days (100.2 ± 1.4 versus 106.1 ± 1.2 mm; $T(124) = -3.30$, $P = .001$). Growth rates of infected fish were 48% greater in hot than cold treatments. Greater variation in the size of infected fish resulted in no statistical differences between mean fish sizes in different temperature treatments by day 52, though fish in the hot treatment were also nearly 6 mm larger on average than those in the cold treatment (97.9 ± 1.4 versus 103.9 ± 4.1 mm; $T(15) = -1.38$, $P = .188$).

Reduced somatic growth in the infected fish did not fully account for their lower total energy content, however, as energy densities were also lower in infected fish for each temperature (Figure 4, Table 2). Interaction effects of lipid content and temperature also contributed to decreased energy in infected herring. In the infected fish, lipid content remained depressed by day 31 in all temperatures and did not increase until day 52 ($F_{(6,33)} = 1.85$, $P = .120$). A similar delay in the recovery of lipid content was observed in the cold treatment for controls, though the delay did not occur in the warmer temperatures ($F_{(6,51)} = 4.10$, $P = .002$).

4. Discussion

4.1. Energetic Cost of Infection in "Autumn Herring" (Ambient Water Temperature). Juvenile herring infected with *Ichthyophonus* incurred significant energetic costs, the magnitude of which depended on fish condition and consequently season at the time of infection. "Autumn herring" that fed continually and were in relatively good condition at the time of infection were representative of wild juvenile

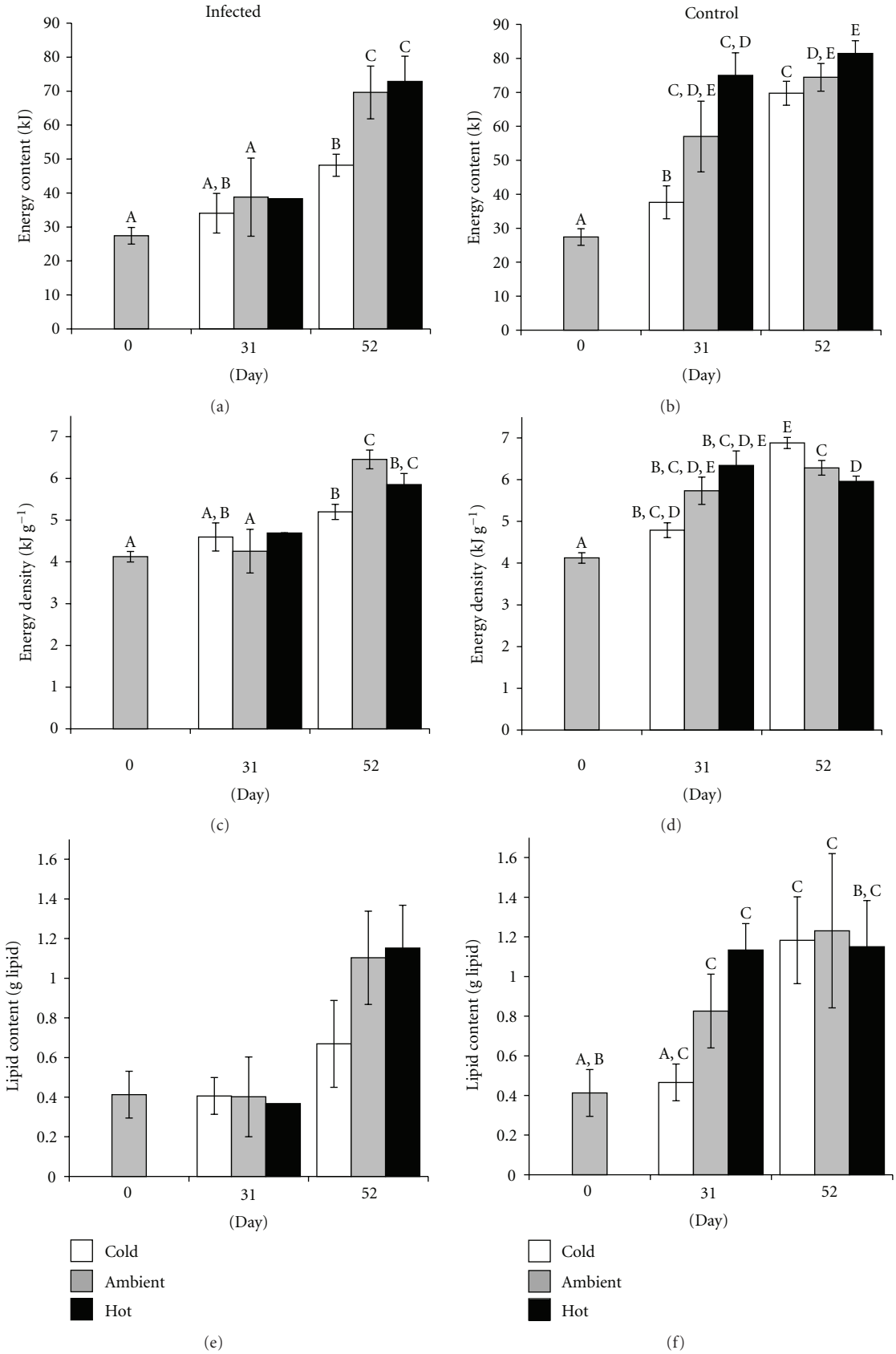


Figure 4: Temperature influence on the energetic cost of *Ichthyophonus* infection in "spring" young-of-the-year Pacific herring depicted by total energy content, energy density, and lipid content (g lipid). Water temperatures were cold (9.5°C), ambient (12.0°C), and hot (15.0°C). Different letters within a panel represent statistical differentiation. Lack of letters indicates no statistical differentiation. Low sample size of infected fish on day 31 precludes statistical tests.

herring during peak energetic condition in the fall. The energy density of juvenile herring in our experiment at the time of infection ($5.2 \pm 0.3\,\mathrm{kJ\,g^{-1}}$) lies within peak seasonal values reported in November in Prince William Sound (4.9–$7.5\,\mathrm{kJ\,g^{-1}}$) [21, 22]. Fifty-two days after infection, juvenile herring incurred a 20% reduction in energy density. To put this in perspective, the energetic cost of *Ichthyophonus* infection we measured over 52 days is similar to the energy density metabolized by fasting herring in a laboratory study of similar duration (23% loss from $5.2\,\mathrm{kJ\,g^{-1}}$ over 55 days) [15]. Likewise, energetic costs incurred from *Ichthyophonus* infection are equal to 50–100% of the overwinter energy loss measured in the wild over approximately 120 days [15, 21, 27]. We conclude that herring infected with *Ichthyophonus* had no energy available for storage.

4.2. Compensatory Response in "Spring Herring" (Ambient Water Temperature). Following food deprivation, *Ichthyophonus* infection caused an initial delay in the compensatory response of "spring herring". Thirty-one days after infection and feeding commenced, lipid stores of infected fish continued to decline by an additional 22% despite *ad libitum* food supplies. This represents a 10% accelerated rate beyond the lipid catabolism associated with fasting alone. Coincidentally, control fish incurred a 38% increase in lipid content, demonstrating the initial cost of infection superseded compensatory response. Over the ensuing 21 days, however, the body composition of infected herring recovered to values similar for continually fed fish and "spring controls", demonstrating herring's high capacity for compensatory response. Similar immediate reductions in lipid stores and energy content have been observed in juvenile halibut (*Hippoglossus stenolepis*) and channel catfish (*Ictalurus punctatus*). Initial declines in lipid and energy content facilitated rapid growth during the compensation phase [28, 29]. Similarly, infected herring had disproportionally greater growth rates than controls over the first 31 days post-infection. This was the only instance in which infected herring were larger than controls (Figure 5, day 31, ambient). As in the juvenile halibut and channel catfish [28, 29], lipid and energy content of infected herring was restored subsequently. This energetic strategy contrasts with other fish species such as juvenile Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) which initially prioritize recovery of energy reserves and secondarily prioritize structural growth [30, 31].

The various energy allocation strategies suggest life history differences among species and maturation states. For example, initial restoration of energy and fat stores by juvenile Atlantic salmon may be a mechanism to enhance spawning [31] as suspended maturation has been demonstrated in males that fail to build sufficient energy reserves prior to the maturation process [32]. Juvenile herring likely elect to replenish energy and fat stores relatively quickly in order to provide endogenous energy for winter when prey is scarce. Energy stores in the autumn have been identified as a major factor in their overwinter survival [21, 22].

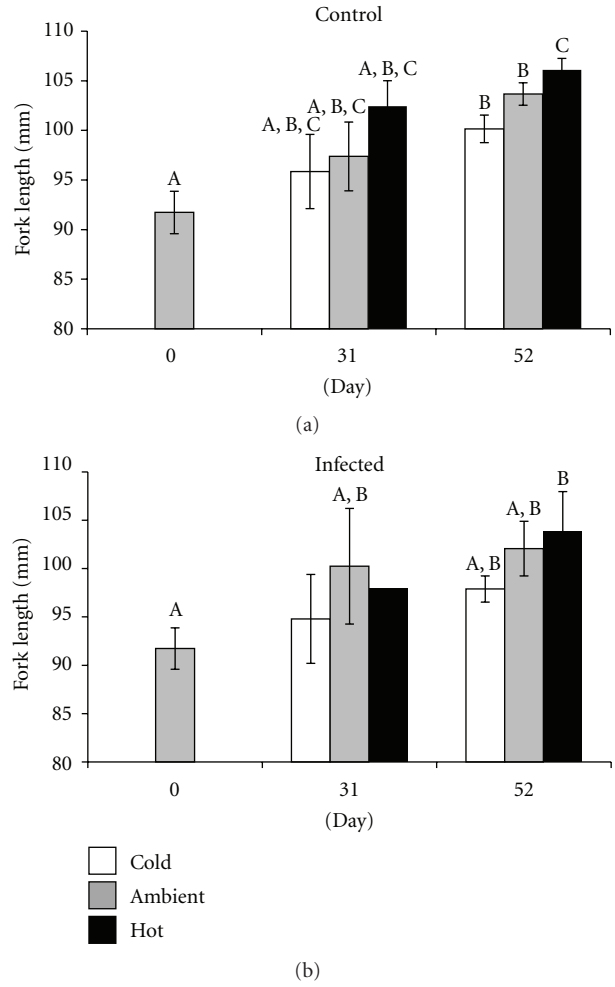


Figure 5: Growth rates of "spring" young-of-the-year herring over the course of the experiment under variable temperature conditions: cold (9.5°C), ambient (12.0°C), and hot (15.0°C). Different letters represent statistical differentiation. Lack of letters indicates no statistical differentiation. Low sample size of infected fish on day 31 precludes statistical tests.

The capacity to rapidly recover energetic condition and size during a compensatory phase following food or growth restrictions does not come without long-term costs. Of greatest concern are potential reductions in immune capacity [33]. By spring, herring have undergone several months of severe food restrictions. Spring plankton blooms likely stimulate a compensatory response. Coincidentally, excluding periods of epidemics, *Ichthyophonus* prevalence in herring is generally greatest in spring, albeit in spawning schools [1, 8]. If spawning schools and juvenile nursery areas overlap spatially, spring may be the time in which herring are most susceptible to infection due to the combined effects of (1) reduced energy state from fasting and (2) decreased immune capacity from compensatory growth.

Other deleterious effects of compensatory growth include prolonged periods of poor growth and condition despite initial restoration of lipid reserves [31]. Poor

growth may result in reduced swimming capacity [34, 35]. *Ichthyophonus* infection alone can also reduce swimming performance [11], thus there is potential for additive or synergistic effects of infection and compensatory response on swimming performance, especially as water temperatures increase [12]. Reduced swimming capacity could lead to increased vulnerability to predation [36] or declines in foraging efficiency and thus starvation. Some fish species have demonstrated the capacity to suppress metabolism under conditions of food deprivation, however, which could mediate costs associated with compensatory growth [37].

4.3. Temperature Effects on "Spring Herring". Over the temperature range we examined (9.5–15.0°C), temperature had a significant impact on the energetic costs of *Ichthyophonus* infection in "spring herring". Increasing water temperatures accommodated greater lipid and energy acquisition in juvenile herring. As a result, we observed the greatest impact of infection in cold temperatures in which energy acquisition rates were lowest. Water temperature ranges used in this study were typical of Puget Sound, however, they encompassed the optimal water temperature for juvenile Pacific herring growth of 12.2°C [38]. Alaskan water temperatures are cooler than those of Puget Sound, seasonally ranging from 4.5–10°C at 25 m water depth in Prince William Sound [22] and 6.5–13.5°C in southeast Alaska [39]. Thus the impacts of *Ichthyophonus* may be of greater consequence in colder Alaskan waters.

Ichthyophonus infection confers significant energetic costs to juvenile herring, though the mechanism behind the costs is unknown. Reduced consumption rates of infected fish likely contribute to their decreased body condition. Though consumption was not directly measured in this experiment, observational evidence suggests that infection did reduce feeding rates. Temperature-dependent consumption rates could additionally confound differences in body condition by infection and temperature. However, energetic costs incurred by infected herring were greater than would otherwise be incurred by starvation alone, suggesting additional costs likely associated with immune function are at play.

4.4. Summary. We found differential energetic impacts imposed by *Icthyophonus* infection depending on herring's condition at the time of infection. Among "autumn fish" feeding *ad libitum* with good body condition, infection caused a 30% decrease in total energy content after 52 days which is comparable in magnitude to overwinter losses in the wild which occur in less than half the time. Among "spring herring" having undergone extensive fasting with poor body condition, the energetic toll of *Ichthyophonus* infection initially delayed recovery of body composition. One month after re-feeding infected fish showed no sign of compensatory response and their total energy content was reduced by 32% relative to controls. During the subsequent 21 days compensatory response became evident, however, and the energetic costs of infection were significantly reduced such that infected fish only had a 6% reduction in total energy content relative to controls. Despite initial delays, compensatory response appears to mediate energetic costs of infection eventually, though long term consequences are unknown. Energetic impacts of infection were greatest in colder temperatures, suggesting *Ichthyophonus*-induced reductions in body condition may have greater consequences in the northern extent of herring's range where juveniles use most of their energy reserves to survive their first winter.

Acknowledgments

Funding was provided by the Exxon Valdez Oil Spill Trustee Council, projects nos. 090806, 100806, and the National Marine Fisheries Service (NMFS). This project was not possible without the herring culturing expertise and facility at the US Geological Survey, Marrowstone Marine Field Station. Significant chemistry laboratory contributions were made by Robert Bradshaw, Fletcher Sewall, and Andrew Eller (NMFS). Courtney Grady contributed significantly to fish culture and disease analysis (USGS). The findings and conclusions in the paper are those of the authors and do not necessarily represent the views of the National Marine Fisheries Service, NOAA.

References

[1] H. Rahimian and J. Thulin, "Epizootiology of *Ichthyophonus hoferi* in herring populations off the Swedish west coast," *Disease of Aquatic Organisms*, vol. 27, no. 3, pp. 187–195, 1996.

[2] A. H. McVicar, "*Ichthyophonus* and related organisms," in *Fish Diseases and Disorders, Viral, Bacterial and Fungal Infections*, P. T. K. Woo and D. W. Bruno, Eds., pp. 661–687, CABI, New York, NY, USA, 1999.

[3] R. A. Herr, L. Ajello, J. W. Taylor, S. N. Arseculeratne, and L. Mendoza, "Phylogenetic analysis of *Rhinosporidium seeberi's* 18S small-subunit ribosomal DNA groups this pathogen among members of the protoctistan mesomycetozoa clade," *Journal of Clinical Microbiology*, vol. 37, no. 9, pp. 2750–2754, 1999.

[4] R. M. Kocan, P. Hershberger, T. Mehl et al., "Pathogenicity of *Ichthyophonus hoferi* for laboratory-reared Pacific herring *Clupea pallasi* and its early appearance in wild Puget Sound herring," *Diseases of Aquatic Organisms*, vol. 35, no. 1, pp. 23–29, 1999.

[5] P. K. Hershberger, K. Stick, B. Bui et al., "Incidence of *Ichthyophonus hoferi* in Puget Sound fishes and its increase with age of Pacific herring," *Journal of Aquatic Animal Health*, vol. 14, no. 1, pp. 50–56, 2002.

[6] G. D. Marty, E. F. Freiberg, and T. R. Meyers, "Viral hemorrhagic septicemia virus, *Ichthyophonus hoferi*, and other causes of morbidity in Pacific herring *Clupea pallasi* spawning in Prince William Sound, Alaska, USA," *Diseases of Aquatic Organisms*, vol. 32, no. 1, pp. 15–40, 1998.

[7] S. R. M. Jones and S. C. Dawe, "*Ichthyophonus hoferi* Plehn & Mulsow in British Columbia stocks of Pacific herring, *Clupea pallasi Valenciennes*, and its infectivity to chinook salmon, *Oncorhynchus tshawytscha* (Walbaum)," *Journal of Fish Diseases*, vol. 25, no. 7, pp. 415–421, 2002.

[8] G. D. Marty, P. J. F. Hulson, and S. E. Miller, "Failure of population recovery in relation to disease in Pacific herring," *Diseases of Aquatic Organisms*, vol. 90, no. 1, pp. 1–14, 2010.

[9] K. R. Patterson, "Modelling the impact of disease-induced mortality in an exploited population: the outbreak of the fungal parasite *Ichthyophonus hoferi* in the North Sea herring (*Clupea harengus*)," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 53, no. 12, pp. 2870–2887, 1996.

[10] A. H. McVicar and H. A. McLay, "Tissue response of plaice, haddock, and rainbow trout to the systemic fungus Ichthyophonus," in *Fish and Shellfish Pathology*, A. E. Ellis, Ed., pp. 329–346, Academic Press, London, UK, 1985.

[11] R. Kocan, S. LaPatra, J. Gregg, J. Winton, and P. Hershberger, "Ichthyophonus-induced cardiac damage: a mechanism for reduced swimming stamina in salmonids," *Journal of Fish Diseases*, vol. 29, no. 9, pp. 521–527, 2006.

[12] R. Kocan, P. Hershberger, G. Sanders, and J. Winton, "Effects of temperature on disease progression and swimming stamina in *Ichthyophonus*-infected rainbow trout, *Oncorhynchus mykiss* (Walbaum)," *Journal of Fish Diseases*, vol. 32, no. 10, pp. 835–843, 2009.

[13] E. T. Schultz and D. O. Conover, "The allometry of energy reserve depletion: test of a mechanism for size-dependent winter mortality," *Oecologia*, vol. 119, no. 4, pp. 474–483, 1999.

[14] R. J. Beamish and C. Mahnken, "A critical size and period hypothesis to explain natural regulation of salmon abundance and the linkage to climate and climate change," *Progress in Oceanography*, vol. 49, no. 1–4, pp. 423–437, 2001.

[15] A. J. Paul and J. M. Paul, "Comparisons of whole body energy content of captive fasting age zero Alaskan Pacific herring (*Clupea pallasi Valenciennes*) and cohorts over- wintering in nature," *Journal of Experimental Marine Biology and Ecology*, vol. 226, no. 1, pp. 75–86, 1998.

[16] J. R. Post and E. A. Parkinson, "Energy allocation strategy in young fish: allometry and survival," *Ecology*, vol. 82, no. 4, pp. 1040–1051, 2001.

[17] J. N. Womble and M. F. Sigler, "Seasonal availability of abundant, energy-rich prey influences the abundance and diet of a marine predator, the Steller sea lion *Eumetopias jubatus*," *Marine Ecology Progress Series*, vol. 325, pp. 281–293, 2006.

[18] P. G. R. Jodice, D. D. Roby, K. R. Turco et al., "Assessing the nutritional stress hypothesis: relative influence of diet quantity and quality on seabird productivity," *Marine Ecology Progress Series*, vol. 325, pp. 267–279, 2006.

[19] M. Yang, "Food habits of the commercially important groundfishes in the Gulf of Alaska in 1990," *NOAA Technical Memorandum, NMFS-AFSC*, vol. 22, 59 pages, 1993.

[20] R. J. Foy and B. L. Norcross, "Spatial and temporal variability in the diet of juvenile Pacific herring (*Clupea pallasi*) in Prince William Sound, Alaska," *Canadian Journal of Zoology*, vol. 77, no. 5, pp. 697–706, 1999.

[21] R. J. Foy and A. J. Paul, "Winter feeding and changes in somatic energy content of age-0 Pacific herring in Prince William Sound, Alaska," *Transactions of the American Fisheries Society*, vol. 128, no. 6, pp. 1193–1200, 1999.

[22] B. L. Norcross, E. D. Brown, R. J. Foy et al., "A synthesis of the life history and ecology of juvenile Pacific herring in Prince William Sound, Alaska," *Fisheries Oceanography*, vol. 10, supplement 1, pp. 42–57, 2001.

[23] J. J. Vollenweider, R. A. Heintz, L. Schaufler et al., "Seasonal cycles in whole-body proximate composition and energy content of forage fish vary with water depth," *Marine Biology*, vol. 158, pp. 413–427, 2011.

[24] W. W. Christie, *Lipid Analysis: Isolation, Separation, Identification, and Structural Analysis of Lipids*, Oily Press, Bridgwater, England, 3rd edition, 2003.

[25] R. A. Sweeney and P. R. Rexroad, "Comparison of LECO FP-228 nitrogen determinator with AOAC copper catalyst Kjeldahl method for crude protein," *Journal of the Association of Official Analytical Chemists*, vol. 70, no. 6, pp. 1028–1030, 1987.

[26] J. R. Brett, "Energetics," in *Physiological Ecology of Pacific Salmon*, C. Groot, L. Margolis, and W. C. Clarke, Eds., chapter 1, UBC Press, Vancouver, Canada, 1995.

[27] A. J. Paul, J. M. Paul, and E. D. Brown, "Fall and spring somatic energy content for Alaskan Pacific herring (*Clupea pallasi Valenciennes* 1847) relative to age, size and sex," *Journal of Experimental Marine Biology and Ecology*, vol. 223, no. 1, pp. 133–142, 1998.

[28] T. P. Hurst, M. L. Spencer, S. M. Sogard, and A. W. Stoner, "Compensatory growth, energy storage and behavior of juvenile Pacific halibut *Hippoglossus stenolepis* following thermally induced growth reduction," *Marine Ecology Progress Series*, vol. 293, pp. 233–240, 2005.

[29] M. K. Kim and R. T. Lovell, "Effect of restricted feeding regimens on compensatory weight gain and body tissue changes in channel catfish *Ictalurus punctatus* in ponds," *Aquaculture*, vol. 135, no. 4, pp. 285–293, 1995.

[30] D. Álvarez and A. G. Nicieza, "Compensatory response 'defends' energy levels but not growth trajectories in brown trout, *Salmo trutta* L," *Proceedings of the Royal Society B*, vol. 272, no. 1563, pp. 601–607, 2005.

[31] I. J. Morgan and N. B. Metcalfe, "Deferred costs of compensatory growth after autumnal food shortage in juvenile salmon," *Proceedings of the Royal Society B*, vol. 268, no. 1464, pp. 295–301, 2001.

[32] D. K. Rowe, J. E. Thorpe, and A. M. Shanks, "Role of fat stores in the maturation of male Atlantic salmon (*Salmo salar*) parr," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 48, no. 3, pp. 405–413, 1991.

[33] J. D. Arendt, "Adaptive intrinsic growth rates: an integration across taxa," *Quarterly Review of Biology*, vol. 72, no. 2, pp. 149–177, 1997.

[34] J. M. Billerbeck, T. E. Lankford Jr., and D. O. Conover, "Evolution of intrinsic growth and energy acquisition rates. I. Trade-offs with swimming performance in *Menidia menidia*," *Evolution*, vol. 55, no. 9, pp. 1863–1872, 2001.

[35] N. J. Royle, J. Lindström, and N. B. Metcalfe, "Effect of growth compensation on subsequent physical fitness in green swordtails *Xiphophorus helleri*," *Biology Letters*, vol. 2, no. 1, pp. 39–42, 2006.

[36] S. B. Munch and D. O. Conover, "Rapid growth results in increased susceptibility to predation in *Menidia menidia*," *Evolution*, vol. 57, no. 9, pp. 2119–2127, 2003.

[37] K. I. O'Connor, A. C. Taylor, and N. B. Metcalfe, "The stability of standard metabolic rate during a period of food deprivation in juvenile Atlantic salmon," *Journal of Fish Biology*, vol. 57, no. 1, pp. 41–51, 2000.

[38] V. Haist and M. Stocker, "Growth and maturation of Pacific herring (*Clupea harengus pallasi*) in the Strait of Georgia," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 42, no. 1, pp. 138–146, 1985.

[39] National Data Buoy Center, 2010, http://www.ndbc.noaa.gov/.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 942616, 8 pages
doi:10.1155/2011/942616

Review Article

Helminth Parasites and the Modulation of Joint Inflammation

Chelsea E. Matisz,[1] Jason J. McDougall,[2] Keith A. Sharkey,[1,3] and Derek M. McKay[1]

[1] *Gastrointestinal Research Group, Department of Physiology and Pharmacology, The Calvin, Phoebe and Joan Snyder Institute of Infection Immunity and Inflammation, 1877 HSC University of Calgary, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1*
[2] *Inflammation Research Network, Department of Physiology and Pharmacology, The Calvin, Phoebe and Joan Snyder Institute of Infection Immunity and Inflammation, University of Calgary, 1877 HSC, 3330 Hospital Drive NW, Calgary, AB, Canada T2N 4N1*
[3] *Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada T2N 4N1*

Correspondence should be addressed to Derek M. McKay, dmckay@ucalgary.ca

Received 21 December 2010; Accepted 14 February 2011

Academic Editor: Renato A. Mortara

There is an urgent need to develop better therapeutics for autoimmune and autoinflammatory diseases, of which musculoskeletal disorders such as rheumatoid arthritis are particularly prevalent and debilitating. Helminth parasites are accomplished masters at modifying their hosts' immune activity, and so attention has focused on rodent-helminth model systems to uncover the workings of the mammalian immune response to metazoan parasites, with the hope of revealing molecules and/or mechanisms that can be translated into better treatments for human autoimmune and idiopathic disorders. Substantial proof-of-principal data supporting the concept that infection with helminth parasites can reduce the severity of concomitant disease has been amassed from models of mucosal inflammation. Indeed, infection with helminth parasites has been tried as a therapy in inflammatory bowel disease, and there are case reports relating to other conditions (e.g., autism); however, the impact of infection with parasitic helminths on musculoskeletal diseases has not been extensively studied. Here, we present the view that such a strategy should be applied to the amelioration of joint inflammation and review the literature that supports this contention.

1. Introduction

Infection with helminth parasites results in a conserved series of immune events that are orchestrated and dominated by T helper cell type 2 (Th2) events [1]. Given the reciprocity in immune regulation where, for example, Th2 cell-derived mediators inhibit the activity of Th1 cells, the hypothesis arises that individuals infected with helminth parasites could be less susceptible to other inflammatory diseases. By extrapolation, infection with helminth parasites could be used to treat disease driven by Th1 cells. Many species of parasitic helminths reside at mucosal surfaces, and it has repeatedly been shown that infection with trematode, cestode, or nematode parasites can reduce the severity of colitis and airway inflammation in murine models [2]. The impact of infection with helminths on organs outside the parasite's location has received less attention. Here, we review the effect of infection with helminth parasites on joint inflammation.

The last four decades have seen an alarming increase in the prevalence of autoimmune diseases such as inflammatory bowel disease (IBD), diabetes, multiple sclerosis, and rheumatoid arthritis (RA) in Western societies. Rheumatoid arthritis is a painful and debilitating disease that affects ~1% of the North American population [3]. The socioeconomic burden of this disease is substantial; in 2003 arthritis and other rheumatic conditions cost the United States $127.8 billion per year, an amount equivalent to 1.2% of the GDP and tantamount to a small, chronic recession [4].

Current therapies for RA include nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids (GCs), and disease-modifying antirheumatic drugs (DMARDs). As their name implies NSAIDs inhibit inflammation, although their mild-to-moderate effects do not fully alleviate the symptoms of RA. Moreover, these drugs do not inhibit the progression of arthritic disease; thus joint damage persists. Adverse reactions in the gastrointestinal tract (GIT), kidney, and central nervous systems are frequently reported with

the use of NSAIDs [5]. Glucocorticoids possess potent immunosuppressive and anti-inflammatory properties [6], but their use comes with considerable long-term risks: for example, GCs decrease calcium absorption and impair bone formation, leading to a significant decline in bone mineral density within the first 6–12 months of therapy [6]. Additional side effects of sustained GC use include weight gain, redistribution of adipose tissue, and increased risk of developing diabetes mellitus, peptic ulcers, pancreatitis, cataracts, and glaucoma [6]. While DMARDs have been successful in slowing the progression of bone and cartilage erosion, these drugs (e.g., methotrexate (MTX)), even at low doses, can generate a variety of toxic side effects [7]. Toxic effects of MTX have been observed in the liver and GIT, mucous and skin membranes, and the respiratory and hematopoietic systems [7].

Despite many pharmacological advances, the limited effectiveness and adverse side effects of current therapies for arthritic diseases highlight the urgent need for alternative treatment(s), which can only be generated through a better understanding of the mediators and mechanisms that generate pain and inflammation in the joint [8]. While animal models do not fully replicate human RA, they are invaluable tools for elucidating immunopathological processes that can cause arthritis. For example, the inhibition of arthritic disease in mice by neutralizing the proinflammatory cytokine, tumor necrosis factor (TNF)-α, has led to the introduction of anti-TNFα therapy in clinical practice [9].

2. Animal Models of Joint Inflammation

Numerous studies suggest that RA is more accurately classified as a syndrome rather than one specific disease, as evidenced by the complex pathophysiological pathways involved. There exists a wide diversity of models of joint inflammation that employ rabbits, guinea pigs, and primates, although rodents are, by far, the most commonly used (Table 1). The induction of arthritis in these models typically employs the administration of an adjuvant ± an antigen, with the antigen immunization generating immunological memory. The use of transgenic mouse technology and the development of knock-out strains of mice have provided the opportunity to examine the "spontaneous" development of arthritis, and the genes and proteins that contribute to joint inflammation.

The most commonly used model of arthritis is the type II collagen (Col II) model. Collagen is a key component of cartilage, and immunologic hypersensitivity to Col II results in joint inflammation. In this model, animals are immunized with type II collagen (from the same or a different species) that is emulsified in Freund's adjuvant. This provokes a multijoint inflammation characterized by synovitis, inflammatory cell infiltrate, pannus formation, bone and cartilage erosion, and fibrosis: features that more closely resemble human RA than those observed with adjuvant only-induced arthritis [26]. The source of collagen used for immunization affects the character of the disease: heterologous (from a different species) Col II elicits an acute and severely erosive polyarthritis 2-3 weeks after immunization, while homologous Col II evokes a severe and more chronic form of arthritis [24].

Arthritis is commonly induced through the use of Freund's adjuvant (mineral oil) with (Freund's Complete adjuvant; CFA) or without (Freund's incomplete adjuvant; IFA) the addition of an inactivated bacterium (frequently *Mycobacterium tuberculosis*). CFA is effective in stimulating cell-mediated immunity, leading to the production of antibodies. Depending on the route of administration, a systemic or local arthritic effect can be induced. Intra-articular injection of CFA results in a monoarthritis which spares the other joints, and while not fully recapitulating RA this offers the advantage of a localised inflammation with reduced systemic involvement and allows for pain assessment studies in which the contralateral, noninjected joint acts as an internal control. The CFA model is often used for testing potential anti-inflammatory or antinociceptive therapeutic agents.

The most recent addition to the panoply of models of arthritis has been the use of transgenic animals engineered to either overexpress or to lack a particular gene either globally or in a specific tissue. For example, overexpression of TNF-α resulted in the spontaneous development of a chronic erosive arthritis in 100% of the animals, with lesions similar to RA [27].

Before considering the impact of infection with helminths on joint inflammation, we must first briefly outline the immune response to infection with these metazoan parasites, as this is important to subsequent analyses of how "bystander' diseases can be modified by the parasite or the host response to the infection [1].

3. Mammalian Immune Response to Helminth Parasites

Although the precise nature of the host immune response varies considerably between species, a Th2 phenotype is a conserved response to helminth infection in mice and humans and is marked by the production of significant amounts of interleukins (IL)-4, -5, -9, -10, and -13 [1, 28], and the more recently identified IL-21 and IL-33. Mucosal mast cell hyperplasia, eosinophilia, IgE production, and increased expression of goblet cells are immunologic responses characteristic of infection with parasitic helminths [29]. Basically, there exist two potential mechanisms by which parasites can affect host immunity in a manner that would modify concomitant disease. First, products secreted from the parasite could have immunomodulatory effects; that is, they exert the capacity to directly modulate host immune functions. Second, the immune response generated to combat the infection could counteract the immunopathological reactions that drive autoimmune diseases. A widely recognized feature of helper T cells is that, in the presence of Th2 effector responses, Th1 responses are suppressed and vice versa. Thus, Th2-type reactions evoked in response to helminth infection would in theory have the ability to suppress proinflammatory Th1 responses that generate

Table 1: Summary of some of the most common rodent models of musclo-skeletal disease.

Principle	Model	Induction	Immunological features	Reference
Cartilage complex autoimmunity	Collagen-induced arthritis (CIA)	Immunization with homologous or heterologous type II collagen	(i) Increases in anticollagen AB (ii) Increases in complement, neutrophils, macrophages, B cells, and α/β $TCR^+/CD4^+$ T cells (iii) Increases in TNFα, TGFβ_2, IL-1, IL-6, IL-17 and IFNγ, and IL-12 (later phases)	[10–13]
	Proteoglycan-induced arthritis	IP injections in FCA at 0 and 1 weeks; IP injections in FIA at week 4 and 7	(i) Increases in $CD4^+$ T cells and Th1-type cytokines, particularly IFNγ	[14]
	K/BxN serum transfer	IP injection of serum from KRNxNOD strain	(i) Increases in GPI antibodies, mast cells, neutrophils, TNFα, and IL-1	[15]
Infection	Mycoplasma infection	IV injection with *M. arthriticus*	(i) Increases in Th1 in susceptible mice Increases in Th2 in arthritis-resistant mice (ii) Suppression of T-lymphocyte proliferation and IL-2 Increases in IL-4 and IL-6	[16, 17]
	Staphylococcus infection	IV injection with *S. aureus*	(i) Increases in TNFα, TNFβ, IL-1β, and IL-12	[18]
Bacterial fragment induced	Streptococcal cell wall (SCW)	IP/IA injection of SCW	(i) Increases in TNFα, TNFβ, IL-1, IL-6, IFNβ, and IFNγ (ii) Increases in $CD4^+$ T cells in chronic, not acute arthritis	[19–21]
	Mycobacterium in FCA	IA injection	(i) Increases in circulating neutrophils, TNFα, IL-12p40, and IL-17	[22, 23]
Adjuvantinduced	Avridine	SC injection	(i) Activated macrophages, $CD4^+$ T cells	[24]
	Pristane	SC (rats) or IP (mice) injection + booster 7 weeks later	(ii) Increases in TNFα, IL-1β, and IL-6	[25]
	Oil induced FIA	SC injection	(iii) Increases in macrophages, neutrophils, $CD4^+$ T cells, B cells, TNFα, and IL-6	[24]
Genetic manipulation	TNFα	TNF transgenic strain; spontaneous	(i) Increases in TNFα, IL-6, and IL-1β	[21]

(AB: antibodies; CFA: Freund's complete adjuvant; FIA: Freund's incomplete adjuvant; GPI: glucose-6 phosphate isomerase; IA: intra-articular; IFNγ: interferon-gamma; IL: interleukin; IP: intraperitoneal; IV: intravenous; KXN: T-cell receptor transgene mouse strain; NOD: nonobese diabetic mouse strain; RA: rheumatoid arthritis; SC: subcutaneous; TGFβ: transforming growth factor-beta; TNFα: tumour necrosis factor-alpha).

immunopathology. Models that incorporate infection with helminth parasites in conjunction with the expression of autoimmune diseases are proving to be extremely useful in elucidating immuno-regulatory pathways. Indeed, a picture is emerging in which infection with helminth parasites leads to a general dampening of host immunity or the creation of an immuno-regulatory environment (dominated by IL-10, transforming-growth factor (TGF-β), regulatory T cells, and alternatively activated macrophages) that can explain the seemingly paradoxical observation that the "helminth therapy" may reduce Th2-dominated conditions such as allergy and atopy [1, 2]. The goal of research with these animal models is that characterization of the host-parasite relationship will lead to the identification of therapeutic agents to control proinflammatory events.

3.1. Parasite-Derived Immunomodulatory Molecules. Virtually, all helminths studied to date express one or more molecules that can modify the activity of their host's immune response; however, the majority of these are poorly characterized [30, 31]. A notable exception is ES-62, a glycoprotein derived from *Acanthocheilonema vitea*. The numerous immuno-regulatory capacities of ES-62 are attributed to the presence of phosphorylcholine [32]. Features of ES-62-induced immune modulation include altered B-cell and cytokine proliferation, a shift towards an anti-inflammatory phenotype of antigen presenting cells, and reduced mast cell degranulation. Mechanistically, the action of ES-62 is in part attributed to its ability to downregulate protein kinase C, a signalling enzyme that plays a role in B cell proliferation and mast cell degranulation [33, 34]. In murine models, ES-62 induces a cytokine and antibody shift characterized by increased levels of IL-10, reduced levels of proinflammatory cytokines (IL-12, TNFα), and the production of IgG1 molecules [34]. Thus, ES-62 does not simply function as an immunosuppressant; rather it induces active anti-inflammatory responses via Th2-driven events.

Administration of ES-62 can protect mice from collagen-induced arthritis through its inhibition of the production and activity of proinflammatory cytokines (e.g., IL-6, TNFα, and IFNγ) and collagen-specific antibodies and evoking the increased synthesis of IL-10 [35]. In terms of the relief of the symptoms of arthritis, ES-62 treatment resulted in reduced progression of knee swelling with the involvement of fewer joints, and synovial hyperplasia, cellular infiltration, and bone erosion were suppressed. In the presence of ES-62, cultures from human RA synovial fluid and membranes expressed reduced levels of TNFα, IL-6 [35], and IFNγ [36], while peripheral blood mononuclear cells from patients with RA exhibited low IFNγ secretion following ES-62 challenge [36].

Despite the myriad of partially purified helminth-derived molecules that can suppress or redirect mammalian immune responses, the promise of helminths as a source of anti-inflammatory or immunomodulatory drugs has yet to materialize [30, 31]. One reason for this has been the inability to isolate pure antigens/molecules from small amounts of parasite tissue; however, we contend that this should not deter the search for these molecules. Technological advances in recent years (e.g., mass spectrometry) have enhanced our ability to isolate, characterize, and purify molecules from miniscule amounts of starting material. Additionally, a wealth of simple bioassays is available to screen the potential immunomodulatory properties of these molecules.

4. Suppression of Joint Inflammation by Helminth Parasites

Numerous proof-of-principal studies have shown that the severity of concomitant disease in mice (e.g., colitis, airway hyper-reactivity, and experimental allergic encephalitis) can be reduced by prophylactic or therapeutic infection with parasitic helminths [2]. The mechanism of the reduction in disease has been, in a species- and model-specific manner, ascribed to the inhibition of Th1 cytokine production [37], induction of regulatory cytokines (e.g., IL-10 and TGFβ) [38, 39], production of Foxp3$^+$ regulatory T cells [40], and activity of alternatively activated macrophages [41]. (A smaller number of reports have presented the caveat that infection with helminth parasites can exaggerate other disease conditions [42].) To date we are aware of only five publications that have assessed the effect of infection with helminth parasites on joint inflammation.

To our knowledge, the first report on reduced arthritic disease in helminth-infected rodents was the serendipitous observation that rats infected with the nematode *Syphacia obvelata* had less CFA-induced arthritis (as gauged by the number of joints involved and degree of swelling) [43]. The identification of the helminth was made following the observation that some of the CFA-treated rats had limited disease—that is, the investigators had not purposefully infected a cohort of animals; rather the infection was unintended.

Spontaneous arthritis develops in Murphy Roths Large (MRL)/lpr mice. Infection of these mice with either of the nematodes *Heligmosomoides polygyrus* or *Nippostrongylus brasiliensis* resulted in a slightly reduced incidence of arthritis and a reduction in synovial hyperplasia. Other significant features of arthritic disease, including pannus formation, cartilage erosion, and bone destruction, were not attenuated by infection with either helminth [44]. While attesting to the potential of infection with helminth parasites to reduce the severity of arthritic disease, this study revealed no specific anti-inflammatory mechanism of action.

Recently, Osada and colleagues demonstrated that infection with the trematode *Schistosoma mansoni* attenuated Col II-induced arthritis in mice [45]. In that study, naïve mice or those infected two weeks previously with *S. mansoni* were immunized with heterologous Col II. The infected animals developed less severe arthritis, as indicated by reduced limb involvement and swelling. While typical arthritic features such as synovial hyperplasia, inflammatory cell recruitment, and bone/cartilage destruction were present in uninfected Col II immunized mice, mice infected with *S. mansoni* were protected from arthritis as assessed by these measures [45]. Dose studies indicated that 4-5 pairs of *S. mansoni*

were sufficient to provide an antiarthritic effect. By 8 and 12 weeks after immunization the levels of IgG_{2a}, which are involved in the pathology of Col II-induced arthritis, were significantly lowered in *S. mansoni*-infected mice. Polyclonal stimulated splenic T cells from Col II-injected mice produced substantial amounts of IFNγ, IL-17A, and TNFα, and comparatively little IL-4 and IL-10. The reverse is true of T cells retrieved from Col II+*S. mansoni*-treated mice. In addition, quantitative PCR revealed upregulation of IL-1β and IL-6, and receptor activator of nuclear factor κ-B ligand (RANKL) in the paws of Col II arthritic mice that was not observed in the *S. mansoni*-infected mice. Analysis of IL-10 and TGFβ mRNA revealed no differences between the groups, and somewhat, counterintuitively, there was a subtle increase in Foxp3 mRNA in paws from immunized mice that was not observed in Col II+*S. mansoni*-treated mice. Collectively, these data demonstrate the capacity of a prophylactic infection with *S. mansoni* to attenuate the severity of collagen-induced arthritic disease, which may be due to systemic suppression of proinflammatory cytokine production and enhanced synthesis of IL-10. Very similar results have been reported by He et al. [46], who showed inhibition of Col II-induced arthritis in mice infected seven weeks, but not two weeks, previously with *Schistosoma japonicum*.

Most recently it was found that infection with the rat tapeworm, *Hymenolepis diminuta* significantly attenuated CFA-induced arthritis [23]. In this model, CFA was injected directly into the knee of naïve Balb/c or C57/Bl6 mice. A subset of animals was infected 8 days previously with *H. diminuta*, a time-point at which immunological expulsion of the helminth is underway. CFA-treated mice displayed an increase in knee diameter of 20–40%, increased synovial blood flow, increased myeloperoxidase (MPO) activity (a measure of granulocyte infiltrate, typically neutrophils), and increased pain sensitivity; all of these signs and symptoms of joint inflammation were reduced in the parasitized mice [23]. Moreover, infection with *H. diminuta* was as effective as treatment with either the steroid dexamethasone or the NSAID indomethacin, at inhibiting the proarthritic effects of intra-articular injection of CFA. In addition, the antiarthritic effect of infection with *H. diminuta* was absent in mice lacking T and B cells or the α-chain of the IL-4 receptor, suggesting that the active adaptive immune response against the parasite had the bystander effect of antagonising the effects of CFA. Typical of the response to infection with helminth parasites, spleen cells from the infected mice produced significantly more IL-4 and IL-10 when challenged with the T cell-mitogen, concanavalin A, than splenocytes from CFA-only-treated or naïve mice [23].

A subsequent series of mechanistic studies employed (i) an *in vivo* neutralizing antibody against IL-10 and (ii) IL-10 KO mice and (iii) adoptive transfer of $CD4^+$ T cells from *H. diminuta*-infected wild-type or IL-10 KO mice [23]. The outcome of these investigations supports the conclusions that the IL-10 component of the response to infection with *H. diminuta* is important for the inhibition of CFA-induced joint inflammation and that $CD4^+$ T cells (which could be a source of IL-10) from infected mice can transfer protection against the proarthritic effects of CFA injection [23].

5. The Challenges Ahead

The concept of "helminth therapy" has generated significant interest among scientists, the public, and some patients for whom standard therapies have either limited effectiveness or have failed [47, 48]. While the study of helminth parasites in models of arthritis has lagged behind those of other diseases (e.g., IBD [49]), data from murine model systems indicate that infection with helminth parasites can alleviate the severity of joint inflammation. Further, studies in other models of arthritis are warranted. These should be complemented with clinical data and observations and ultimately carefully controlled double-blind clinical trails. At this juncture, beyond the suggestion of the value of small open-label studies with a defined helminth in a well-characterized group of patients with arthritis, it is, in our view, premature to advocate helminth therapy for joint inflammation. A major challenge in the field will be the identification and characterization of specific antigens from a given helminth that drives the protective response and those antigens/molecules that can serve as blueprints for drug design in the development of novel anti-inflammatories or analgesics for the treatment of an array of human maladies.

There are potential drawbacks to helminth therapy that need to be carefully assessed: (i) certain parasites (e.g., *S. mansoni*) are not viable candidates because of the disease they cause (this does not diminish their value in animal models for the elucidation of anti-inflammatory mechanisms and immunological principles), (ii) the spectre of iatrogenic infection, and (iii) the possibility that infection with specific parasites could be additive to an existing condition or promote the clinical manifestation of a disease or comorbidities. For instance, recent data suggest that helminth therapy could be contraindicated in treating a disease characterized by increased numbers of eosinophils [50]. Moreover, despite the many reports of infection with helminth parasites blocking disease in animal models, the exact mechanism of action is not well defined, and potential side effects [51], dosing regimens, the impact of other helminth or protozoan infections, and the immunological and nutritional status of the host are important issues that remain unexplored.

Assessments of the anti-inflammatory effect(s) of infection with helminth parasites have focused on immune factors: an obvious and important starting point [1, 2]. However, lipid-derived molecules, stromal (e.g., fibroblasts) and endocrine cells, and modulation of the normal microflora of the gut all have the potential to dampen disease propagating events. The role of these factors in helminth inhibition of concomitant inflammatory disease is unknown and should be the focus of future research endeavours.

6. Conclusions

While a few studies have shown that infection with helminth parasites can exaggerate existing disease, the consensus is that

the generation of an immunoregulatory environment as a consequence of infection with parasitic helminths can reduce the severity of concomitant disease (an arrangement that would benefit parasites while they complete their lifecycle). The consideration of the impact of infection with helminth parasites on arthritic disease is limited, but the available data support the general concept of "helminth therapy"—or rather, that data obtained from models of arthritis and concomitant helminth infection have the potential to reveal novel approaches to treat arthritis. While a number of obvious caveats pertain to helminth therapy, there is no doubt that there is much to be learned from analyses of rodent-helminth model systems, and a "tolerant" view of "our wormy friends" may be warranted. This is an exciting area of translational research that will eventually generate novel data on anti-inflammatory, proresolution, or immunoregulatory mediators, cells and pathways that can be converted into new approaches to autoinflammatory and idiopathic human diseases. The notion of being able to prescribe a specific parasitic helminth to treat a disease in an individual whose immunological status has been carefully characterized could be a tantalizing aspect of future "personalised medicine." However, we see this as a future possibility and not a future certainty and caution individuals to resist any temptation to infect themselves with a helminth parasite. Finally, we would emphasize that animal models need to be more rigorously employed to comprehensively define all immuneregulatory ramifications and potential side effects of infection with parasitic helminths in the context of other disease.

Conflict of Interests

The authors have no financial or other conflicts to declare.

Acknowledgments

The work from the authors' laboratory was supported by a Canadian Institutes of Health Research Grant (no. MOP-218990). D. M. McKay and K. A. Sharkey hold Scientist Awards from the Alberta Heritage Foundation for Medical Research (AHFMR), J. J. McDougall is an AHFMR Senior Scholar. D. M. McKay is recipient of a Canada Research Chair (Tier 1), K. A. Sharkey holds the Crohn's and Colitis Foundation of Canada (CCFC) Chair in Inflammatory Bowel Disease (IBD) Research at the University of Calgary, J. J. McDougall is an Arthritis Society of Canada Investigator, and C. E. Matisz is a recipient of an Achievers in Medical Science (AIMS) graduate studentship from the University of Calgary and an Alberta Innovates-Health Solutions (AI-HS) Graduate Studentship.

References

[1] R. M. Maizels, "Exploring the immunology of parasitism from surface antigens to the hygiene hypothesis," *Parasitology*, vol. 136, no. 12, pp. 1549–1564, 2009.

[2] D. M. McKay, "The therapeutic helminth?" *Trends in Parasitology*, vol. 25, no. 3, pp. 109–114, 2009.

[3] A. M. Abdel-Nasser, J. J. Rasker, and H. A. Valkenburg, "Epidemiological and clinical aspects relating to the variability of rheumatoid arthritis," *Seminars in Arthritis and Rheumatism*, vol. 27, no. 2, pp. 123–140, 1997.

[4] M. G. Cisternas, L. B. Murphy, E. H. Yelin, A. J. Foreman, D. J. Pasta, and C. G. Helmick, "Trends in medical care expenditures of US adults with arthritis and other rheumatic conditions 1997 to 2005," *Journal of Rheumatology*, vol. 36, no. 11, pp. 2531–2538, 2009.

[5] R. O'Day, L. M. March, G. G. Graham, K. F. Scott, and K. M. Williams, "NSAIDs and analgesics," in *Rheumatoid Arthritis*, G. S. Firestein, G. S. Panayi, and F. A. Wollheim, Eds., pp. 317–336, Oxford University Press, New York, NY, USA, 2006.

[6] J. W. J. Biljsma and W. G. Jacobs, "Systemic and intra-articular glucocorticoids in rheumatoid arthritis," in *Rheumatoid Arthritis*, G. S. Firestein, G. S. Panayi, and F. A. Wollheim, Eds., pp. 337–354, Oxford University Press, New York, NY, USA, 2006.

[7] R. Rau, "The new frontier in pathogenesis and treatment: methotrexate and leflunomide," in *Rheumatoid Arthritis*, G. S. Firestein, G. S. Panayi, and F. A. Wollheim, Eds., pp. 355–368, Oxford Universty Press, New York, NY, USA, 2006.

[8] J. J. McDougall, "Arthritis and pain. Neurogenic origin of joint pain," *Arthritis Research and Therapy*, vol. 8, article 220, 2006.

[9] P. C. Taylor and M. Feldmann, "Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis," *Nature Reviews Rheumatology*, vol. 5, no. 10, pp. 578–582, 2009.

[10] A. Mussener, M. J. Litton, E. Lindroos, and L. Klareskog, "Cytokine production in synovial tissue of mice with collagen-induced arthritis (CIA)," *Clinical and Experimental Immunology*, vol. 107, no. 3, pp. 485–493, 1997.

[11] L. Svensson, J. Jirholt, R. Holmdahl, and L. Jansson, "B cell-deficient mice do not develop type II collagen-induced arthritis (CIA)," *Clinical and Experimental Immunology*, vol. 111, no. 3, pp. 521–526, 1998.

[12] S. Thornton, L. E. Duwel, G. P. Boivin, Y. Ma, and R. Hirsch, "Association of the course of collagen-induced arthritis with distinct patterns of cytokine and chemokine messenger RNA expression," *Arthritis and Rheumatism*, vol. 42, no. 6, pp. 1109–1118, 1999.

[13] E. Lubberts, L. A. B. Joosten, B. Oppers et al., "IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis," *Journal of Immunology*, vol. 167, no. 2, pp. 1004–1013, 2001.

[14] A. Finnegan, K. Mikecz, P. Tao, and T. T. Glant, "Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines," *Journal of Immunology*, vol. 163, no. 10, pp. 5383–5390, 1999.

[15] H. J. Ditzel, "The K/BxN mouse: a model of human inflammatory arthritis," *Trends in Molecular Medicine*, vol. 10, no. 1, pp. 40–45, 2004.

[16] B. C. Cole, E. Ahmed, B. A. Araneo et al., "Immunomodulation *in vivo* by the *Mycoplasma arthritidis* superantigen, MAM," *Clinical Infectious Diseases*, vol. 17, no. 1, pp. S163–S169, 1993.

[17] M. Hong-Hua, A. D. Sawitzke, and B. C. Cole, "Modulation of cytokine profiles by the mycoplasma superantigen *Mycoplasma arthritidis* mitogen parallels susceptibility to arthritis induced by *M. arthritidis*," *Infection and Immunity*, vol. 68, no. 3, pp. 1142–1149, 2000.

[18] Y. X. Zhao, A. Ljungdahl, T. Olsson, and A. Tarkowski, "*In situ* hybridization analysis of synovial and systemic cytokine

messenger RNA expression in superantigen-mediated Staphylococcus aureus arthritis," *Arthritis and Rheumatism*, vol. 39, no. 6, pp. 959–967, 1996.

[19] S. M. Wahl, J. B. Allen, K. Ohura, D. E. Chenoweth, and A. R. Hand, "IFN-γ inhibits inflammatory cell recruitment and the evolution of bacterial cell wall-induced arthritis," *Journal of Immunology*, vol. 146, no. 1, pp. 95–100, 1991.

[20] R. C. Schimmer, D. J. Schrier, C. M. Flory et al., "Streptococcal cell wall-induced arthritis: requirements for neutrophils, P-selectin, intercellular adhesion molecule-1, and macrophage-inflammatory protein-2," *Journal of Immunology*, vol. 159, no. 8, pp. 4103–4108, 1997.

[21] K. Kannan, R. A. Ortmann, and D. Kimpel, "Animal models of rheumatoid arthritis and their relevance to human disease," *Pathophysiology*, vol. 12, no. 3, pp. 167–181, 2005.

[22] K. A. Bush, K. M. Farmer, J. S. Walker, and B. W. Kirkham, "Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein," *Arthritis and Rheumatism*, vol. 46, no. 3, pp. 802–805, 2002.

[23] M. Shi, A. Wang, D. Prescott et al., "Infection with an intestinal helminth parasite reduces Freund's complete adjuvant-induced monoarthritis in mice," *Arthritis and Rheumatism*, vol. 63, no. 2, pp. 434–444, 2011.

[24] R. Holmdahl, "Experimental models for RA," in *Rheumatoid Arthritis*, G. S. Firestein, G. S. Panayi, and F. A. Wollheim, Eds., pp. 41–54, Oxford University Press, New York, NY, USA, 2006.

[25] C. Patten, K. Bush, I. Rioja et al., "Characterization of pristane-induced arthritis, a murine model of chronic disease: response to antirheumatic agents, expression of joint cytokines, and immunopathology," *Arthritis and Rheumatism*, vol. 50, no. 10, pp. 3334–3345, 2004.

[26] R. O. Williams, "Rodent models of arthritis: relevance for human disease," *Clinical and Experimental Immunology*, vol. 114, no. 3, pp. 330–332, 1998.

[27] A. M. Bendele, "Animal models of rheumatoid arthritis," *Journal of Musculoskeletal and Neuronal Interactions*, vol. 1, no. 4, pp. 377–385, 2001.

[28] D. Artis, "New weapons in the war on worms: identification of putative mechanisms of immune-mediated expulsion of gastrointestinal nematodes," *International Journal for Parasitology*, vol. 36, no. 6, pp. 723–733, 2006.

[29] W. C. Gause, J. F. Urban, and M. J. Stadecker, "I-The immune response to parasitic helminths: insights from murine models," *Trends in Immunology*, vol. 24, no. 5, pp. 269–277, 2003.

[30] M. J. G. Johnston, J. A. MacDonald, and D. M. McKay, "Parasitic helminths: a pharmacopeia of anti-inflammatory molecules," *Parasitology*, vol. 136, no. 2, pp. 125–147, 2009.

[31] W. Harnett and M. M. Harnett, "Helminth-derived immunomodulators: can understanding the worm produce the pill?" *Nature Reviews Immunology*, vol. 10, no. 4, pp. 278–284, 2010.

[32] W. Harnett, M. M. Harnett, B. P. Leung, J. A. Gracie, and I. B. McInnes, "The anti-inflammatory potential of the filarial nematode secreted product, ES-62," *Current Topics in Medicinal Chemistry*, vol. 4, no. 5, pp. 553–559, 2004.

[33] A. J. Melendez, M. M. Harnett, P. N. Pushparaj et al., "Inhibition of FcεRI-mediated mast cell responses by ES-62, a product of parasitic filarial nematodes," *Nature Medicine*, vol. 13, no. 11, pp. 1375–1381, 2007.

[34] M. M. Harnett, A. J. Melendez, and W. Harnett, "The therapeutic potential of the filarial nematode-derived immunodulator, ES-62 in inflammatory disease," *Clinical and Experimental Immunology*, vol. 159, no. 3, pp. 256–267, 2010.

[35] I. B. McInnes, B. P. Leung, M. Harnett, J. A. Gracie, F. Y. Liew, and W. Harnett, "A novel therapeutic approach targeting articular inflammation using the filarial nematode-derived phosphorylcholine-containing glycoprotein ES-62," *Journal of Immunology*, vol. 171, no. 4, pp. 2127–2133, 2003.

[36] I. B. McInnes and G. Schett, "Cytokines in the pathogenesis of rheumatoid arthritis," *Nature Reviews Immunology*, vol. 7, no. 6, pp. 429–442, 2007.

[37] W. I. Khan, P. A. Blennerhasset, A. K. Varghese et al., "Intestinal nematode infection ameliorates experimental colitis in mice," *Infection and Immunity*, vol. 70, no. 11, pp. 5931–5937, 2002.

[38] M. M. Hunter, A. Wang, C. L. Hirota, and D. M. McKay, "Neutralizing anti-IL-10 antibody blocks the protective effect of tapeworm infection in a murine model of chemically induced colitis," *Journal of Immunology*, vol. 174, no. 11, pp. 7368–7375, 2005.

[39] T. Setiawan, A. Metwali, A. M. Blum et al., "Heligmosomoides polygyrus promotes regulatory T-cell cytokine production in the murine normal distal intestine," *Infection and Immunity*, vol. 75, no. 9, pp. 4655–4663, 2007.

[40] J. R. Grainger, K. A. Smith, J. P. Hewitson et al., "Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-β pathway," *Journal of Experimental Medicine*, vol. 207, no. 11, pp. 2331–2341, 2010.

[41] A. Espinoza-Jiménez, I. Rivera-Montoya, R. Cárdenas-Arreola, L. Morán, and L. I. Terrazas, "*Taenia crassiceps* infection attenuates multiple low-dose streptozotocin-induced diabetes," *Journal of Biomedicine & Biotechnology*, vol. 2010, Article ID 850541, 11 pages, 2010.

[42] M. M. Hunter, A. Wang, and D. M. Mckay, "Helminth infection enhances disease in a murine TH2 model of colitis," *Gastroenterology*, vol. 132, no. 4, pp. 1320–1330, 2007.

[43] D. J. Pearson and G. Taylor, "The influence of the nematode *Syphacia oblevata* on adjuvant arthritis in the rat," *Immunological Communications*, vol. 29, no. 2, pp. 391–396, 1975.

[44] M. C. Salinas-Carmona, G. de la Cruz-Galicia, I. Pérez-Rivera et al., "Spontaneous arthritis in MRL/lpr mice is aggravated by *Staphylococcus aureus* and ameliorated by *Nippostrongylus brasiliensis* infections," *Autoimmunity*, vol. 42, no. 1, pp. 25–32, 2009.

[45] Y. Osada, S. Shimizu, T. Kumagai, S. Yamada, and T. Kanazawa, "*Schistosoma mansoni* infection reduces severity of collagen-induced arthritis via down-regulation of pro-inflammatory mediators," *International Journal for Parasitology*, vol. 39, no. 4, pp. 457–464, 2009.

[46] Y. He, J. Li, W. Zhuang et al., "The inhibitory effect against collagen-induced arthritis by Schistosoma japonicum infection is infection stage-dependent," *BMC Immunology*, vol. 11, article 28, pp. 128–133, 2010.

[47] Z. Cormier, "Can hookworms cure allergies?" 2010, http://www.besthealthmag.ca/get-healthy/health/can-hookworms-cure-allergies.

[48] N. Seppa, "Worming your way to better health," *Science News*, pp. 26–29, 2011.

[49] J. V. Weinstock and D. E. Elliott, "Helminths and the IBD hygiene hypothesis," *Inflammatory Bowel Diseases*, vol. 15, no. 1, pp. 128–133, 2009.

[50] A. Wang, M. Fernando, G. Leung, V. Phan, D. Smyth, and D. M. McKay, "Exaggeration of oxazolone-induced colitis

by *Hymenolepis diminuta*: involvement of interleukin 5," *American Journal of Pathology*, vol. 177, no. 6, pp. 2850–2859, 2010.

[51] A. Melon, A. Wang, V. Phan, and D. M. McKay, "Infection with *Hymenolepis diminuta* is more effective than daily corticosteroids in blocking chemically induced colitis in mice," *Journal of Biomedicine & Biotechnology*, vol. 2010, Article ID 384523, 7 pages, 2010.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 563412, 9 pages
doi:10.1155/2011/563412

Research Article

Effects of Environmental Temperature on the Dynamics of Ichthyophoniasis in Juvenile Pacific Herring (*Clupea pallasii*)

Jake L. Gregg,[1] Johanna J. Vollenweider,[2] Courtney A. Grady,[1] Ron A. Heintz,[2] and Paul K. Hershberger[1]

[1] *U.S. Geological Survey, Western Fisheries Research Center, Marrowstone Marine Field Station, 616 Marrowstone Point Road, Nordland, WA 98358, USA*
[2] *NOAA National Marine Fisheries Service, Alaska Fisheries Science Center, Auke Bay Laboratories, 17109 Pt. Lena Loop Road, Juneau, AK 99801, USA*

Correspondence should be addressed to Jake L. Gregg, jgregg@usgs.gov

Received 23 November 2010; Accepted 8 February 2011

Academic Editor: Bernard Marchand

The effects of temperature and infection by *Ichthyophonus* were examined in juvenile Pacific herring (*Clupea pallasii*) maintained under simulated overwinter fasting conditions. In addition to defining parameters for a herring bioenergetics model (discussed in Vollenweider et al. this issue), these experiments provided new insights into factors influencing the infectivity and virulence of the parasite *Ichthyophonus*. In groups of fish with established disease, temperature variation had little effect on disease outcome. *Ichthyophonus* mortality outpaced that resulting from starvation alone. In newly infected fish, temperature variation significantly changed the mortality patterns related to disease. Both elevated and lowered temperatures suppressed disease-related mortality relative to ambient treatments. When parasite exposure dose decreased, an inverse relationship between infection prevalence and temperature was detected. These findings suggest interplay between temperature optima for parasite growth and host immune function and have implications for our understanding of how *Ichthyophonus* infections are established in wild fish populations.

1. Introduction

Ichthyophonus hoferi, a highly pathogenic parasite of marine and anadromous fishes, is currently ubiquitous in Pacific herring (*Clupea pallasii*) populations throughout the NE Pacific [1–3]. Phenotypic [4] and genotypic [5, 6] differences among isolates of *I. hoferi* suggest that there are multiple sympatric species in the region. Due to this taxonomic uncertainty, here we refer to the parasite by its generic name. Among wild Pacific herring, the prevalence of infection typically increases with age; consequently, the resulting disease (ichthyophoniasis) is thought to affect primarily older age cohorts [2, 7]. However, the impact of *Ichthyophonus* infections on juvenile Pacific herring has received little attention. In Atlantic herring (*Clupea harengus*), it has been suggested that time to mortality is shorter for juveniles than for adults resulting in relatively low, but highly variable, prevalence [8]. Prevalence of infection among young-of-the-year (YOY) herring is typically 3–13% in the NE Pacific (USGS unpublished data) with infections detected as early as 4 months after hatch [9]. In addition to causing direct mortality from disease, *Ichthyophonus* infection may predispose juvenile herring to indirect mortality through predation [10], presumably as a result of decreased swimming performance [11, 12]. This type of indirect mortality is difficult to demonstrate but should be expected considering the extensive tissue damage that can result from ichthyophoniasis. *Ichthyophonus* invades multiple visceral organs and can appear in the heart within 26 h after exposure [13]. Kocan et al. [11] reported a 40% increase in cardiac weight in infected rainbow trout (*Oncorhynchus mykiss*) resulting from parasite biomass and host inflammatory response. The physiological cost of ichthyophoniasis and resulting reduction in fitness could compound the effects of other environmental and biological stressors experienced by juvenile Pacific herring.

In the NE Pacific, specifically Prince William Sound, the overwinter fast has been identified as a primary factor limiting YOY herring survival [14]. This study and its companion

(see in Vollenweider et al. this issue) were designed to improve our understanding of the bioenergetic costs of overwinter fasting for juvenile Pacific herring. We were specifically interested in the compounding effects of disease, temperature, and starvation on survival, and the effect of temperature on disease processes. Three experiments were conducted to determine (1) effects of temperature on mortality in fasting Pacific herring with established disease, (2) effects of temperature on disease progression and mortality in fasting Pacific herring immediately after parasite exposure, and (3) effects of temperature on disease progression and mortality in Pacific herring recovering from pre-exposure fast.

2. Materials and Methods

2.1. Pacific Herring. To ensure that animals were parasite-free and immunologically naïve at initiation of experiments, a cohort of specific-pathogen-free (SPF) Pacific herring was reared for this study at the USGS Marrowstone Marine Field Station (MMFS) (Nordland, WA, USA). Naturally spawned eggs from wild Pacific herring were collected from the southern Strait of Georgia (48°55.85′N, 122°48.15′W) on 5 May 2008 and transported to MMFS where they were held in 270 L tanks supplied with filtered, UV-irradiated seawater. Eggs hatched on 18 May 2008 and larvae were subsequently moved to 1700 L tanks where they were fed enriched live feeds (i.e., *Brachionus plicatilis* and *Artemia franciscana*) and frozen copepods (Cyclop-eeze, Argent Chemical Laboratories, Redmond, WA, USA) until metamorphosis to juveniles. Juvenile herring were weaned to a krill-meal pellet produced at the USFWS Abernathy Fish Technology Center (Longview, WA, USA) and eventually to commercially available salmon pellet (Bio-Olympic Fry, Bio-Oregon, Longview, WA, USA).

2.2. Experiment Design and Environment. Three experiments were performed over an eleven-month period to investigate the effects of fasting and temperature on disease dynamics in *Ichthyophonus*-infected Pacific herring (Table 1). Two treatments, infected and uninfected, were applied to fasting herring held at each of three temperatures (low, ambient, and high). To control for the effect of fasting, this 2×3 design was modified with the addition of two fed treatments, infected and uninfected, maintained at ambient temperature. Herring were randomly assigned to triplicate 270 L tanks in each treatment. Flow through seawater (low, ambient, and high temperature) was delivered to each tank at the rate of 4 L min^{-1}. The temperature regulating system allowed temperatures to vary with seasonal seawater temperature, but maintained a relatively consistent separation between low, ambient, and high temperature treatments. Seawater temperatures in low and high treatments were adjusted gradually over the first 24 h after fish were distributed to replicate tanks. Water temperature was recorded every 30 min (Hobo Water Temp Pro v2, Onset Computer Corp., Bourne, MA, USA). Temperature treatments will be identified here as the mean temperature that occurred throughout the experimental period.

2.3. Parasite Exposure. *Ichthyophonus* exposure for herring in infected treatments occurred via intraperitoneal (IP) injection. To obtain stock material for *Ichthyophonus* inoculum, heart tissues from *Ichthyophonus*-infected herring (wild and laboratory infected) were cultured in Eagle's minimum essential medium supplemented with fetal bovine serum (5% v/v), penicillin (100 IU mL^{-1}), streptomycin (100 μg mL^{-1}) and gentamycin (100 μg mL^{-1}) and buffered to pH 7.8 with 1 M Tris (MEM). After 2–4 weeks in culture, thousands of *Ichthyophonus* schizonts (i.e., spherical multinucleate bodies) had grown out of the tissues and were free in the media. Host tissues and medium were removed from these cultures with sterile pipettes, several cultures were combined, and this mixture was diluted in sterile phosphate-buffered saline (PBS) to produce an inoculating solution that contained approximately 2000 schizonts mL^{-1}. Fifty μL of inoculum was then injected into each fish with the goal of introducing 100 schizonts/fish regardless of size. For each experiment, three 50 microliter aliquots were collected during the injection process and schizonts were counted at 40X magnification on an inverted microscope. Final inoculum concentration (i.e. dose) for each experiment was determined by dividing the mean number of schizonts by mean fish weight.

2.4. Effects of Temperature on Mortality in Fasting Pacific Herring with Established Disease. Juvenile SPF herring (age = 145 d) were inoculated with *Ichthyophonus* schizonts (mean = 174 schizonts $fish^{-1}$) and placed in a 760 L holding tank. Nineteen days post-exposure (DPE), when external signs of ichthyophoniasis (i.e. black skin ulcerations) were evident in many individuals, herring from this tank and a corresponding tank containing uninfected controls were distributed to triplicate 260 L tanks (n = 47 to 52 fish/tank) at each of three temperatures, 6.7°C (low), 9.3°C (ambient), and 12.3°C (high), and fasting was initiated. Additional 9.3°C (ambient) treatment groups with infected and uninfected herring were fed to satiation daily. The experiment was terminated 80 DPE.

2.5. Effects of Temperature on Disease Progression and Mortality in Fasting Pacific Herring Immediately after Parasite Exposure. Juvenile SPF herring (age = 241 d) were injected with *Ichthyophonus* schizonts (mean = 167 schizonts $fish^{-1}$) or PBS and immediately distributed to triplicate tanks (n = 49 to 51 fish/tank) at each of three temperatures: 5.6°C (low), 7.9°C (ambient), and 12.4°C (high), and fasting was initiated. Additional 7.9°C (ambient) treatment groups with infected and uninfected herring were fed to satiation daily. Treatments were terminated between 111 and 127 DPE.

2.6. Effects of Temperature on Disease Progression and Mortality in Pacific Herring Recovering from Pre-Exposure Fast. Feed was withheld from 607 SPF herring held in two 760 L tanks and a second group of 300 SPF herring in a single 760 L tank were fed to excess 3x $week^{-1}$ (i.e., >10% biomass per feeding). The experiment was initiated after 56 d when herring from the fasted colony were injected with

Table 1: *Clupea pallasii*. Summary data for 3 *Ichthyophonus* experiments conducted in this study. Pacific herring in experiments 1 and 2 fasted during challenge, herring in experiment 3 fasted for 56 d prior to challenge and then fed to satiation during challenge.

Experiment			Herring			*Ichthyophonus*		Temperature (°C)[a]		
No.	Disease state	Duration (d)	Age (d)	FL (mm)	Weight (g)	Schizonts[b] per inoculation	Dose (schizonts g^{-1})[c]	Low	Amb	High
(1)	Diseased: 19 d after exposure	80	164	44	1.0	174	174	6.7	9.3	12.3
(2)	Infected: 1 d after exposure	127	241	70	3.6	167	46	5.6	7.9	12.4
(3)	Infected: 1 d after exposure	54	431	92 (100)[d]	6.5 (9.2)[d]	112	17 (12)[d]	9.3	12.0	15.3

[a]Temperature reported is mean of temperature recordings made every 30 minutes during course of challenge.
[b]Schizonts are multinucleate spherical bodies from *in vitro Ichthyophonus* cultures, number reported is mean of three 50 μL samples.
[c]Dose = mean number of schizonts divided by mean weight of fish.
[d]Values in parentheses are from group of herring that did not go through prechallenge fast in experiment (3).

either *Ichthyophonus* or PBS and transferred to triplicate tanks (N = 27 to 30 fish per tank) at each of three temperatures: 9.3°C (low), 12.0°C (ambient), and 15.3°C (high). Herring from the fed colony were injected with either *Ichthyophonus* or PBS and transferred to triplicate tanks at 12.0°C (ambient). Fish in all treatments (previously fasted and fed) were fed to satiation daily after exposure. Live herring were subsampled (n = 2 $tank^{-1}$) for bioenergetic analysis (see in Vollenweider et al. this issue) 31 DPE, and the experiment was terminated 54 DPE.

2.7. Fish Sampling. A subsample (n = 20 fish) was taken from pre-experiment pools for fork length (FL) and weight (WT) measurements at the initiation of each experiment. Mortalities in all challenges were removed from tanks daily, and survivors were euthanized with an overdose of tricaine methane sulfonate in buffered seawater at the termination of each experiment. Prevalence of infection and disease was determined among mortalities and survivors from all experiments. Prevalence of infection was determined by *in vitro* explant culture of heart tissue in MEM. Heart cultures were incubated at 15°C and examined microscopically (40x magnification) for the presence of *Ichthyophonus* schizonts and/or hyphae after 14 d. Culture-positive samples were considered diseased when visible lesions indicative of ichthyophoniasis were observed on the skin and/or heart.

2.8. Statistics. Cumulative mortality was compared between treatments at the midpoint and end of each experiment using a single factor (treatment) ANOVA followed by a Tukey Test for multiple comparisons. Infection and disease prevalence were similarly compared between the *Ichthyophonus*-exposed treatments. All analyses were conducted using arcsine transformed data. Statistical significance was assigned to comparisons with $P \leq .05$.

3. Results

3.1. Mortality in Fasting Pacific Herring with Established Disease. Herring were 44 mm FL (SD = 4.7) and 1.0 g WT (SD = 0.38) at the initiation of this challenge. A dose of 174 schizonts g^{-1} successfully established *Ichthyophonus* infections, and a majority of the infections progressed to overt disease. In *Ichthyophonus*-exposed groups, infection prevalence ranged from 95.9 to 98.6% with no significant differences between treatments ($F_{0.05(2),3,8} = 1.749$, $P < .23$). Similarly, prevalence of disease (i.e., clinical signs) among infected fish ranged from 97.0 to 99.3% with no significant differences between treatments ($F_{0.05(2),3,8} = 1.089$, $P < .41$). *Ichthyophonus* was not detected in any herring from unexposed control groups.

Cumulative mortality in infected treatments generally followed a sigmoid pattern reaching 44% to 55% by the midpoint of the experiment and 65% to 98% by the end (Figure 1). Cumulative mortality varied significantly with treatment at the midpoint of the experiment (day 40; $F_{0.05(2),7,16} = 102.2$, $P < 4.4 \times 10^{-12}$). The Tukey test for multiple comparisons grouped all infected treatments separate from uninfected controls on 40 DPE, with no separation due to temperature. Starvation mortality in fasting, uninfected treatments followed a predictable pattern in relation to temperature. At 52, 61, and 72 DPE, cumulative mortality reached 10% in 12.3°C (high), 9.3°C (ambient), and 6.7°C (low) treatments, respectively, and increased exponentially until termination of the experiment (Figures 1(a), 1(b), and 1(c)). Starvation mortality continued in infected treatments late into the challenge preventing a plateau that appeared to be developing in infected treatments around 60% to 70%. This plateau did develop in the single infected treatment (9.3°C) that was fed throughout the challenge (Figure 1(d)). At termination of the experiment, significant differences in cumulative mortality did exist between treatments (day 80; $F_{0.05(2),7,16} = 55.3$, $P < 5.0 \times 10^{-10}$), but the Tukey test did not group the treatments by infection status as on day 40. At termination cumulative mortality in fasting uninfected treatments approached or surpassed that resulting from disease alone. Cumulative mortality in the uninfected, fed treatment (9.3°C) remained below 5% (Figure 1(d)).

3.2. Disease Progression and Mortality in Fasting Pacific Herring Immediately after Parasite Exposure. Herring were

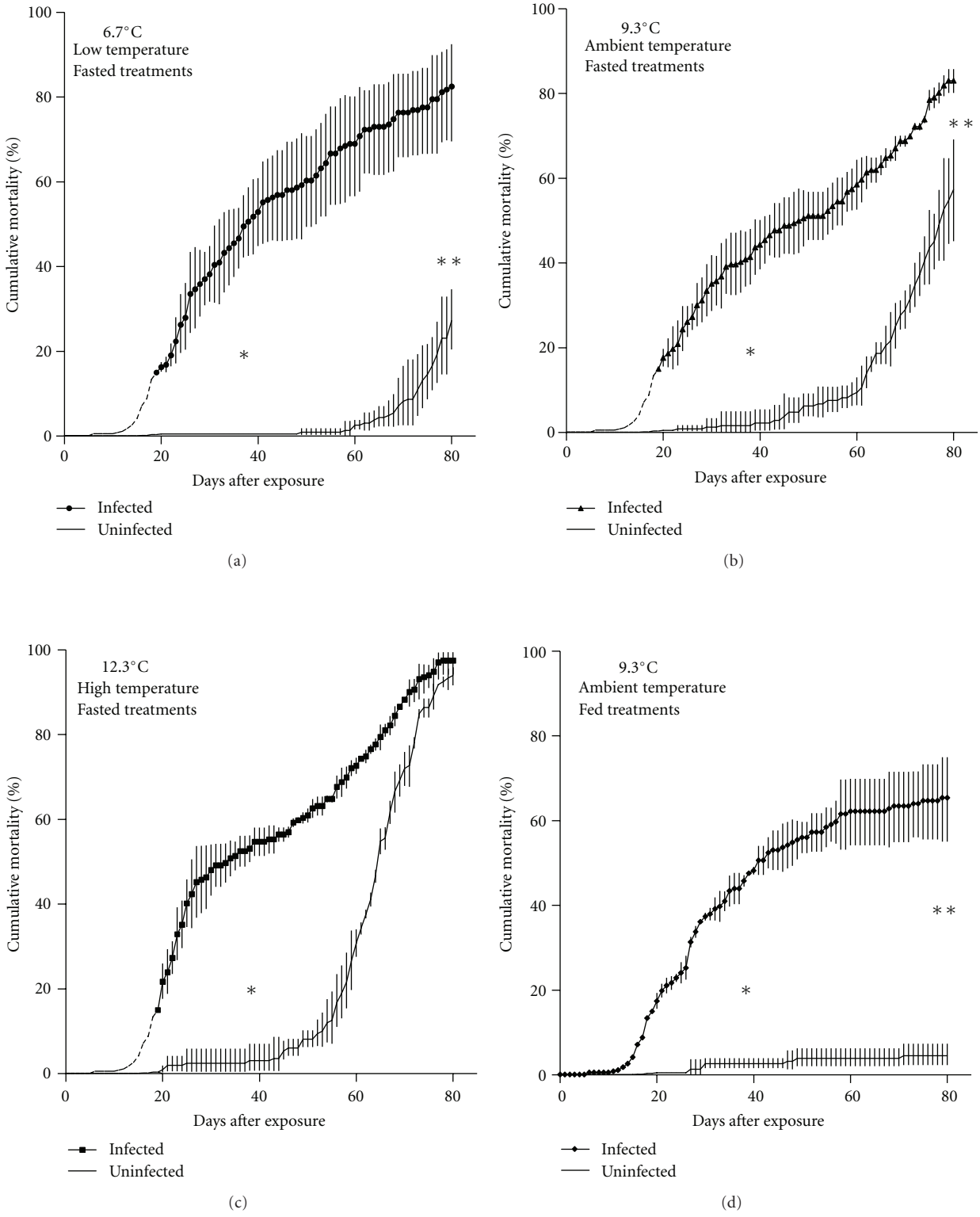


Figure 1: *Clupea pallasii*. Cumulative mortality of diseased (ichthyophoniasis) and control Pacific herring in three temperature treatments. Data in (a), (b), and (c) are from fish undergoing simulated overwinter fast. Data in (d) are from fed treatments. Temperature adjustments were made 19 days after inoculation with *Ichthyophonus* schizonts. Data are means of 3 replicate tanks in each treatment. Error bars are one SD above and below the mean. Means and SD were calculated from arcsine-transformed data. Mortality data prior to day 19 are from the pools of infected and control fish prior to separation into treatment. Significant differences between infected and uninfected groups (Tukey multiple comparisons) indicated by $*$ and $**$ for midpoint and end of challenge, respectively.

70 mm FL (SD = 6.3) and 3.6 g WT (SD = 0.97) at the initiation of this challenge. A dose of 46 schizonts g^{-1} successfully established *Ichthyophonus* infections, and a majority of the infections progressed to overt disease. In *Ichthyophonus*-exposed groups, infection prevalence ranged from 95.4% to 98.0% with no significant differences between treatments ($F_{0.05(2),3,8} = 1.005$, $P < .44$). Similarly, prevalence of disease (i.e., clinical signs) among infected fish ranged from 95.1% to 99.3% with no significant differences between treatments ($F_{0.05(2),3,8} = 1.977$, $P < .20$). *Ichthyophonus* was not detected in any herring from PBS-injected control groups.

Disease-related mortality was negated in fasting Pacific herring moved to 5.6°C and 12.4°C shortly after *Ichthyophonus* exposure (Figures 2(a) and 2(c)), while the newly exposed Pacific herring remaining at 7.9°C displayed mortality kinetics (Figures 2(b) and 2(d)) similar to fish with established disease (Figures 1(b) and 1(d)). Significant difference did occur between treatments at midpoint ($F_{0.05(2),7,16} = 11.83$, $P < 2.8 \times 10^{-5}$) and end ($F_{0.05(2),7,16} = 30.45$, $P < 4.3 \times 10^{-8}$) of the challenge, but these differences were not simply the result of differences between infected and uninfected treatments. Cumulative mortality at 5.6°C reached 48.7% and 45.3% in infected and uninfected treatments, respectively. Little separation occurred between these treatments at the midpoint or end of the experiment. Similarly, there were no significant differences between uninfected and infected treatments at 12.4°C, where mortality reached 92.0% and 96.5%, respectively, by the end of the experiment. Mortality curves in infected groups held at 5.6°C and 12.4°C were similar to those which resulted from starvation alone (Figures 2(a) and 2(c)), a simple exponential increase. Mortality in herring held at 7.9°C after exposure varied significantly with infection status (Figures 2(b) and 2(d)). Cumulative mortality in fed infected herring reached 25.3% 55 DPE and plateaued just below 50% around 90 DPE. Mortality of fasted infected herring reached 34.6% 55 DPE but did not plateau as starvation mortality (evident in uninfected groups) ensued, pushing cumulative mortality to 83.1% by the end of the experiment. Fed uninfected fish experienced less than 5% mortality.

3.3. Disease Progression and Mortality in Pacific Herring Recovering from Pre-Exposure Fast. Prevalence of *Ichthyophonus* infection after IP exposure to schizonts was inversely related to water temperature. Pre-exposure fasting resulted in herring that were 92 mm FL (SD = 11.7) and 6.5 g WT (SD = 2.4). A dose of 17 schizonts g^{-1} established infection in 76.3%, 53.9%, and 23.9% of these fish in 9.3°C, 12.0°C, and 15.3°C (low, ambient, and high) treatments, respectively. The pre-exposure fed group was larger (100 mm, SD = 12.3; 9.2 g, SD = 3.2) resulting in a lower dose of 12 schizonts g^{-1}. This dose established infections in 30.9% of the fish at 12.0°C. Prevalence of infection varied significantly with treatment (Figure 3; $F_{0.05(2),3,8} = 5.956$, $P < .02$). Among infected herring, prevalence of disease (i.e., clinical signs) ranged from 95.0% to 100% with no significant differences between treatments ($F_{0.05(2),3,8} = 0.668$, $P < .59$). *Ichthyophonus* was not detected in any control herring injected with PBS.

Mortality resulting from handling and injection of fish at the initiation of this experiment was very high, most likely due to the weakened condition of the fish at the end of the prechallenge fast, some treatments lost as many as 28% of fish in first few days after exposure. For this reason, we calculated cumulative mortality based on the fish that remained in the tanks on day 10 and only compared cumulative mortality (ANOVA) at the end of the challenge. Overall, adjusted cumulative mortality was lower and more variable than in the previous two challenges; 6.1% to 15.9% in uninfected treatments and 11.8% to 24.9% in infected treatments; ANOVA detected no significant differences between treatments ($F_{0.05(2),7,16} = 2.23$, $P < .09$).

4. Discussion

Disease processes do vary with temperature in juvenile Pacific herring infected with *Ichthyophonus*. However, the degree to which changes in temperature alter the outcome (i.e, infection prevalence or cumulative mortality) for *Ichthyophonus*-infected groups depends on the stage/intensity of the infection. Temperature manipulation had no detectable effect on *Ichthyophonus* infections that had progressed to overt disease. Mortality in these cases outpaced that which resulted from starvation alone (Figure 1). However, disease outcome was affected when temperature manipulation occurred within 24 h of *Ichthyophonus* exposure. Cumulative mortality in high (12.4°C) and low (5.6°C) temperature infected treatments was not significantly different from that of corresponding uninfected groups, while mortality kinetics in ambient treatments (7.9°C) did differ with infection status (Figure 2). Suppression of *Ichthyophonus* mortality was so complete in high and low temperatures that net *Ichthyophonus* mortality (i.e., disease mortality—control mortality) remained low throughout the study and at some time points was negative. Slower parasite growth could explain the lower mortality in low temperature treatments, as our experiments were conducted in a range where temperature and *Ichthyophonus* growth (*in vitro*) are directly related [15, 16]. However, if the relationship was simply based on parasite growth, the trend would hold and mortality would increase with increasing temperature, as has been demonstrated in some bacteria-salmonid systems [17, 18]. Suppressed mortality from ichthyophoniasis in both low and high temperature treatments suggests interplay of temperature optima for parasite growth and host immune function.

Innate and adaptive immune functions of fish are significantly affected by environmental temperature [19–22]. Immunocompetence is generally considered to vary directly with temperature within the homeostatic range of the host; however, there is suggestion that at low temperatures some nonspecific immune functions may increase to compensate for suppression of adaptive immune responses [22]. Complement-mediated lytic activity [23], production of macrophage activating factor [24], natural antibody activity [19], and protection resulting from vaccination [25] have all been shown to increase with increased temperature. The immune response to histozoic parasites includes an innate response at the mucous layer, an innate response in tissues,

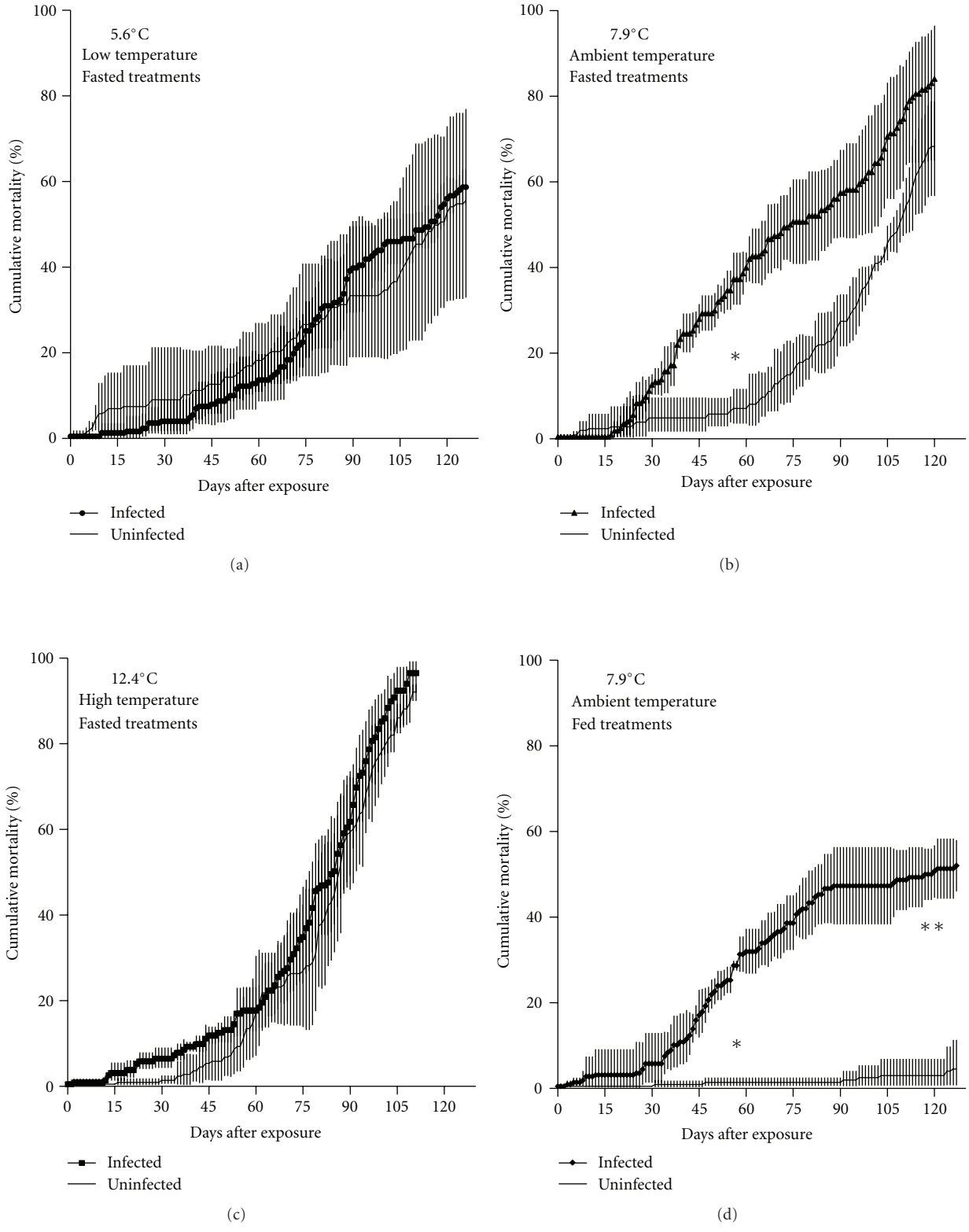


Figure 2: *Clupea pallasii*. Cumulative mortality of infected (*Ichthyophonus*) and control Pacific herring in three temperature treatments. Data in (a), (b), and (c) are from fish undergoing simulated overwinter fast. Data in (d) are from fed treatments. *Ichthyophonus* inoculation occurred on day 0, temperature adjustments on day 1. Data are means of 3 replicate tanks in each treatment. Error bars are one SD above and below the mean. Means and SD were calculated from arcsine-transformed data. Significant differences between infected and uninfected groups (Tukey multiple comparisons) indicated by $*$ and $**$ for midpoint and end of challenge, respectively.

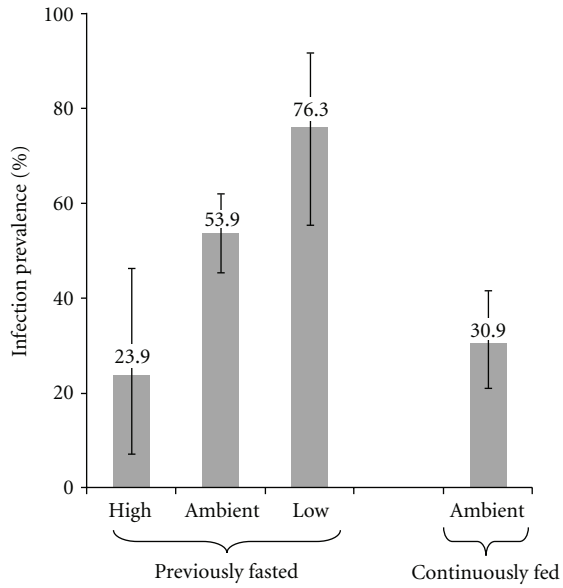


Figure 3: *Clupea pallasii. Ichthyophonus* infection prevalence in groups of Pacific herring held at three temperatures. Data are means of arcsine transformed data from 3 replicate tanks; error bars are one SD above and below the mean. Mean temperatures were 9.3°C, 12.0°C, and 15.3°C for low, ambient, and high treatments, respectively. Prechallenge fast lasted 56 days, after which fish were inoculated with *Ichthyophonus*, separated to treatments, and fed to satiation daily. ANOVA and Tukey test for multiple comparisons indicates that high-temperature treatment and continuously fed treatments are significantly different from low-temperature treatment.

and eventually an adaptive response in tissues [26, 27]. *Ichthyophonus* has been shown to elicit a cell mediate innate immune response in plaice (*Pleuronectes platessa*), haddock (*Melanogrammus aeglefinus*), and rainbow trout (*O. mykiss*) with persistent infections resulting in focal granulomata that encapsulate the parasite [13]. An antibody response has been demonstrated in plaice, but there is no evidence that it is protective [28].

The suppression of *Ichthyophonus* by a temperature-enhanced innate response is a plausible explanation for the reduced *Ichthyophonus* mortality that we observed in the newly exposed high-temperature treatment. This hypothesis is further supported by prevalence data from the pre-exposure fast challenge, where 76%, 54%, and 24% of fish were infected in the low, ambient, and high temperature fasted/fed, treatments, respectively (Figure 3). The relative dose of *Ichthyophonus* decreased over the course of this study as fish mass increased (Table 1). In the third challenge, fasted fish received 17 schizonts g^{-1}. At this dose, a fraction of the fish were able to clear the schizonts and prevent establishment of infection, and the number that was able to do so was markedly (3x) higher in the high-temperature treatment. This phenomenon was not evident in the second challenge where inoculation dose was 46 schizonts g^{-1}, suggesting that high-parasite load can mask temperature effects. The importance of parasite load/dose in determining disease outcome is also evident in comparison of ambient treatments in the third challenge. Previously fasted fish received 17 schizonts g^{-1}, while continuously fed received 12 schizonts g^{-1}, resulting in mean infection prevalence of 54% and 31%, respectively (Figure 3).

The temperature-driven variation demonstrated in this study has implications for our understanding of disease processes in wild herring populations. The temperatures used (5.6°C to 15.3°C) are within those that the host species can experience across its geographic distribution [29–32], and the magnitude of temperature variation used in each challenge (5-6°C) could plausibly be experienced by a single individual as the result of season temperature fluctuation and migration [14, 33]. We did not see temperature effects in diseased fish but did see differences at early stages of infection, especially when exposure dose was low, suggesting that seawater temperature at time of exposure could be an important factor that determines disease prevalence over ensuing months and years. Unfortunately, the complete life cycle of *Ichthyophonus* is not known, and whether or not the disease is monoxenous in Pacific herring is still unclear. Other species such as rainbow trout, haddock, and plaice can become infected by eating infected fish tissues [11, 13], but the route of infection in Pacific herring is still in question. Juveniles captured shortly after metamorphosis are infected when they are still obligate planktivores [9]. Future field surveys and empirical studies should focus on closing the life history gaps that exist for *Ichthyophonus*, specifically the modality and timing of infection in Pacific herring. Once these variables are understood, more focused work could incorporate the findings of this study to mimic possible environmental processes at the time of infection.

Acknowledgments

Funding was provided by the *Exxon Valdez* Oil Spill Trustee Council, Projects nos. 1010086, 090806, 080806, and 070819 and U.S. Geological Survey Fisheries and Aquatic Resources Program. Technical assistance was provided by L. Taylor and M. Denny (USGS-MMFS). Custom made fish feed was offered in kind by Ann Gannam (USFWS). The use of trade, firm, or corporation names in this paper is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Interior or the U.S. Geological Survey of any product or service to the exclusion of others that may be suitable.

References

[1] G. D. Marty, E. F. Freiberg, T. R. Meyers, J. Wilcock, T. B. Farver, and D. E. Hinton, "Viral hemorrhagic septicemia virus, *Ichthyophonus hoferi*, and other causes of morbidity in Pacific herring *Clupea pallasi* spawning in Prince William Sound, Alaska, USA," *Diseases of Aquatic Organisms*, vol. 32, no. 1, pp. 15–40, 1998.

[2] P. K. Hershberger, K. Stick, B. Bui et al., "Incidence of *Ichthyophonus hoferi* in Puget Sound fishes and its increase with age of Pacific herring," *Journal of Aquatic Animal Health*, vol. 14, no. 1, pp. 50–56, 2002.

[3] S. R. M. Jones and S. C. Dawe, "*Ichthyophonus hoferi* Plehn & Mulsow in British Columbia stocks of Pacific herring, *Clupea pallasi* Valenciennes, and its infectivity to chinook salmon, *Oncorhynchus tshawytscha* (Walbaum)," *Journal of Fish Diseases*, vol. 25, no. 7, pp. 415–421, 2002.

[4] P. K. Hershberger, C. A. Pacheco, J. L. Gregg, M. K. Purcell, and S. E. LaPatra, "Differential survival of *Ichthyophonus* isolates indicates parasite adaptation to its host environment," *Journal of Parasitology*, vol. 94, no. 5, pp. 1055–1059, 2008.

[5] C. Rasmussen, M. K. Purcell, J. L. Gregg, S. E. LaPatra, J. R. Winton, and P. K. Hershberger, "Sequence analysis of the internal transcribed spacer (ITS) region reveals a novel clade of *Ichthyophonus* sp. from rainbow trout," *Diseases of Aquatic Organisms*, vol. 89, no. 2, pp. 179–183, 2010.

[6] C. D. Criscione, V. Watral, C. M. Whipps, M. S. Blouin, S. R. M. Jones, and M. L. Kent, "Ribosomal DNA sequences indicate isolated populations of *Ichthyophonus hoferi* in geographic sympatry in the north-eastern Pacific Ocean," *Journal of Fish Diseases*, vol. 25, no. 10, pp. 575–582, 2002.

[7] G. D. Marty, T. J. Quinn, G. Carpenter, T. R. Meyers, and N. H. Willits, "Role of disease in abundance of a Pacific herring (*Clupea pallasi*) population," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 60, no. 10, pp. 1258–1265, 2003.

[8] H. Rahimian and J. Thulin, "Epizootiology of *Ichthyophonus hoferi* in herring populations off the Swedish west coast," *Disease of Aquatic Organisms*, vol. 27, no. 3, pp. 187–195, 1996.

[9] R. M. Kocan, P. Hershberger, T. Mehl et al., "Pathogenicity of *Ichthyophonus hoferi* for laboratory-reared Pacific herring *Clupea pallasi* and its early appearance in wild Puget Sound herring," *Diseases of Aquatic Organisms*, vol. 35, no. 1, pp. 23–29, 1999.

[10] S. Kramer-Schadt, J. C. Holst, and D. Skagen, "Analysis of variables associated with the *Ichthyophonus hoferi* epizootics in Norwegian spring spawning herring, 1992–2008," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 67, no. 11, pp. 1862–1873, 2010.

[11] R. Kocan, S. LaPatra, J. Gregg, J. Winton, and P. Hershberger, "*Ichthyophonus*-induced cardiac damage: a mechanism for reduced swimming stamina in salmonids," *Journal of Fish Diseases*, vol. 29, no. 9, pp. 521–527, 2006.

[12] R. Kocan, P. Hershberger, G. Sanders, and J. Winton, "Effects of temperature on disease progression and swimming stamina in *Ichthyophonus*-infected rainbow trout, *Oncor-hynchus mykiss* (Walbaum)," *Journal of Fish Diseases*, vol. 32, no. 10, pp. 835–843, 2009.

[13] A. H. McVicar and H. A. McLay, "Tissue response of plaice, haddock, and rainbow trout to the systemic fungus *Ichthyophonus*," *Fish and Shellfish Pathology*, pp. 329–346, 1985.

[14] B. L. Norcross, E. D. Brown, R. J. Foy et al., "A synthesis of the life history and ecology of juvenile Pacific herring in Prince William Sound, Alaska," *Fisheries Oceanography*, vol. 10, no. 1, pp. 42–57, 2001.

[15] S. A. Hart, "The effect of temperature and media type on the production of *Ichthyophonus* in vitro," unpublished Student Papers, Emerging Diseases and Latent Infections in Aquatic Organisms. University of Washington, Friday Harbor Laboratories: Friday Harbor, Wash, USA, 2003.

[16] B. Spanggaard and H. H. Huss, "Growth of the fish parasite *Ichthyophonus hoferi* under food relevant conditions," *International Journal of Food Science and Technology*, vol. 31, no. 5, pp. 427–432, 1996.

[17] R. A. Holt, J. E. Sanders, J. L. Zinn, J. L. Fryer, and K. S. Pilcher, "Relation of water temperature to *Flexibacter columnaris* infection in steelhead trout (*Salmo gairdneri*), coho (*Oncorhynchus kisutch*) and chinook (*O. tshawytscha*) salmon," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 32, no. 9, pp. 1553–1559, 1975.

[18] W. J. Groberg Jr., R. H. McCoy, K. S. Pilcher, and J. L. Fryer, "Relation of water temperature to infections of coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tsawytscha*) and steelhead trout (*Salmo gairdneri*) with *Aeromonas salmonicida* and *A. hydrophila*," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 35, no. 1, pp. 1–7, 1978.

[19] B. Magnadóttir, "Innate immunity of fish (overview)," *Fish & Shellfish Immunology*, vol. 20, no. 2, pp. 137–151, 2006.

[20] M. Watts, B. L. Munday, and C. M. Burke, "Immune responses of teleost fish," *Australian Veterinary Journal*, vol. 79, no. 8, pp. 570–574, 2001.

[21] T. J. Bowden, K. D. Thompson, A. L. Morgan, R. M. L. Gratacap, and S. Nikoskelainen, "Seasonal variation and the immune response: a fish perspective," *Fish & Shellfish Immunology*, vol. 22, no. 6, pp. 695–706, 2007.

[22] C. Le Morvan, D. Troutaud, and P. Deschaux, "Differential effects of temperature on specific and nonspecific immune defences in fish," *Journal of Experimental Biology*, vol. 201, no. 2, pp. 165–168, 1998.

[23] H. Boshra, J. Li, and J. O. Sunyer, "Recent advances on the complement system of teleost fish," *Fish & Shellfish Immunology*, vol. 20, no. 2, pp. 239–262, 2006.

[24] L. J. Hardie, T. C. Fletcher, and C. J. Secombes, "Effect of temperature on macrophage activation and the production of macrophage activating factor by rainbow trout (*Oncorhynchus mykiss*) leucocytes," *Developmental and Comparative Immunology*, vol. 18, no. 1, pp. 57–66, 1994.

[25] M. Alishahi and K. Buchmann, "Temperature-dependent protection against *Ichthyophthirius multifiliis* following immunisation of rainbow trout using live theronts," *Diseases of Aquatic Organisms*, vol. 72, no. 3, pp. 269–273, 2006.

[26] K. Buchmann, J. Sigh, C. V. Nielsen, and M. Dalgaard, "Host responses against the fish parasitizing ciliate *Ichthyophthirius multifiliis*," *Veterinary Parasitology*, vol. 100, no. 1-2, pp. 105–116, 2001.

[27] S. R. M. Jones, "The occurrence and mechanisms of innate immunity against parasites in fish," *Developmental and Comparative Immunology*, vol. 25, no. 8-9, pp. 841–852, 2001.

[28] A. H. McVicar, "*Ichthyophonus* and related organisms," in *Fish Diseases and Disorders, Viral, Bacterial and Fungal Infections*, P. T. K. Woo and D. W. Bruno, Eds., pp. 661–687, CABI Publishing, New York, NY, USA, 1999.

[29] D. R. Lassuy, "Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Pacific Northwest)—Pacific herring," *Biological Report—US Fish & Wildlife Service*, vol. 82, no. 11, 1989.

[30] A. Huyer, "Seasonal variation in temperature, salinity, and density over the continental shelf off Oregon," *Limnology and Oceanography*, vol. 22, no. 3, pp. 442–453, 1977.

[31] S. K. Moore, N. J. Mantua, J. A. Newton, M. Kawase, M. J. Warner, and J. P. Kellogg, "A descriptive analysis of temporal and spatial patterns of variability in Puget Sound oceanographic properties," *Estuarine, Coastal and Shelf Science*, vol. 80, no. 4, pp. 545–554, 2008.

[32] B. C. Weare, A. R. Navato, and R. E. Newell, "Empirical orthogonal analysis of pacific sea surface temperatures," *Journal of Physical Oceanography*, vol. 6, no. 5, pp. 671–678, 1976.

[33] D. J. Miller and J. Schmidtke, "Report on the distribution and abundance of Pacific herring (*Clupea pallasi*) along the coast of Central and Southern California," *California Fish and Game*, vol. 42, pp. 163–187, 1956.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 479146, 7 pages
doi:10.1155/2011/479146

Review Article

Possible Roles of Ectophosphatases in Host-Parasite Interactions

Marta T. Gomes,[1,2] Angela H. Lopes,[3] and José Roberto Meyer-Fernandes[1,2]

[1] *Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, CCS, Bloco H, Cidade Universitária, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil*
[2] *Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem (INCTBEB), CCS, Bloco H, Cidade Universitária, 21941-902 Rio de Janeiro, RJ, Brazil*
[3] *Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, CCS, Bloco H, Cidade Universitária, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to José Roberto Meyer-Fernandes, meyer@bioqmed.ufrj.br

Received 22 November 2010; Revised 7 February 2011; Accepted 1 March 2011

Academic Editor: Barbara Papadopoulou

The interaction and survival of pathogens in hostile environments and in confrontation with host immune responses are important mechanisms for the establishment of infection. Ectophosphatases are enzymes localized at the plasma membrane of cells, and their active sites face the external medium rather than the cytoplasm. Once activated, these enzymes are able to hydrolyze phosphorylated substrates in the extracellular milieu. Several studies demonstrated the presence of surface-located ecto-phosphatases in a vast number of pathogenic organisms, including bacteria, protozoa, and fungi. Little is known about the role of ecto-phosphatases in host-pathogen interactions. The present paper provides an overview of recent findings related to the virulence induced by these surface molecules in protozoa and fungi.

1. Introduction

Cells are exposed to diverse environmental stimuli throughout their cycles in all biological systems. Protein phosphorylation and dephosphorylation are central events in cell recognition of external and internal signals, leading to specific responses. While protein kinases transfer a phosphate group from ATP to a protein (i.e., phosphorylate), protein phosphatases catalyze the removal of phosphate groups from specific residues of proteins (i.e., dephosphorylate) [1, 2]. The balance between the antagonistic activities of protein kinases and phosphatases are responsible for many cellular functions, including metabolic pathways, cell-cell communication, proliferation, and gene transcription [3].

The complete genome sequencing of various microorganisms made it possible to assemble the kinome and phosphatome of a few trypanosomatids [4, 5]. These strategies have brought new perspectives of researches in the areas of biochemistry, physiology, and genetics, providing knowledge about the microorganisms' life cycles, as well as predicting diagnostic biomarkers, novel drug targets and vaccine candidates against parasitic infections.

Parasites engage a plethora of surface and secreted molecules in order to attach and enter mammalian cells. Some of these molecules are involved in triggering specific signaling pathways both in the parasite and the host cell, which are critical for parasite entry and survival [6]. Plasma membranes of cells contain enzymes that are oriented with their active sites facing the external medium rather than the cytoplasm, which are important for host-parasite interactions [7, 8]. In the case of an ectoenzyme other criteria can be included as: (1) the enzyme has to act on extracellular substrate, (2) cellular integrity is maintained during enzyme activity, (3) the products are released extracellularly, (4) the enzyme is not released to the extracellular environment; and (5) the enzyme activity can be modified by nonpenetrating reagents [7, 8]. Supporting this idea, the presence of surface-located phosphatases, called ecto or extracytoplasmic phosphatases have been characterized in several microorganisms. However, the physiological roles of these enzymes in these cells are not well established yet. In eukaryotes, the most predominant phosphorylation sites are detected on serine, threonine and tyrosine residues. Thus, catalytic signature

motifs and substrate preferences classified these proteins into four major groups: phosphoprotein phosphatases (PPPs), metallo-dependent protein phosphatases (PPMs), aspartate-based phosphatases with a DxDxT/V motif (the members of these three groups are Ser/Thr specific phosphatases) and the distinct group of protein tyrosine phosphatases (PTPs) [9]. Protein tyrosine phosphatases belong to three evolutionarily unrelated classes: protein tyrosine phosphatases (PTPs), Cdc25 and low molecular weight phosphatases (LMW-PTPs), which have a common motif (CX5R) in their catalytic sites [10]. The classical PTPs are classified, depending on the presence or absence of transmembrane domains, into receptor or nonreceptor type phosphatase groups.

The use of inhibitors, divalent cations, metal chelators and different pH range has also been an important tool for classification of these enzymes. Likewise, phosphatases may be acid or alkaline according to their pH range for activity. The optimum pH for acid ectophosphatases lies on the acid range (pH values between 4.5 and 5.5), while the optimum pH for alkaline ectophosphatases lies on the alkaline range (pH values between 8.0 and 9.0) [9, 10]. The inhibitors classically used include: phosphotyrosine phosphatase inhibitors ammonium molybdate and sodium orthovanadate; acid phosphatase inhibitor sodium fluoride (NaF); secreted phosphatase inhibitor sodium tartrate; alkaline phosphatase inhibitor levamisole and phosphoserine/threonine phosphatases inhibitors okadaic acid and microcystin-LR [11–13]. Several biological roles for ectophosphatases have been proposed. These enzymes may provide microorganisms with a source of inorganic phosphate by hydrolyzing phosphomonoester metabolites [13–15] protect them upon entering the macrophage by suppressing the respiratory burst [16], as well as play a role in cell differentiation [17], infection of host cells [18–20] and protecting the cells from acidic conditions by buffering the periplasmic space with phosphate released from polyphosphates [21]. Some protein phosphatases have been described as being active towards low molecular weight nonproteic phosphoesters, such as alkyl and aryl phosphates, including the phosphotyrosine analog, *p*-nitrophenylphosphate [18]. From a general standpoint, the surface accessibility of ectophosphatases, along with protein phosphorylated on serine/threonine/tyrosine residues at the cell surface make this set of enzymes a key tool for the survival of pathogens in hostile environments and escaping the host immune responses [19, 22–24]. In this review, we describe the role of ectophosphatase activities in host-parasite interactions, particularly ectophosphatases in parasitic protozoa and fungi.

2. Ectophosphatase Activities in Protozoa Infection

Little is still known about the physiological role of protein phosphatase activity in trypanosomatids, even though the first demonstration of this activity in *Trypanosoma brucei* and *T. cruzi* took place in 1972 [25]. The kinetoplastid parasites have complex life cycles and some of their life forms are difficult to grow in culture, which may represent a problem for studying ectophosphatases. Pathogenic trypanosomatids have at least two different host environments in their life cycles, an insect vector and a mammal. Also, each trypanosomatid genus has different abilities to survive and reproduce in such hosts. For instance, *Leishmania* spp. are intracellular parasites, seeking to invade macrophages. On the other hand, *T. cruzi* invades and replicates in many cell-types, including macrophages, fibroblasts and myocytes. *T. brucei* is an exclusively extracellular parasite that resides in the bloodstream of the mammalian host. As the life cycles of these parasites take place through widely different environments, frequent and substantial adaptive changes are required in many cell processes, resulting in changes in gene expression, protein levels and protein modifications [26, 27]. Along with those, cell surface components play a key role in the survival of protozoan parasites in hostile environments and in confrontation with host immune responses. Since, these flagellates have an unusual composition of phosphatases with the PTP family being greatly reduced while the STP family is expanded by comparison with human phosphatases. The low similarity to their vertebrate counterparts indicates that these enzymes may be potentially suitable targets for development of potent inhibitors with minimal effects on the physiology of mammalian hosts [5].

Under these conditions, ectophosphatases play an important role in the interaction of cells with their surroundings, especially because their catalytic sites face the extracellular milieu. Ecto-phosphatases has been reported in some protozoa parasites, such as *T. rhodesiense* [28], *T. congolense* [29], *T. brucei* [30, 31], *T. cruzi* [32], *T. rangeli* [13, 33], some *Leishmania* species [11, 34], *Herptomonas muscarum muscarum* [35], *Phytomonas* spp. [36, 37], *Entamoeba histolytica* [38], *Giardia lamblia* [39] and *Trichomonas vaginalis* [40]. In general, these ectoenzymes are usually reported to have optimum activities in the acidic pH range, and they are therefore also known as membrane-bound acid phosphatases [28, 29]. In trypanosomatids, the low optimum pH and the surface location of these enzymes suggest its role in an acidic microenvironment and/or a close relationship with lysosomal digestion, possibly reflecting an adaptation of the parasite to the intracellular or phagosomal environment [41, 42].

Cloning and purification of an acidic phosphatase in *T. brucei* suggest that these enzymes may represent a new ectophosphatase class lacking homology to other known phosphatases [31]. It seems that these proteins are related to the regulation of *T. brucei* development, since these acidic phosphatases are expressed in bloodstream forms, but not in the insect procyclic form [31]. Likewise, an ectophosphatase activity on the surface of intact procyclic and bloodstream forms of *T. brucei* was demonstrated by Fernandes et al. [43, 44]. These enzymes show different behavior, like sensitivity to inhibitors and metal interference. Similarly, an ectophosphatase was also cloned and purified in *L. mexicana*, where it was located in the endosomal/lysosomal compartment between the flagellar pocket and the nucleus in wild-type promastigotes, and the overexpression of this protein leads to its abundant exposure on the cell surface [45, 46]. The same was seen with membrane-bound acid

phosphatase from the bloodstream form of *T. brucei*, where the enzyme is supposed to participate in the maintenance of endocytosis/exocytosis and in differentiation to the insect stage [47]. The wide distribution of acid phosphatases on the cell may reflect some physiological adaptation for parasite survival within the host.

In this scenario, ectophosphatase activities were identified at the cell surface of all *T. cruzi* development stages: epimastigote [48], trypomastigote [18] and amastigote forms [18, 32]. It seems that in amastigote forms these enzymes are magnesium-dependent and can hydrolyse phosphoaminoacids and phosphoproteins under physiological conditions [18, 32]. This behavior could facilitate the interaction between parasite and host cells, once *T. cruzi* phosphatases leads to dephosphorylation of proteins important in the signal transduction pathway or cycle regulation of this protozoan parasite. Supporting this idea, Y strain presents Mg^{2+}-dependent ectophosphatase activity, while Colombiana strain expresses Mg^{2+}-independent activity [48]. Among other characteristics, members of these two groups have different patterns of behavior considering their ability to infect mammalian host cells. Parasites from the Colombiana strain appeared to be more infective to myoblasts than those from the Y strain, while the latter is more infective towards macrophages than the parasites of the Colombiana strain [49]. Intriguingly, platelet-activating factor (PAF), a phospholipid mediator involved in differentiation cellular in *T. cruzi*, induces the secretion of an ectophosphatase in these parasites, associating this event with the infectivity of the parasite [50].

Addition of sodium orthovanadate (a protein tyrosine phosphatase inhibitor) in the interaction medium from *L. amazonensis* and macrophages significantly increased parasite binding and internalization, suggesting that *Leishmania* induces tyrosine phosphorylation [24, 51]. Under these conditions, protein tyrosine kinase-linked pathways regulate the *Leishmania* promastigote invasion, which ectophosphatase activity upregulate *L. amazonensis* binding ligands for macrophage receptors and intracellular survival within these cells [24, 51, 52]. It seems that during macrophage infection by *Leishmania* the parasite attenuates MAP kinase signaling, as well as c-FOS and iNOS expression in macrophages, stimulating the phosphotyrosine phosphatase activity in these cells [53–55]. These findings suggest a mechanism for macrophage deactivation used by *Leishmania* spp. and possibly by other intracellular pathogens as a strategy of the parasites to interact and survive within their hosts. In *L. donovani* tyrosine phosphatase activity was also detected, suggesting that tyrosine phosphorylation occurs, though not via receptor tyrosine kinase or tyrosine kinase-like activities but very likely due to the activity of atypical and/or dual specific kinases [56]. Futhermore, a membrane-bound PTP has been describe in *L. major* metacyclic promastigote forms, which is translocated to the cytoplasm in promastigotes. In spite of the increased level of the molecule in metacyclic promastigotes compared to the procyclic forms, the specific activity of the enzyme was lower in metacyclic than in procyclic promastigotes [42]. Interestingly, a protein tyrosine phosphatase, has been identified in *L. major* (*Lm*PTP1) that allows amastigotes forms to survive in mice [57]. Although its biological function is unclear, this may be an important factor in virulence, enabling the invading pathogen to survive in a host. Ecto-phosphatase isolated from *L. donovani* promastigotes inhibits the production of superoxide anions in intact human neutrophils [16]. This activity could contribute to the survival of the parasite within the host, we can hypothesize that parasites with greater ectophosphatase activity would be more resistant to oxidative bursts from the host's immune system.

The role of ectophosphatases in invasive amoebiasis is still unknown, even though two acid phosphatases have been characterized in these parasites: a membrane-bound acid phosphatase (MAP) [58, 59] and a phosphatase that is secreted to the culture medium (SAP), as well as to the cell interface in amoebic liver abscess [60, 61]. These enzymes may be associated with cellular adhesion processes, since the invasive *E. histolytica* showed much higher ectophosphatase activity when compared to the noninvasive counterpart and the free-living *E. moshkovskii* [62].

3. Ectophosphatase Activities in Fungi Infection

The fungal cell wall is a compact albeit dynamic structure that plays important roles in several biological processes determining cell shape, morphogenesis, reproduction, cell-cell and cell-matrix interactions, osmotic and physical protection. Several different cell wall components have been characterized such as specific enzymatic activities, heat-shock proteins, glycosphingolipids (GSL), melanin, histone and integrin-like proteins [63]. These components have been exhaustively studied as putative targets for drug and immunotherapy.

Even though the roles of ectophosphatases in fungi are still largely unknown, the cellular distribution of ectophosphatases, together with their ability to interfere with physiologic processes through the removal of phosphate groups of regulatory proteins, suggest a task for these molecules during the infection of host cells. The presence of surface-located acid phosphatases, called ecto or extracytoplasmic phosphatases has been demonstrated in nonpathogenic yeast *Saccharomyces cerevisiae* [64] and in pathogenic species such as *Candida albicans* [20], *Candida parapsilosis* [19, 65], *Sporothrix schenckii* [66], *Aspergillus fumigatus* [67], *Fonsecaea pedrosoi* [22, 68], *Cryptococcus neoformans* [23] and *Pseudallescheria boydii* [69].

Futhermore, most of the phosphatases synthesized under Pi-limiting conditions are either located on the extracellular medium or are associated with the plasma membrane or cell wall [15, 22]. Corroborating with this hypothesis, Kneipp et al. [22] demonstrated that conidial forms of *F. pedrosoi* has an ectophosphatase activity modulated by exogenous phosphate. It seems that in *F. pedrosoi,* conidial cells that were cultivated in a Pi-depleted medium had an ectophosphatase

activity significantly higher than that of fungal cells grown in the complete medium. These cells expressing high phosphatase activity were significantly more capable of adhering to epithelial cells and fibroblasts than fungi expressing basal levels of enzyme activity [22]. It was then proposed that the removal of phosphate groups from surface proteins in host cells could result in conformational transitions and in an attenuated electrostatic repulsion between fungal and epithelial cells. Probably, the removal of inorganic phosphate could therefore expose at the host surface additional sites for interaction with infectious agents. It seems that ectophosphatases may contain adhesive domains that could directly promote the attachment of fungal cells to their hosts, therefore functioning similarly to the well-characterized microbial adhesins. Probably, they could regulate the functional activation of surface adhesins, which would be the key structures mediating fungal attachment. Intriguingly, known activators of signaling pathways and cell differentiation, PAF and propanolol, promoted an enhancement of *F. pedrosoi* ectophosphatase activity [17] suggesting that *F. pedrosoi* ectophosphatase may be considered a surface marker for morphological transition and infection.

In the fungus *C. neoformans* a thick capsule composed of neutral and charged polysaccharides [70], can be modulated by different environmental conditions, including the sites of fungal infection inside the host. It seems that the molecules coating the outer layer of the cell wall could be relevant during the interaction of poorly encapsulated cells with host tissues. Ectoenzymes possibly have their accessibility to external receptors masked by the capsule polysaccharides of *C. neoformans*, diminishing the potential of these structures to be surface molecules influencing the interaction between fungal and host cells. In fact, different isolates of *C. neoformans* express ectophosphatase activity [23]. However, the levels of enzyme activity, varied considerably among the isolates and no correlation between enzyme activity and capsular size or serotype was observed. Evidences show that isolates with capsular polysaccharides of the same serotype varied greatly in ectophosphatase activity. In addition, the strain, which is poorly encapsulated, removed phosphate groups much more efficiently than strain, which expresses a large capsule, indicating that the presence of the capsule impairs enzyme activity in this process. On the other hand, some encapsulated strains presented levels of ectophosphatase activity higher than that observed in the acapsular mutant. Moreover, some strains that had very similar levels of enzyme activity, but differ greatly in capsule size were also found [23]. Taken together, these data indicate that differences observed in enzyme activity should be derived from natural variation of ectophosphatase expression in different *C. noeformans* strains. Corroborating with the previous findings, Kiffer-Moreira et al. [19] investigated three different isolates of *C. parapsilosis*, including a laboratory-adapted strain (CCT 3834) and two recently isolated strains (RFO and H297). They observed that the RFO strain exhibits the highest levels of enzyme activity and adhesion to CHO cells, followed by the H297 and the CCT 3834 isolates. Pretreatment of yeasts with the irreversible inhibitor sodium orthovanadate caused a significant reduction in the ability of these fungi to attach to epithelial cells [19]. Although sodium orthovanadate can affect different biological processes [71] and inhibit ATPases involved in cation transport [72, 73], its major biological activity in living cells occur on the cell surface, as the oxidation–reduction reactions that take place in the cytoplasm diminish its inhibitory effect. *C.parapsilosis* ectophosphatase may be considered an important virulence factor. Similarly, *C. albicans* isolate from oral cavities of HIV-infected children (HIV^+) present an ectophosphatase activity significantly higher than the HIV-negative children (HIV^-) [20]. The *C. albicans* yeasts from HIV^+ patients showed higher indices of adhesion to epithelial cells, which suggests that the activity of fungal acidic surface phosphatases may contribute to the early mechanisms required for disease establishment [20]. It is reasonable the hypothesis that ectophosphatases represent a virulence marker, since these enzymes represent part of the outer layer and are linked to cell differentiation and host cell-pathogen interactions.

4. Concluding Remarks

The balance of phosphorylation-dephosphorylation of serine, threonine and tyrosine residues modulates signaling pathways critical for determining the outcome of multiple cellular functions [74]. Ecto-phosphatases are enzymes able to hydrolyze phosphorylated substrates in the extracellular medium. Further studies are warranted to resolve the roles of ectophosphatases in host-pathogen interactions, as well as the possible correlations between the expression of these enzymes and the clinical manifestation of the diseases.

Acknowledgments

Part of the work mentioned in this review was supported by grants from the Brazilian Agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), and Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem (INCTBEB).

References

[1] C. J. Oliver and S. Shenolikar, "Physiologic importance of protein phosphatase inhibitors," *Frontiers in Bioscience*, vol. 3, pp. D961–D972, 1998.

[2] P. Cohen, "The origins of protein phosphorylation," *Nature Cell Biology*, vol. 4, no. 5, pp. E127–E130, 2002.

[3] T. Hunter, "Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling," *Cell*, vol. 80, no. 2, pp. 225–236, 1995.

[4] M. Parsons, E. A. Worthey, P. N. Ward, and J. C. Mottram, "Comparative analysis of the kinomes of three pathogenic trypanosomatids: *Leishmania major*, *Trypanosoma brucei* and Trypanosoma cruzi," *BMC Genomics*, vol. 6, pp. 127–145, 2005.

[5] R. Brenchley, H. Tariq, H. McElhinney et al., "The TriTryp phosphatome: analysis of the protein phosphatase catalytic domains," *BMC Genomics*, vol. 8, pp. 434–455, 2007.

[6] M. J. M. Alves and W. Colli, "*Trypanosoma cruzi*: adhesion to the host cell and intracellular survival," *IUBMB Life*, vol. 59, no. 4-5, pp. 274–279, 2007.

[7] J. R. Meyer-Fernandes, "Ecto-ATPases in protozoa parasites: looking for a function," *Parasitology International*, vol. 51, no. 3, pp. 299–303, 2002.

[8] J. R. Meyer-Fernandes, D. Cosentino-Gomes, D. P. Vieira, and A. H. Lopes, "Ecto-nucleoside triphosphate diphosphohydrolase activities in trypanosomatids: possible roles in infection, virulence and purine recycling," *The Open Parasitology Journal*, vol. 4, pp. 116–119, 2010.

[9] B. Szöör, "Trypanosomatid protein phosphatases," *Molecular and Biochemical Parasitology*, vol. 173, pp. 53–63, 2010.

[10] A. V. Andreeva and M. A. Kutuzov, "Protozoan protein tyrosine phosphatases," *International Journal for Parasitology*, vol. 38, no. 11, pp. 1279–1295, 2008.

[11] E. E. D. Almeida-Amaral, R. Belmont-Firpo, M. A. Vannier-Santos, and J. R. Meyer-Fernandes, "*Leishmania amazonensis*: characterization of an ecto-phosphatase activity," *Experimental Parasitology*, vol. 114, no. 4, pp. 334–340, 2006.

[12] S. A. O. Gomes, A. L. Fonseca-de-Souza, B. A. Silva et al., "*Trypanosoma rangeli*: differential expression of cell surface polypeptides and ecto-phosphatase activity in short and long epimastigote forms," *Experimental Parasitology*, vol. 112, no. 4, pp. 253–262, 2006.

[13] A. L. Fonseca-de-Souza, C. F. Dick, A. L. A. dos Santos, and J. R. Meyer-Fernandes, "A Mg^{2+}-dependent ecto-phosphatase activity on the external surface of Trypanosoma rangeli modulated by exogenous inorganic phosphate," *Acta Tropica*, vol. 107, no. 2, pp. 153–158, 2008.

[14] A. L. Fonseca-de-Souza, C. F. Dick, A. L. A. dos Santos, F. V. Fonseca, and J. R. Meyer-Fernandes, "*Trypanosoma rangeli*: a possible role for ecto-phosphatase activity on cell proliferation," *Experimental Parasitology*, vol. 122, no. 3, pp. 242–246, 2009.

[15] C. F. Dick, A. L. A. dos-Santos, A. L. Fonseca-de-Souza, J. Rocha-Ferreira, and J. R. Meyer-Fernandes, "*Trypanosoma rangeli*: differential expression of ecto-phosphatase activities in response to inorganic phosphate starvation," *Experimental Parasitology*, vol. 124, no. 4, pp. 386–393, 2010.

[16] A. T. Remaley, D. B. Kuhns, and R. E. Basford, "Leishmanial phosphatase blocks neutrophil O-2 production," *The Journal of Biological Chemistry*, vol. 259, no. 18, pp. 11173–11175, 1984.

[17] D. S. Alviano, L. F. Kneipp, A. H. Lopes et al., "Differentiation of *Fonsecaea pedrosoi* mycelial forms into sclerotic cells is induced by platelet-activating factor," *Research in Microbiology*, vol. 154, no. 10, pp. 689–695, 2003.

[18] T. Furuya, L. I. Zhong, J. R. Meyer-Fernandes, H. G. Lu, S. N. J. Moreno, and R. Docampo, "Ecto-protein tyrosine phosphatase activity in *Trypanosoma cruzi* infective stages," *Molecular and Biochemical Parasitology*, vol. 92, no. 2, pp. 339–348, 1998.

[19] T. Kiffer-Moreira, A. A. D. S. Pinheiro, W. S. Alviano et al., "An ectophosphatase activity in *Candida parapsilosis* influences the interaction of fungi with epithelial cells," *FEMS Yeast Research*, vol. 7, no. 4, pp. 621–628, 2007.

[20] M. B. Portela, L. F. Kneipp, I. P. Ribeiro De Souza et al., "Ectophosphatase activity in *Candida albicans* influences fungal adhesion: study between HIV-positive and HIV-negative isolates," *Oral Diseases*, vol. 16, no. 5, pp. 431–437, 2010.

[21] E. Touati, E. Dassa, J. Dassa, and P. L. Boquet, "Acid phosphatase (pH2.5) of *Escherichia coli*: regulatory characteristics," in *Microorganisms*, A. Torriani- Gorini, F. G. Rothman, S. Silver, A. Wright, and E. Yagil, Eds., pp. 31–40, ASM Press, Washington, DC, USA, 1987.

[22] L. F. Kneipp, M. L. Rodrigues, C. Holandino et al., "Ectophosphatase activity in conidial forms of Fonsecaea pedrosoi is modulated by exogenous phosphate and mediates fungal adhesion to epithelial cells," *Microbiology*, vol. 150, pp. 3355–3362, 2004.

[23] I. Collopy-Junior, F. F. Esteves, L. Nimrichter, M. L. Rodrigues, C. S. Alviano, and J. R. Meyer-Fernandes, "An ectophosphatase activity in *Cryptococcus neoformans*," *FEMS Yeast Research*, vol. 6, no. 7, pp. 1010–1017, 2006.

[24] A. Martiny, M. A. Vannier-Santos, V. M. Borges et al., "*Leishmania*-induced tyrosine phosphorylation in the host macrophage and its implication to infection," *European Journal of Cell Biology*, vol. 71, no. 2, pp. 206–215, 1996.

[25] J. M. Jadin and J. Creemers, "The role of excretion of acid phosphatase in *Trypanosoma brucei* and *T. cruzi*," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 66, no. 1, pp. 8–9, 1972.

[26] L. O. Andrade and N. W. Andrews, "Opinion: the Trypanosoma cruzi-host-cell interplay: location, invasion, retention," *Nature Reviews Microbiology*, vol. 3, no. 10, pp. 819–823, 2005.

[27] J. A. Atwood, D. B. Weatherly, T. A. Minning et al., "Microbiology: the Trypanosoma cruzi proteome," *Science*, vol. 309, no. 5733, pp. 473–476, 2005.

[28] J. McLaughlin, "The association of distinct acid phosphatases with the flagella pocket and surface membrane fractions obtained from bloodstream forms of *Trypanosoma rhodesiense*," *Molecular and Cellular Biochemistry*, vol. 70, no. 2, pp. 177–184, 1986.

[29] O. M. Tosomba, T. H. T. Coetzer, and J. D. Lonsdale-Eccles, "Localisation of acid phosphatase activity on the surface of bloodstream forms of *Trypanosoma congolense*," *Experimental Parasitology*, vol. 84, no. 3, pp. 429–438, 1996.

[30] E. C. Fernandes, J. R. Meyer-Fernandes, M. A. C. Silva-Neto, and A. E. Vercesi, "*Trypanosoma brucei*: ecto-phosphatase activity present on the surface of intact procyclic forms," *Zeitschrift fur Naturforschung Section C—Journal of Biosciences*, vol. 52, no. 5-6, pp. 351–358, 1997.

[31] N. Bakalara, X. Santarelli, C. Davis, and T. Baltz, "Purification, cloning, and characterization of an acidic ectoprotein phosphatase differentially expressed in the infectious bloodstream form of *Trypanosoma brucei*," *The Journal of Biological Chemistry*, vol. 275, no. 12, pp. 8863–8871, 2000.

[32] J. R. Meyer-Fernandes, M. A. Da Silva-Neto, M. Dos Santos Soares, E. Fernandas, A. E. Vercesi, and M. M. de Oliveira, "Ecto-phosphatase activities on the cell surface of the amastigote forms of *Trypanosoma cruzi*," *Zeitschrift fur Naturforschung—Section C Journal of Biosciences*, vol. 54, no. 11, pp. 977–984, 1999.

[33] D. Cosentino-Gomes, T. Russo-Abrahão, A. L. Fonseca-de-Souza, C. R. Ferreira, A. Galina, and J. R. Meyer-Fernandes, "Modulation of *Trypanosoma rangeli* ecto-phosphatase activity by hydrogen peroxide," *Free Radical Biology and Medicine*, vol. 47, no. 2, pp. 152–158, 2009.

[34] N. Bakalara, A. Seyfang, C. Davis, and T. Baltz, "Characterization of a life-cycle-stage-regulated membrane protein tyrosine phosphatase in *Trypanosoma brucei*," *European Journal of Biochemistry*, vol. 234, no. 3, pp. 871–877, 1995.

[35] P. M. L. Dutra, C. O. Rodrigues, J. B. Jesus, A. H. C. S. Lopes, T. Souto-Padrón, and J. R. Meyer-Fernandes, "A

novel ecto-phosphatase activity of herpetomonas *muscarum muscarum* inhibited by platelet-activating factor," *Biochemical and Biophysical Research Communications*, vol. 253, no. 1, pp. 164–169, 1998.

[36] P. M. L. Dutra, C. O. Rodrigues, A. Romeiro et al., "Characterization of ectophosphatase activities in trypanosomatid parasites of plants," *Phytopathology*, vol. 90, no. 9, pp. 1032–1038, 2000.

[37] P. M. L. Dutra, F. A. Dias, M. A. A. Santos et al., "Secreted phosphatase activities in trypanosomatid parasites of plants modulated by platelet-activating factor," *Phytopathology*, vol. 91, no. 4, pp. 408–414, 2001.

[38] M. M. Aguirre-García, J. Cerbón, and P. Talamás-Rohana, "Purification and properties of an acid phosphatase from *Entamoeba histolytica* HM-1:IMSS," *International Journal for Parasitology*, vol. 30, no. 5, pp. 585–591, 2000.

[39] J. N. Amazonas, D. Cosentino-Gomes, A. Werneck-Lacerda et al., "Giardia lamblia: characterization of ecto-phosphatase activities," *Experimental Parasitology*, vol. 121, no. 1, pp. 15–21, 2009.

[40] J. De Jesus, T. Podlyska, A. Hampshire, C. Lopes, M. Vannier-Santos, and J. Meyer-Fernandes, "Characterization of an ecto-phosphatase activity in the human parasite *Trichomonas vaginalis*," *Parasitology Research*, vol. 88, no. 11, pp. 991–997, 2002.

[41] A. M. Shakarian, M. B. Joshi, E. Ghedin, and D. M. Dwyer, "Molecular dissection of the functional domains of a unique, tartrate-resistant, surface membrane acid phosphatase in the primitive human pathogen *Leishmania donovani*," *The Journal of Biological Chemistry*, vol. 277, no. 20, pp. 17994–18001, 2002.

[42] M. M. Aguirre-García, A. R. Escalona-Montaño, N. Bakalara, A. Pérez-Torres, L. Gutiérrez-Kobeh, and I. Becker, "*Leishmania major*: detection of membrane-bound protein tyrosine phosphatase," *Parasitology*, vol. 132, no. 5, pp. 641–649, 2006.

[43] E. C. Fernandes, J. M. Granjeiro, E. M. Taga, J. R. Meyer-Fernandes, and H. Aoyama, "Phosphatase activity characterization on the surface of intact bloodstream forms of *Trypanosoma brucei*," *FEMS Microbiology Letters*, vol. 220, no. 2, pp. 197–206, 2003.

[44] E. C. Fernandes, J. M. Granjeiro, H. Aoyama, F. V. Fonseca, J. R. Meyer-Fernandes, and A. E. Vercesi, "A metallo phosphatase activity present on the surface of *Trypanosoma brucei* procyclic forms," *Veterinary Parasitology*, vol. 118, no. 1-2, pp. 19–28, 2003.

[45] B. Menz, G. Winter, T. Ilg, F. Lottspeich, and P. Overath, "Purification and characterization of a membrane-bound acid phosphatase of *Leishmania mexicana*," *Molecular and Biochemical Parasitology*, vol. 47, no. 1, pp. 101–108, 1991.

[46] M. Wiese, O. Berger, Y. D. Stierhof, M. Wolfram, M. Fuchs, and P. Overath, "Gene cloning and cellular localization of a membrane-bound acid phosphatase of *Leishmania mexicana*," *Molecular and Biochemical Parasitology*, vol. 82, no. 2, pp. 153–165, 1996.

[47] M. Engstler, F. Weise, K. Bopp et al., "The membrane-bound histidine acid phosphatase TbMBAP1 is essential for endocytosis and membrane recycling in *Trypanosoma brucei*," *Journal of Cell Science*, vol. 118, no. 10, pp. 2105–2118, 2005.

[48] P. M. L. Dutra, L. C. Couto, A. H. C. S. Lopes, and J. R. Meyer-Fernandes, "Characterization of ecto-phosphatase activities of *Trypanosoma cruzi*: a comparative study between Colombiana and Y strains," *Acta Tropica*, vol. 100, no. 1-2, pp. 88–95, 2006.

[49] T. C. A. Jorge, H. S. Barbosa, A. L. Moreira, W. De Souza, and M. N. L. Meirelles, "The interaction of myotropic and macrophagotropic strains of *Trypanosoma cruzi* with myoblasts and fibers of skeletal muscle," *Zeitschrift für Parasitenkunde Parasitology Research*, vol. 72, no. 5, pp. 577–584, 1986.

[50] C. O. Rodrigues, P. M. L. Dutra, F. S. Barros, T. Souto-Padrón, J. R. Meyer-Fernandes, and A. H. C. S. Lopes, "Platelet-activating factor induction of secreted phosphatase activity in *Trypanosoma cruzi*," *Biochemical and Biophysical Research Communications*, vol. 266, no. 1, pp. 36–42, 1999.

[51] A. Martiny, J. R. Meyer-Fernandes, W. De Souza, and M. A. Vannier-Santos, "Altered tyrosine phosphorylation of ERK1 MAP kinase and other macrophage molecules caused by *Leishmania amastigotes*," *Molecular and Biochemical Parasitology*, vol. 102, no. 1, pp. 1–12, 1999.

[52] M. A. Vannier-Santos, A. Martiny, J. R. Meyer-Fernandes, and W. De Souza, "Leishmanial protein kinase C modulates host cell infection via secreted acid phosphatase," *European Journal of Cell Biology*, vol. 67, no. 2, pp. 112–119, 1995.

[53] D. Nandan, R. Lo, and N. E. Reiner, "Activation of phosphotyrosine phosphatase activity attenuates mitogen- activated protein kinase signaling and inhibits c-FOS and nitric oxide synthase expression in macrophages infected with *Leishmania donovani*," *Infection and Immunity*, vol. 67, no. 8, pp. 4055–4063, 1999.

[54] M. A. Gomez, I. Contreras, M. Hallé, M. L. Tremblay, R. W. McMaster, and M. Olivier, "Leishmania GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases," *Science Signaling*, vol. 2, no. 90, p. ra58, 2009.

[55] I. Abu-Dayyeh, K. Hassani, E. R. Westra, J. C. Mottram, and M. Olivier, "Comparative study of the ability of *Leishmania mexicana* promastigotes and amastigotes to alter macrophage signaling and functions," *Infection and Immunity*, vol. 78, no. 6, pp. 2438–2445, 2010.

[56] D. E. Cool and J. J. Blum, "Protein tyrosine phosphatase activity in *Leishmania donovani*," *Molecular and Cellular Biochemistry*, vol. 127-128, pp. 143–149, 1993.

[57] M. Nascimento, W. W. Zhang, A. Ghosh et al., "Identification and characterization of a protein-tyrosine phosphatase in *Leishmania*: involvement in virulence," *The Journal of Biological Chemistry*, vol. 281, no. 47, pp. 36257–36268, 2006.

[58] M. Anaya-Ruiz, J. L. Rosales-Encina, and P. Talamas-Rohana, "Membrane acid phosphatase (MAP) from *Entamoeba histolytica*," *Archives of Medical Research*, vol. 28, pp. 182–183, 1997.

[59] M. M. Aguirre-García, J. Cerbón, and P. Talamás-Rohana, "Purification and properties of an acid phosphatase from *Entamoeba histolytica* HM-1:IMSS," *International Journal for Parasitology*, vol. 30, no. 5, pp. 585–591, 2000.

[60] M. M. Aguirre-Garcia, J. L. Rosales-Encina, and P. Talamas-Rohana, "Secreted *Entamoeba histolytica* acid phosphatase (SAP)," *Archives of Medical Research*, vol. 28, pp. 184–185, 1997.

[61] J. Ventura-Juarez, M. M. Aguirre-Garcia, and P. Talamas-Rohana, "Subcellular distribution and in situ localization of the acid phosphatase of *Entamoeba histolytica*," *Archives of Medical Research*, vol. 31, no. 4, pp. S183–S184, 2000.

[62] A. A. de Sá Pinheiro, J. N. Amazonas, F. de Souza Barros et al., "*Entamoeba histolytica*: an ecto-phosphatase activity regulated by oxidation-reduction reactions," *Experimental Parasitology*, vol. 115, no. 4, pp. 352–358, 2007.

[63] L. Nimrichter, M. L. Rodrigues, E. G. Rodrigues, and L. R. Travassos, "The multitude of targets for the immune system and drug therapy in the fungal cell wall," *Microbes and Infection*, vol. 7, no. 4, pp. 789–798, 2005.

[64] P. Mildner, B. Ries, and S. Barbaric, "Acid phosphatase and adenosine triphosphatase activities in the cell wall of baker's yeast," *Biochimica et Biophysica Acta*, vol. 391, no. 1, pp. 67–74, 1975.

[65] P. H. Fernanado, G. J. Panagoda, and L. P. Samaranayake, "The relationship between the acid and alkaline phosphatase activity and the adherence of clinical isolates of *Candida parapsilosis* to human buccal epithelial cells," *APMIS*, vol. 107, pp. 1034–1042, 1999.

[66] W. N. Arnold, L. C. Mann, and K. H. Sakai, "Acid phosphatases of *Sporothrix schenckii*," *Journal of General Microbiology*, vol. 132, no. 12, pp. 3421–3432, 1986.

[67] M. Bernard, I. Mouyna, G. Dubreucq et al., "Characterization of a cell-wall acid phosphatase (PhoAp) in *Aspergillus fumigatus*," *Microbiology*, vol. 148, no. 9, pp. 2819–2829, 2002.

[68] L. F. Kneipp, V. F. Palmeira, A. A. S. Pinheiro et al., "Phosphatase activity on the cell wall of *Fonsecaea pedrosoi*," *Medical Mycology*, vol. 41, no. 6, pp. 469–477, 2003.

[69] T. Kiffer-Moreira, A. A. S. Pinheiro, M. R. Pinto et al., "Mycelial forms of *Pseudallescheria boydii* present ectophosphatase activities," *Archives of Microbiology*, vol. 188, no. 2, pp. 159–166, 2007.

[70] I. Bose, A. J. Reese, J. J. Ory, G. Janbon, and T. L. Doering, "A yeast under cover: The capsule of *Cryptococcus neoformans*," *Eukaryotic Cell*, vol. 2, no. 4, pp. 655–663, 2003.

[71] J. K. Klarlund, "Transformation of cells by an inhibitor of phosphatases acting on phosphotyrosine in proteins," *Cell*, vol. 41, no. 3, pp. 707–717, 1985.

[72] C. L. Sodré, B. L. M. Moreira, F. B. Nobrega et al., "Characterization of the intracellular Ca^{2+} pools involved in the calcium homeostasis in *Herpetomonas* sp. promastigotes," *Archives of Biochemistry and Biophysics*, vol. 380, no. 1, pp. 85–91, 2000.

[73] E. E. de Almeida-Amaral, C. Caruso-Neves, V. M. P. Pires, and J. R. Meyer-Fernandes, "*Leishmania amazonensis*: characterization of an ouabain-insensitive Na^{+}-ATPase activity," *Experimental Parasitology*, vol. 118, no. 2, pp. 165–171, 2008.

[74] D. Cosentino-Gomes and J. R. Meyer-Fernandes, "Ecto-phosphatases in protozoan parasites: possible roles in nutrition, growth and ROS sensing," *Journal of Bioenergetics and Biomembranes*, vol. 43, no. 1, pp. 89–92, 2011.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 534697, 11 pages
doi:10.1155/2011/534697

Research Article

Acute Disruption of Bone Marrow B Lymphopoiesis and Apoptosis of Transitional and Marginal Zone B Cells in the Spleen following a Blood-Stage *Plasmodium chabaudi* Infection in Mice

Viki Bockstal,[1,2] Nathalie Geurts,[3] and Stefan Magez[1,2]

[1] *Laboratory for Cellular and Molecular Immunology, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium*
[2] *Department of Molecular and Cellular Interactions, VIB, Brussels, Belgium*
[3] *Laboratory of Immunobiology, Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium*

Correspondence should be addressed to Viki Bockstal, viki.bockstal@vub.ac.be

Received 31 December 2010; Accepted 25 February 2011

Academic Editor: Alvin A. Gajadhar

B cells and antibodies are essential for the protective immune response against a blood-stage *Plasmodium* infection. Although extensive research has focused on memory as well as plasma B-cell responses during infection, little is known about how malaria affects B-cell development and splenic maturation into marginal zone B (MZB) and follicular B (FoB) cells. In this study, we show that acute *Plasmodium chabaudi* AS infection in C57Bl/6 mice causes severe disruption of B lymphopoiesis in the bone marrow, affecting in particular pro-, pre-, and immature B cells as well as the expression of the bone marrow B-cell retention chemokine CXCL12. In addition, elevated apoptosis of transitional T2 and marginal zone (MZ) B cells was observed during and subsequent to the control of the first wave of parasitemia. In contrast, Folllicular (Fo) B cells levels were retained in the spleen throughout the infection, suggesting that these are essential for parasite clearance and proper infection control.

1. Introduction

Malaria is a major health problem in developing countries, affecting each year at least 300–500 million individuals of which more than 1 million people die of serious complications. Primarily in children beyond 5 years, parasite-mediated processes and excessive or uncontrolled inflammation cause malaria pathogenesis, characterized by severe malarial anemia (SMA), cerebral malaria (CM), malaria-associated acute lung injury (ALI) and its more severe form malaria-associated acute respiratory distress syndrome (MA-ARDS) [1].

During a malaria infection, pre-erythrocytic liver stages are mainly attacked by $CD8^+$ effector cells and IFN-γ, whereas antibodies are key components against the asexual blood stage of the *Plasmodium* life cycle [1, 2]. Studies in mice lacking B cells revealed that they were unable to clear a *Plasmodium chabaudi* AS (*Pc*AS) infection and instead displayed chronic unresolved parasitemia levels for periods as long as 120 days [3]. Hence, B cells and malaria specific antibodies are, in addition to $CD4^+$T cells, required for effective antimalarial immunity [4–6]. Passive serum transfer studies in human corroborate these findings [7, 8]. Despite the key role of antibodies in immunity to malaria, there is a gap in our knowledge on the cellular basis of these humoral responses during infection. In malaria, several experimental studies report that a single *Pc*AS infection induces both short-lived and long-lived plasma cells including the generation of functional memory B-cells [9–11]. In addition, some recent human malaria studies report the generation of long-lived plasma cells and memory B-cells, whether or not in conditions of frequent re-exposure. However, Wykes et al. revealed that *Plasmodium yoelii* is capable of destroying vaccine-induced memory [12], suggesting defects in the B-cell lineage might be induced during a malaria infection.

B-cell development of cells of the B2 lineage under normal conditions occurs via a series of bone marrow (BM) stromal cell-facilitated processes that begin within

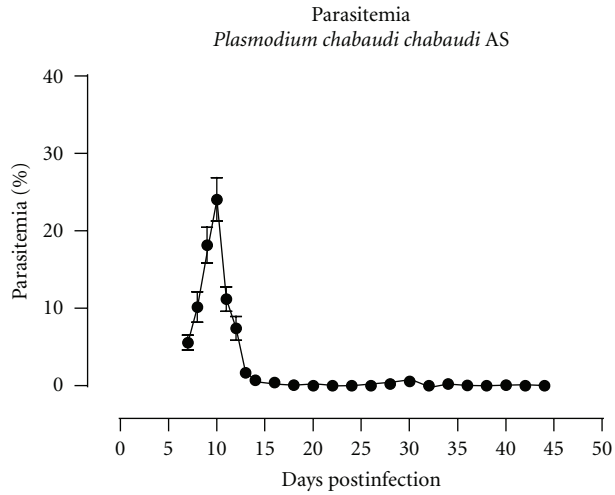


Figure 1: Parasitemia profile of *Pc*AS infection. Course of malaria infection in C57Bl/6 mice infected with blood stages of *Pc*AS. Data are representative of at least 3 independent experiments with $n > 10$ for each experiment (mean ± SEM).

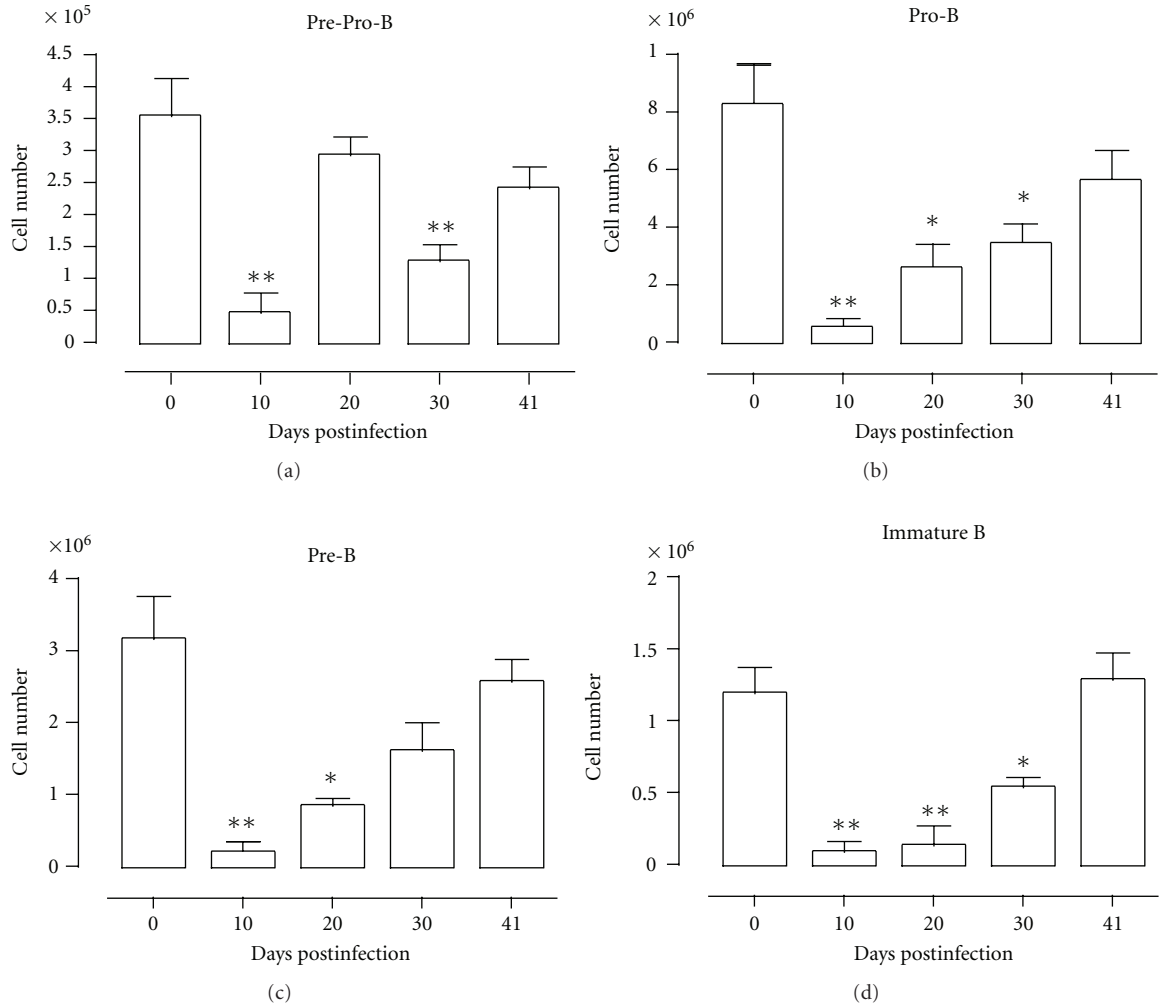


Figure 2: B dyslymphopoiesis in bone marrow during *Pc*AS infection. Bone marrow cells from uninfected mice and mice infected with *Pc*AS for 10–41 days were stained for surface markers commonly used to define developing B cells and analyzed using FACS. Data are represented as mean of three mice per group ± SEM, two independent repeat experiments were performed and statistics are compared to uninfected controls (*) $P < .05$, (**) $P < .01$.

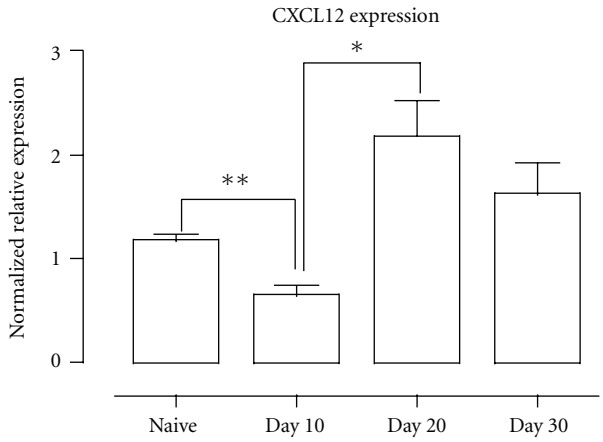


Figure 3: Bone marrow CXCL12 mRNA expression during *Pc*AS infection. BM was isolated and amplified using intron-spanning primers specific for CXCL12 via Quantitative PCR. Data were normalized to S12 expression and are presented as relative expression compared to uninfected controls. Data are represented as mean of 3 mice ± SEM and are representative of two separate experiments were performed. (*) $P < .05$, (**) $P < .01$.

the hematopoietic stem cell pool and proceed in hierarchical steps of lineage commitment [13, 14]. B lymphopoiesis yields several developmental stages of pre-pro-B, pro-B, pre-B and eventually immature B cells, which show a high expression of the IgM form of the antigen receptor and low or no expression of the IgD maturation marker [15, 16]. To complete their development, immature B cells migrate through the periphery; however, only 10% reaches the spleen as transitional B cells of the T1 type. Important is the fact that under inflammatory immune conditions, BM lymphopoiesis is often severely reduced and is compensated for by a splenic cell differentiation process that involves the same B-cell differentiation steps, referred to as extramedullary lymphopoiesis [17, 18]. Once the transitional T1 stage has been reached, B cells develop further into T2 transitional B cells that in turn can mature into either Marginal Zone B (MZB) cells or Follicular B (FoB) cells [19]. Each of these populations is distinguished by a unique set of cell surface antigens, allowing monoclonal antibody (mAb) phenotyping by multicolor flow cytometry [20–22].

Although several studies have focused on analyzing the T- and B-cell memory response in murine models for malaria as well as the plasma B-cell response, little is known about how a *Plasmodium* infection affects the process of B lymphopoiesis in the bone marrow and the maturation into naive, resting cells in the spleen. Recently, it has been reported that acute *Pc*AS infection induces the transient depletion of functional myeloid-erythroid progenitors and loss of common lymphocyte progenitors (CLPs), which under normal circumstances sustain both T- and B-cell development [23]. Because of the essential protective role for B cells in blood-stage malaria, it is important to understand the kinetics and regulation of the whole B-cell cycle from development to differentiation into plasma and memory B cells during infection. In this study, we demonstrate that during the acute phase of a *Pc*AS infection B lymphopoiesis is severely compromised in the bone marrow and infection-induced apoptosis of T2 and MZB cells depletes these populations from the spleen. The retention of splenic FoB cells in the spleen, being a source for high-affinity, parasite-specific-class-switched plasma B-cell responses, is most likely essential for the proper parasitemia clearance following the acute phase of infection. During the chronic phase of infection, when parasitemia levels are low and controlled, B lymphopoiesis in the bone marrow and maturation into transitional T2 and MZB cells in the spleen recovers completely and most likely this is crucial for the further control of infection.

2. Material and Methods

2.1. Parasites and Infection in Mice. Male C57BL/6 wild type (Janvier, Le Genest Saint-Isle, France; 6–8 weeks old) were infected by intraperitoneal (i.p.) injection of blood containing 10^4 *Pc*AS infected red blood cells (iRBC) (a kind gift of the late Professor Dr. D. Walliker, University of Edingburgh, Scotland, UK). Parasitemia was analyzed using Giemsa staining on a blood smear collected from the tail vein during infection. All mice were housed under barrier conditions and all experimental animal procedures were approved by the appropriate university's ethics committees.

2.2. Cell Isolation and Flow Cytometric Analysis. B-cell populations were analyzed by flowcytometry. Both spleen and bone marrow from femur and tibia were harvested from noninfected control and *Pc*AS infected mice 10, 20, 30 and 41 days postinfection (pi). Cell suspensions were prepared in FACS buffer (1.0% BSA (Sigma, St. Louis, MO) in DPBS) and red blood cells were lysed using lysis buffer (0.15 M NH4Cl, 1.0 mM KHCO3, 0.1 mM Na2-EDTA). Nonspecific binding sites were blocked using Fc block (CD16/CD32 Fcγ III/II, BD biosciences, Erembodegem, Belgium) for 30 minutes at 4°C. Cells were washed twice with FACS buffer and stained with biotin- or fluorochrome-conjugated primary antibodies (Section 2.3) for 30 minutes at 4°C. After washing twice, cell suspensions stained with biotin-conjugated antibodies were incubated with streptavidin-conjugated fluorochromes. which detects cell bound biotinylated antibodies, and incubated for an additional 30 minutes at 4°C. Finally, cells were resuspended in FACS buffer with 1 μg of 7-amino-actinomycin D (7AAD), a fluorescent DNA dye that binds to membrane permeable dead or dying cells, (BD biosciences). Analyses were performed using a FACS Canto II flow cytometer (BD Biosciences) and data were processed using FLOWJO software (Tree Star Inc., Ashland, OR). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each tissue.

2.3. Antibodies and Detection Reagents. The following antibodies were added to 100 μL aliquots of 10^6 Fc-blocked leukocytes prepared as described above: 0.5 μg anti-CD23-FITC (clone B3B4), 0.5 μg anti-IL7rα-FITC (clone A7R34),

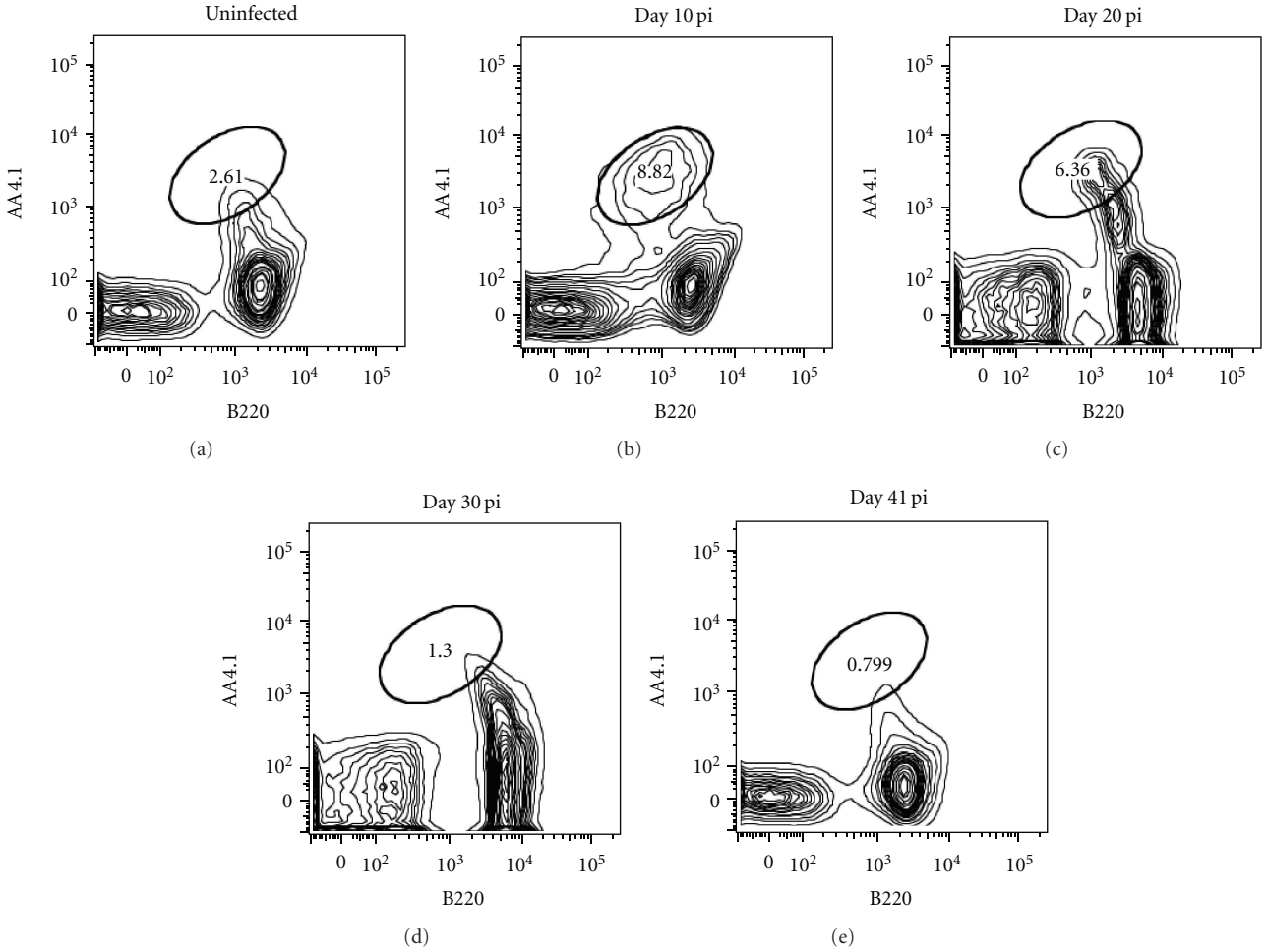


Figure 4: Extramedullary B lymphopoiesis in spleen during *Pc*AS infection. Spleens cells from noninfected mice and mice infected with *Pc*AS for 10–41 days were stained for surface markers used to define developing B cells. The total B cell precursor population, including pre-pro-B, pro-B, pre-B and immature B, was detected as $7AAD^{-}Lin^{-}B220^{int}AA4.1^{hi}$ using FACS. Data are representative of two separate experiments.

0.5 μg anti-CD11b-FITC (clone M1/70; 0.5 mg/mL), 0.5 μg anti-CD45R (B220)-FITC (clone RA3-6B2), 0.2 μg anti-IgM-PE (clone II/41), 0.2 μg anti-CD93–PE (clone AA4.1), 0.5 μg anti-CD95-FITC (clone Jo2), 0.25 μg hamster IgG2, κ isotype control (clone B81-3), 0.2 μg anti-IgM PE-Cy7 (clone II/41), 0.2 μg of anti-CD45R (B220)-PE-Cy7 (clone RA3-6B2), 0.2 μg of anti-CD93-APC (clone AA4.1), and 0.2 μg of anti-CD117 (ckit)-APC (clone 2B8), purchased from eBioscience (San Diego, CA); 0.2 μg anti-CD1d-PE (clone 1b1), 0.2 μg of anti-CD43-PE (clone 1B11), 0.2 μg of anti-CD45R (B220)-APC-Cy7 (clone RA3-6B2), 0.2 μg of anti-CD19-APC-Cy7 (clone 1D3), 0.2 μg of streptavidin-PerCP, and 0.2 μg of streptavidin-PE-Texas Red, purchased from BD Biosciences (Erembodegem, Belgium); 2 μg of each of the following antibodies: CD3ε, CD11b (Mac-1), Gr-1 (Ly-6G and Ly-6C) and Ter-119 (Ly-76) from the Biotin-conjugated Mouse Lineage Panel (BD Biosciences, Erembodegem, Belgium).

2.4. Flow Cytometric Analyses of Apoptosis. Cells were stained as described in Section 2.3 with antibodies. For the polycaspases-based apoptosis assay, labeled cells were further reacted with the FLICA fluorescent inhibitor of caspase-1, -3, -4, -5, -6, -7, -8, and -9, using the FAM Poly Caspases Assay Kit for flow cytometric analysis (Molecular probes, Invitrogen, Merelbeke, Belgium).

2.5. Statistical Analysis. Statistical comparisons were performed by *t*-test or one way ANOVA and means were compared using Tukey and Dunnett's post test when $P \leq .05$ (GraphPad Prism v.4.0, GraphPad Software Inc. San Diego, CA).

3. Results

3.1. PcAS Infection Causes a Transient Suppression of B-Cell Development in the Bone Marrow. To evaluate the kinetics of the different B-cell precursor populations in the bone marrow during *Pc*AS malaria, total bone marrow and spleen cells were isolated from uninfected control mice and mice at 10, 20, 30, and 41 days pi and analyzed using flowcytometry according to Table 1. Parasitemia was measured as percentage of parasitized red blood cells throughout infection and

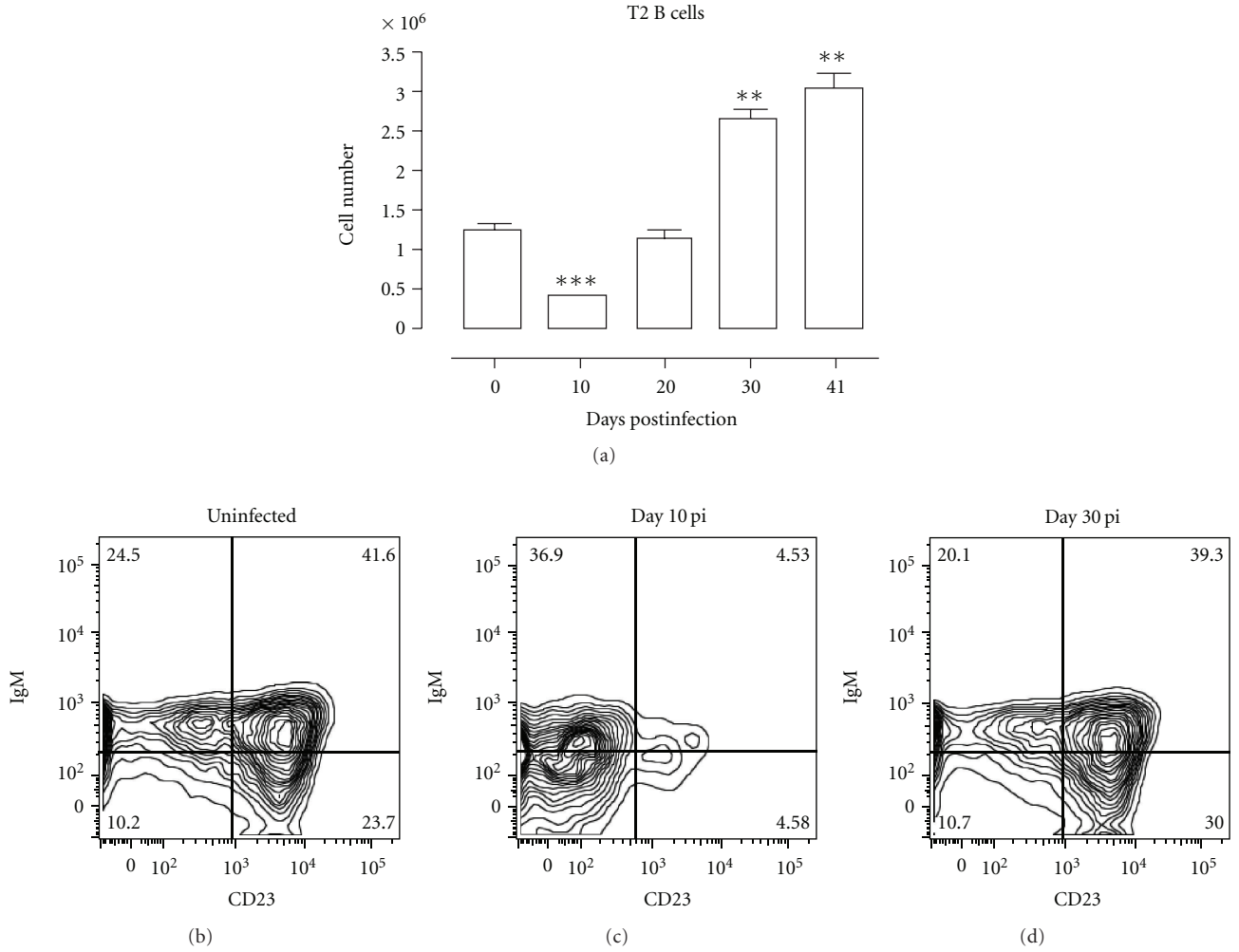


Figure 5: Depletion of transitional type 2 B cells in spleen during *Pc*AS infection. (a) Spleens cells from noninfected mice and mice infected with *Pc*AS for 10–41 days were stained for surface markers commonly used to define transitional T2 B cells and analyzed using FACS. Data are represented as mean of three mice per group ± SEM, two independent repeat experiments were performed and statistics are compared to uninfected controls (**) $P < .01$, (***) $P < .001$. (b), (c), and (d) Transitional T2 B cells were detected as ($AA4.1^+B220^+$) IgM^+CD23^+ in uninfected mice and mice on day 10 and 30 pi.

the representative profile is shown in Figure 1. At day 10 of infection, when parasitemia levels are about to peak, there was a more than 95% depletion of all B-cell specific precursor populations in the bone marrow. Afterwards, B-cell lymphopoiesis slowly recovered until pre-pro-B, pro-B and pre-B cell numbers are back to preinfection levels at day 41 pi (Figure 2).

The interaction between the chemokine CXCL12 and its receptor CXCR4 on developing B cells is crucial for B lymphopoiesis [14, 24], eliciting a stepwise progression of developing B cells through specialized bone marrow niches [25]. Here, we observed that a *Pc*AS infection caused a significant reduction in CXCL12 expression in the bone marrow on day 10 pi, which correlated with the observed reduction in B-cell precursor cells at that time point. At days 20 and 30 of infection, a significant recovery in CXCL12 expression was measured in the bone marrow, coinciding with the observed slow recovery of B lymphopoiesis (Figure 3). Furthermore, when we analyzed the developing B cells in the bone marrow of infected mice for apoptosis induction, there was no difference with uninfected control mice (data not shown), suggesting that apoptosis is not a major contributor to the observed depletion of B-cell precursor populations in the spleen.

Inflammation, characterized by high levels of TNF and other type 1 cytokines, has a negative effect on bone marrow B-cell development. Induction of a reduced CXCL12 ex-pression followed by migration of developing B cells out of the bone marrow has been described to coincide with the occurence of extramedullary B lymphopoiesis in the spleen [17, 18]. Here, on day 10 of a *Pc*AS infection, a distinct population of developing B cells (pre-pro-, pro-, pre- and immature B cells defined here together as $7AAD^-$, Lin^-, $AA4.1^{hi}$, $B220^+$) was found in the spleen, which was significantly increased compared to uninfected controls (Figure 4). However, the number of

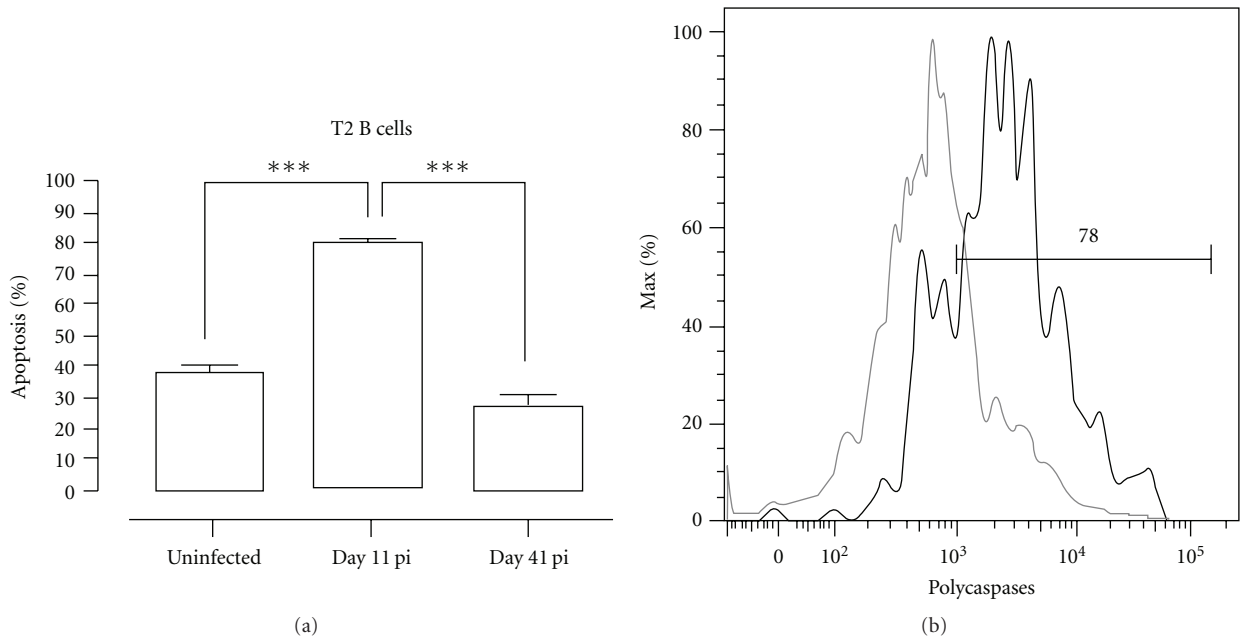


Figure 6: *Pc*AS infection-induced apoptosis of transitional B cells. Spleens cells from uninfected control mice and mice infected with *Pc*AS were stained for surface markers commonly used to define transitional T2 B cells and the amount of active caspase 1, -3, -4, -5, -6, -7, -8 and -9 was measured intracellular by flowcytometry. (a) Percentage of apoptotic cells within T2 transitional B-cell population in uninfected controls versus infected mice on day 11 and 41 pi. Data are represented as mean of three mice per group ± SEM (***) $P < .001$ and representative of two separate experiments. (b) Representative histogram of uninfected controls (grey line) versus a day 11 pi (black line).

Table 1: Differentiation antigen phenotypes of developing and mature B2 B cells.

Cell population	Suface marker phenotype
pre-pro-B	Lin^- (Ter119, CD3ε, CD11b, Gr1, NK1.1), $B220^+$, $AA4.1^+$, IgM^-, $CD19^-$, $CD43^{hi}$
pro-B	Lin^- (Ter119, CD3ε, CD11b, Gr1, NK1.1), $B220^+$, $AA4.1^+$, IgM^-, $CD19^+$, $CD43^{hi}$
pre-B	Lin^- (Ter119, CD3ε, CD11b, Gr1, NK1.1), $B220^+$, $AA4.1^+$, IgM^-, $CD19^+$, $CD43^{lo/-}$
immature B	Lin^- (Ter119, CD3ε, CD11b, Gr1, NK1.1), $B220^+$, $AA4.1^+$, IgM^+, $CD19^+$, $CD43^{lo/-}$
T1 transitional	$B220^+$, $AA4.1^+$, IgM^{hi}, $CD23^-$
T2 transitional	$B220^+$, $AA4.1^+$, IgM^{hi}, $CD23^+$
T3 transitional	$B220^+$, $AA4.1^+$, IgM^{lo}, $CD23^+$
MZB	$B220^+$, $AA4.1^-$, $CD23^{lo}$, $CD21^{hi}$, $CD1d^{hi}$
FoB	$B220^+$, $AA4.1^-$, $CD23^{hi}$, $CD21^{lo}$, $CD1d^{lo}$

developing B cells in the spleen had diminished already by day 20 pi and this population had disappeared completely by day 30 pi (Figure 4), which coincided with the observed recovery of both CXCL12 expression (Figure 3) and B-cell development in the bone marrow at that time (Figure 2).

3.2. PcAS Malaria Results in Apoptosis of Transitional B Cells in the Spleen. Transitional B cells form the cellular link between the B-cell development in the bone marrow and the maturation into mature marginal zone or follicular B cells in the spleen. During *Pc*AS infection in mice, a significant decrease of T2 transitional B cells was observed on day 10 of infection in the spleen, followed by a complete recovery on day 20 pi. (Figure 5).

The induction of apoptosis was analyzed by measuring the amount of active caspases inside the cell using flow cytometry. Interestingly, about 80% of the T2 transitional B-cell populations was triggered to undergo apoptosis on day 11 pi. As a control, on day 41 of infection when T2 B-cell numbers are no longer depleted, the level of apoptosis induction had returned to preinfection level (Figure 6).

3.3. PcAS Infection Causes Apoptosis of Marginal Zone (MZ) B Cells But Not Follicular (Fo) B Cells. Analysis of mice infected with *Pc*AS revealed that there was a more than 95% depletion in MZB cells at days 10 and 20 of infection in the spleen, followed by a strong recovery on day 30 pi. In contrast, there was no significant decrease in FoB cell numbers during the course of infection (Figure 7).

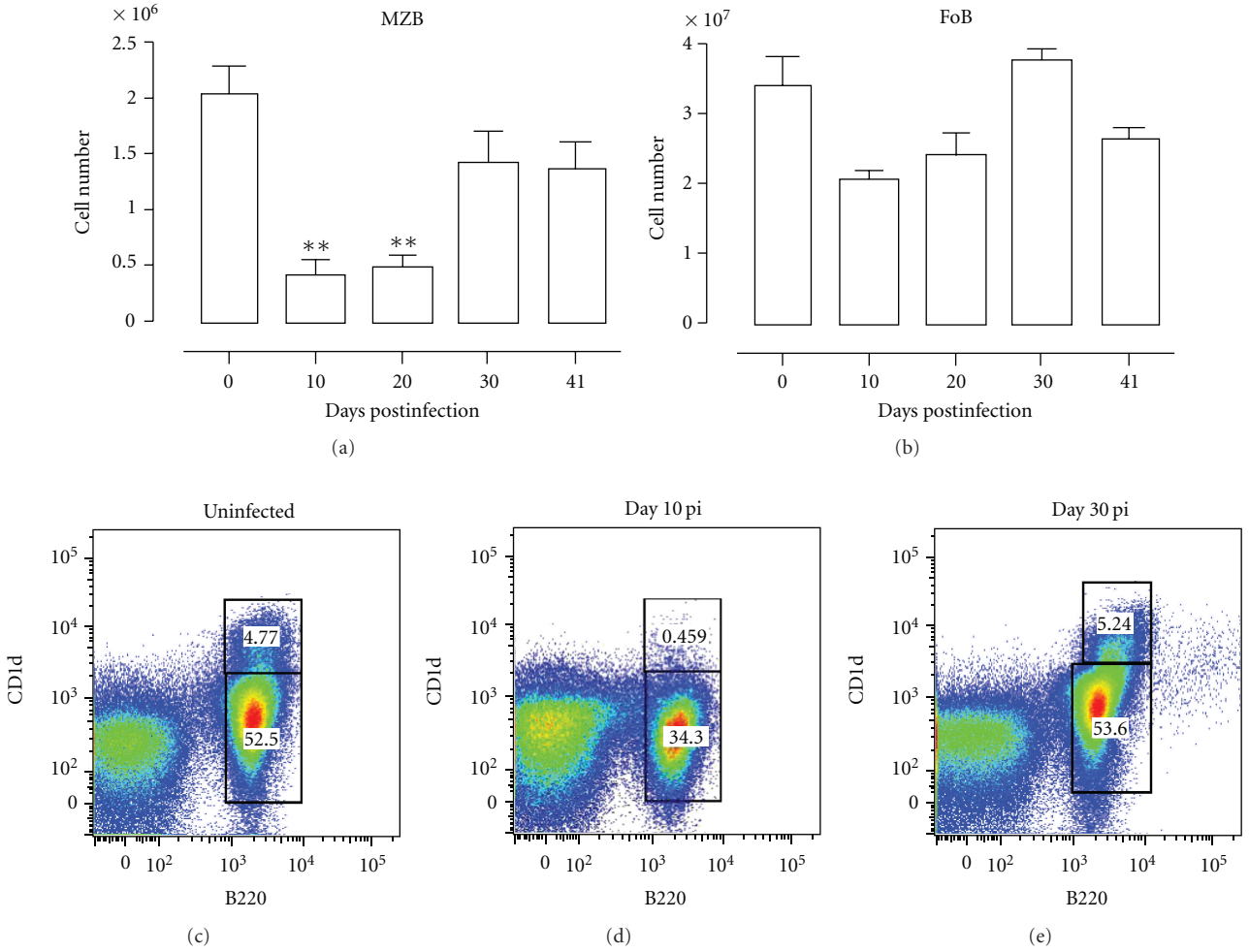


Figure 7: Depletion of MZB but not FoB cells in spleen during *Pc*AS infection. (a) and (b) Spleens cells from noninfected mice and mice infected with *Pc*AS for 10–41 days were stained for surface markers commonly used to define MZB (a) and FoB (b) cells and analyzed using FACS. Data are represented as mean of three mice per group ± SEM, two independent repeat experiments were performed and statistics are compared to uninfected controls (**) $P < .01$. (c), (d), and (e) MZB and FoB cells were detected as (AA4.1$^-$) B220$^+$CD1d$^+$ (CD23^{lo}CD21^{hi}), respectively, (AA4.1$^-$) B220$^+$CD1d$^-$ (CD23^{hi}CD21^{lo}), in noninfected mice and mice on day 10 and 30 pi.

Further analysis revealed that about 80% of the MZB cells present in the spleen on day 11 of infection was undergoing apoptosis (Figure 8). By day 41 of infection the level of caspase activation had returned to preinfection levels, which coincided with the observed recovery in MZB cell numbers in the spleen at that time. In contrast, there was no increased induction of FoB apoptosis during a *Pc*AS infection.

4. Discussion

Polyclonal lymphocyte activation associated with splenomegaly, hypergamma-globulinemia and autoantibody production are common features of *Plasmodium* infections in both humans and experimental murine models. *Pc*AS infections in mice are widely used as a model for *P. falciparum* infections in humans. A primary infection is characterized by parasitemia levels between 20–30% around day 10 of infection, followed by a 2- to 3-month low-grade chronic infection. Hence, this is an appropriate model to study the immunological processes underlying the acquisition of semi-immunity following a malaria infection [9]. Strong innate and Th1 responses are related to the early stage of infection and protection is believed to be associated with IFN-γ, TNF and NO production. Around peak parasitemia a switch to Th2 cytokine production occurs and the decline in acute primary parasitemia and chronic stages of the infection is controlled primarily in a B-cell and antibody-dependent way [26–28]. The protective function of antibodies during blood stage *Plasmodium* infection stretches from being involved in the opsonization of merozoites and parasitized erythrocytes to the stimulation of macrophage and neutrophil phagocytosis, parasite sequestration, and direct merozoite neutralization [29, 30]. There is strong evidence that naturally acquired immunity to blood-stage malaria is strongly dependent on antibodies [29, 31–33]. However, data from both experimental murine and human malaria

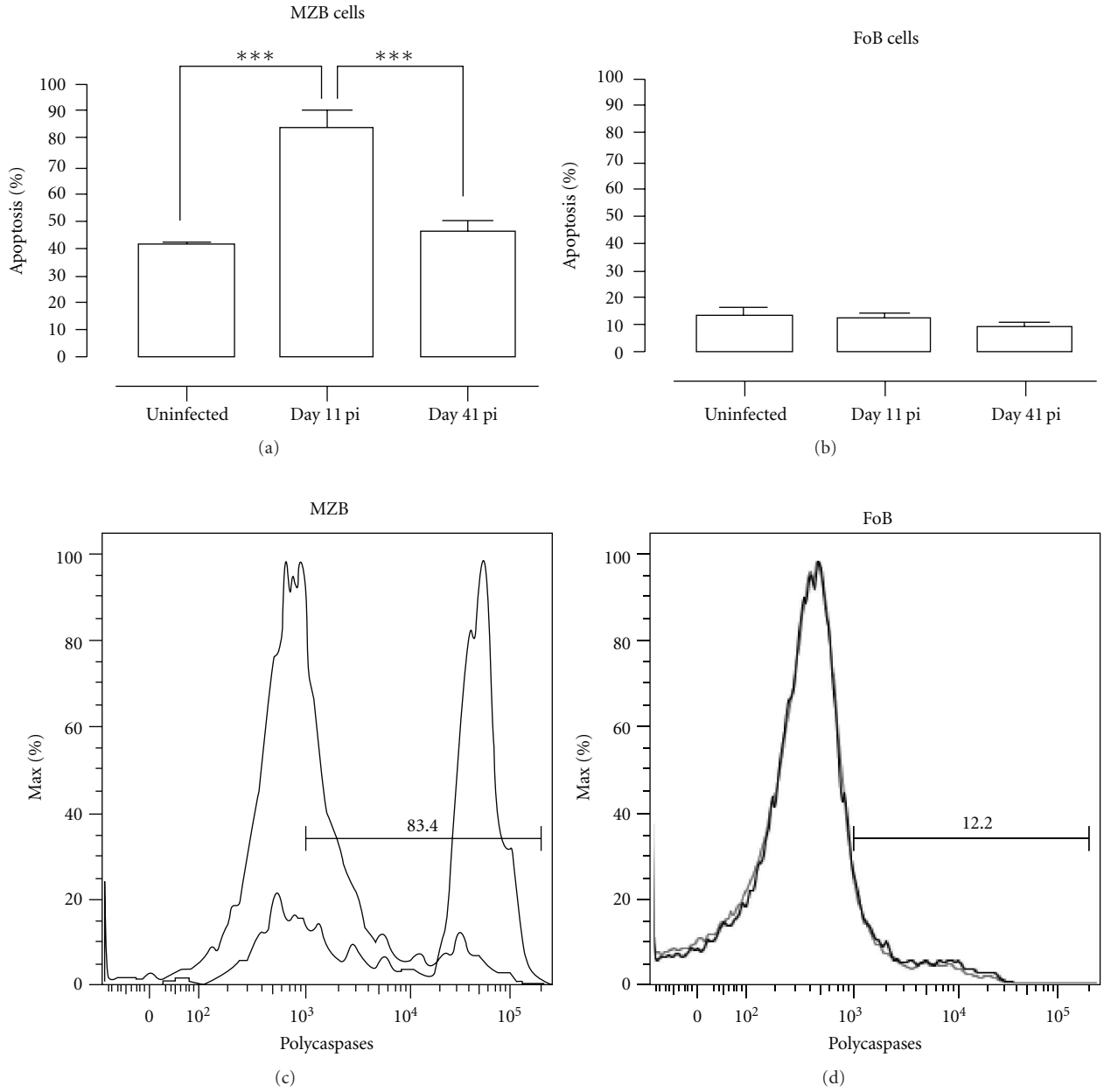


Figure 8: *Pc*AS infection-induced apoptosis of MZB but not FoB cells. Spleens cells from noninfected mice and mice infected with *Pc*AS for 11 and 41 days were stained for surface markers commonly used to define MZB and FoB cells and the amount of active caspase 1, -3, -4, -5, -6, -7, -8, and -9 was measured intracellular by flowcytometry. (a) and (b) Percentage of apoptotic cells within MZB or FoB cell population in uninfected controls versus infected mice on day 11 and 41 pi. Data are represented as mean of three mice per group ± SEM (***) $P < .001$. (c) and (d) Representative histogram of uninfected controls (grey line) versus a day 11 pi (black line).

show loss of activated or memory $CD4^+$T cells, B cells and plasma cells and short-lived malaria-specific antibodies after a primary acute infection [28, 34–36]. Hence, it is possible that the lack of efficient, long lasting protective immunity observed in human malaria is due to defects in the B-cell lineage. Immunization and infection with *Plasmodium* or other pathogens have already been described to transiently suppress and/or alter bone marrow hematopoiesis, followed by an increased migration of immature cells out of the bone marrow, including RAG+ immature B cells [37]. In this context, an increase in GL7-expressing cells was detected in PBMC of *Pc*AS infected mice and these cells also appeared to be mainly immature B cells which had presumably migrated from the bone marrow [38]. In agreement with this, we show in this study that during an acute primary blood stage *Pc*AS infection, B-cell development is seriously impaired with a more than 95% depletion of B-cell precursor cells in the bone marrow. Later, when the acute first peak of infection has been overcome and parasitemia remains below detection levels, B lymphopoiesis slowly but surely recovers in the bone

marrow. Loss of developing B cells from the bone marrow has been correlated before with a reduction in bone marrow CXCL12, resulting in a mobilization of developing B cells to the periphery. In accordance with this, we report here both a reduced CXCL12 production in the bone marrow and an influx of developing B cells in the spleen during the acute phase of infection. This premature migration out of the bone marrow may reflect the physiologic process for replenishing the transitional B-cell pool in the spleen, but it actually results in a transient loss of bone marrow B lymphopoiesis. The inflammatory cytokine TNF has been reported to modulate B-cell development by reducing the ability of the bone marrow to retain developing B cells. ([17], reviewed in [18]). In the serum of *Pc*AS-infected mice, TNF levels are very high around peak parasitemia [39], so it is possible that a TNF-modulated reduction in CXCL12 production in the bone marrow is contributing to the observed impaired B lymphopoiesis.

It had already been described before that the majority of apoptotic cells in the spleen of *Pc*AS infected mice are B cells, most likely comprising polyclonally activated B cells through a mechanism that could possibly involve Fas [40]. Here, we show that a *Pc*AS infection causes a severe depletion of transitional T2 B cells and MZB cells in the spleen but there is no significant change in FoB cell numbers during the early stage of infection. The loss of transitional T2 and MZB cells mainly results from the highly increased occurrence of apoptosis in this population as about 80% of all T2 and MZB cells are undergoing apoptosis in the acute phase of infection. However, most likely MZB cells also serve as an important source for T-cell independently generated IgM+ plasma B cells during the early stage of infection, seen that parasite-specific IgM responses are already high around day 8 and peak at day 14 of infection [41]. In this context, it can be speculated that by inducing massive MZB apoptosis, the parasite is playing in its own advantage since protection during an acute *Pc*AS infection is (partially) dependent on specific IgM and not natural IgM (mostly produced by B-1 cells) [40]. In contrast, there is no infection-induced apoptosis of FoB cells in the spleen. During *Pc*AS infection in mice, there are many class-switched plasma B cells found in both peripheral blood and spleen, comprising mostly IgG2a-secreting cells [42–45] that play an important role in parasite clearance. Most likely the conservation of the FoB cell pool during infection, being the major source for high-affinity malaria-specific class-switched plasma B cells, which in turn are essential for parasite clearance, allows the mouse to control the *Pc*AS infection.

It is believed that periodic reinfection is necessary to maintain acquired immunity to malaria and that *Plasmodium*-specific antibodies are short lived in the absence of reinfection, implying that the B-cell memory response to malaria infection may be defective or suboptimal [1, 46]. However, the development and longevity of malaria-specific B-cell memory is widely debated. Some studies have shown that malaria infection interferes with the development of long-lived plasma cells and memory B cells [35, 47, 48], while others have demonstrated in both human infections and animal models that isotype-switched memory B cells do develop and are detectable for months or years in blood and/or spleen [38, 46, 49]. However, persistence of memory B cells in peripheral blood alone may not correlate with longevity of specific humoral immunity. In this context, in both malaria patients as well as murine models of malaria, both short-lived [50, 51] and long-lived [49] anti-*Plasmodium* antibody responses have been reported in longitudinal and cross-sectional studies. However, in the case of *Plasmodium yoelii*, infection-induced deletion of vaccine-specific memory B cells as well as long-lived plasma cells including those specific for bystander immune responses have been described [12]. Hence, it can be speculated that the prospects for developing an effective vaccine that is protective against human *Plasmodium falciparum* or *Plasmodium vivax* infections are much better than for African trypanosome infections, if further research clarifies for several different infection settings whether there is no continuous destruction of immunological memory during malaria infections or whether there is a situation similar to the one observed for African trypanosome infections [52].

Taken together, our observations fit well with previous studies and elucidate in detail the kinetics of B-cell development and maturation during malaria infection, which is essential for understanding the disease and the future development of antimalaria vaccines.

Acknowledgment

V. Bockstal and N. Geurts are doctoral fellows of the Fund for Scientific Research Flanders (FWO).

References

[1] J. Langhorne, F. M. Ndungu, A. M. Sponaas, and K. Marsh, "Immunity to malaria: more questions than answers," *Nature Immunology*, vol. 9, no. 7, pp. 725–732, 2008.

[2] D. L. Doolan and N. Martinez-Alier, "Immune response to pre-erythrocytic stages of malaria parasites," *Current Molecular Medicine*, vol. 6, no. 2, pp. 169–185, 2006.

[3] T. Von der Weid, D. Kitamura, K. Rajewsky, and J. Langhorne, "A dual role for B cells m *Plasmodium chabaudi chabaudi* (AS) infection?" *Research in Immunology*, vol. 145, no. 6, pp. 412–419, 1994.

[4] C. L. Diggs and A. G. Osler, "Humoral immunity in rodent malaria. II. Inhibition of parasitemia by serum antibody," *Journal of Immunology*, vol. 102, no. 2, pp. 298–305, 1969.

[5] S. J. Meding and J. Langhorne, "CD4+ T cells and B cells are necessary for the transfer of protective immunity to *Plasmodium chabaudi chabaudi*," *European Journal of Immunology*, vol. 21, no. 6, pp. 1433–1438, 1991.

[6] W. Jarra, L. A. Hills, J. C. March, and K. N. Brown, "Protective immunity to malaria. Studies with cloned lines of *Plasmodium chabaudi chabaudi* and P. berghei in CBA/Ca mice. II. The effectiveness and inter- or intra-species specificity of the passive transfer of immunity with serum," *Parasite Immunology*, vol. 8, no. 3, pp. 239–254, 1986.

[7] S. Cohen, I. A. McGregor, and S. Carrington, "Gamma-globulin and acquired immunity to human malaria," *Nature*, vol. 192, no. 4804, pp. 733–737, 1961.

[8] C. L. Diggs, "*Plasmodium falciparum*: passive immunization of Aotus lemurinus griseimembra with immune serum," *Experimental Parasitology*, vol. 80, no. 2, pp. 291–296, 1995.

[9] A. H. Achtman, R. Stephens, E. T. Cadman, V. Harrison, and J. Langhorne, "Malaria-specific antibody responses and parasite persistence after infection of mice with *Plasmodium chabaudi chabaudi*," *Parasite Immunology*, vol. 29, no. 9, pp. 435–444, 2007.

[10] F. M. Ndungu, E. T. Cadman, J. Coulcher et al., "Functional memory B cells and long-lived plasma cells are generated after a single *Plasmodium chabaudi* infection in mice," *PLoS Pathogens*, vol. 5, no. 12, Article ID e1000690, 2009.

[11] R. Stephens, F. M. Ndungu, and J. Langhorne, "Germinal centre and marginal zone B cells expand quickly in a second *Plasmodium chabaudi* malaria infection producing mature plasma cells," *Parasite Immunology*, vol. 31, no. 1, pp. 20–31, 2009.

[12] M. N. Wykes, Y. H. Zhou, X. Q. Liu, and M. F. Good, "*Plasmodium yoelii* can ablate vaccine-induced long-term protection in mice," *Journal of Immunology*, vol. 175, no. 4, pp. 2510–2516, 2005.

[13] M. Qing, D. Jones, and T. A. Springer, "The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment," *Immunity*, vol. 10, no. 4, pp. 463–471, 1999.

[14] T. Nagasawa, "Microenvironmental niches in the bone marrow required for B-cell development," *Nature Reviews Immunology*, vol. 6, no. 2, pp. 107–116, 2006.

[15] E. S. Vitetta and J. W. Uhr, "Immunoglobulin receptors revisited," *Science*, vol. 189, no. 4207, pp. 964–969, 1975.

[16] E. R. Abney, M. D. Cooper, J. F. Kearney, A. R. Lawton, and R. M. Parkhouse, "Sequential expression of immunoglobulin on developing mouse B lymphocytes: a systematic survey that suggests a model for the generation of immunoglobulin isotype diversity," *Journal of Immunology*, vol. 120, no. 6, pp. 2041–2049, 1978.

[17] Y. Ueda, K. Yang, S. J. Foster, M. Kondo, and G. Kelsoe, "Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression," *Journal of Experimental Medicine*, vol. 199, no. 1, pp. 47–57, 2004.

[18] D. Cain, M. Kondo, H. Chen, and G. Kelsoe, "Effects of acute and chronic inflammation on B-cell development and differentiation," *Journal of Investigative Dermatology*, vol. 129, no. 2, pp. 266–277, 2009.

[19] B. Srivastava, R. C. Lindsley, N. Nikbakht, and D. Allman, "Models for peripheral B cell development and homeostasis," *Seminars in Immunology*, vol. 17, no. 3, pp. 175–182, 2005.

[20] J. E. Labrie, A. P. Sah, D. M. Allman, M. P. Cancro, and R. M. Gerstein, "Bone marrow microenvironmental changes underlie reduced RAG-mediated recombination and B cell generation in aged mice," *Journal of Experimental Medicine*, vol. 200, no. 4, pp. 411–423, 2004.

[21] D. Allman, R. C. Lindsley, W. DeMuth, K. Rudd, S. A. Shinton, and R. R. Hardy, "Resolution of three nonproliferate immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation," *Journal of Immunology*, vol. 167, no. 12, pp. 6834–6840, 2001.

[22] L. Gorelik, A. H. Cutler, G. Thill et al., "Cutting Edge: BAFF regulates CD21/35 and CD23 expression independent of its B cell survival function," *Journal of Immunology*, vol. 172, no. 2, pp. 762–766, 2004.

[23] N. N. Belyaev, D. E. Brown, A. I. G. Diaz et al., "Induction of an IL7-R c-Kit myelolymphoid progenitor critically dependent on IFN-γ signaling during acute malaria," *Nature Immunology*, vol. 11, no. 6, pp. 477–485, 2010.

[24] T. Nagasawa, S. Hirota, K. Tachibana et al., "Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1," *Nature*, vol. 382, no. 6592, pp. 635–638, 1996.

[25] K. Tokoyoda, T. Egawa, T. Sugiyama, B. I. Choi, and T. Nagasawa, "Cellular niches controlling B lymphocyte behavior within bone marrow during development," *Immunity*, vol. 20, no. 6, pp. 707–718, 2004.

[26] A. W. Taylor-Robinson and R. S. Phillips, "Functional characterization of protective CD4+ T-cell clones reactive to the murine malaria parasite *Plasmodium chabaudi*," *Immunology*, vol. 77, no. 1, pp. 99–105, 1992.

[27] A. W. Taylor-Robinson, R. S. Phillips, A. Severn, S. Moncada, and F. Y. Liew, "The role of T(H)1 and T(H)2 cells in a rodent malaria infection," *Science*, vol. 260, no. 5116, pp. 1931–1934, 1993.

[28] J. Langhorne, S. J. Meding, K. Eichmann, and S. S. Gillard, "The response of CD4+ T cells to *Plasmodium chabaudi chabaudi*," *Immunological Reviews*, no. 112, pp. 71–94, 1989.

[29] S. Cohen and G. A. Butcher, "Serum antibody in acquired malarial immunity," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 65, no. 2, pp. 125–135, 1971.

[30] P. Druilhe and S. Khusmith, "Epidemiological correlation between levels of antibodies promoting merozoite phagocytosis of *Plasmodium falciparum* and malaria-immune status," *Infection and Immunity*, vol. 55, no. 4, pp. 888–891, 1987.

[31] F. H. A. Osier, G. Fegan, S. D. Polley et al., "Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria," *Infection and Immunity*, vol. 76, no. 5, pp. 2240–2248, 2008.

[32] D. R. Cavanagh, D. Dodoo, L. Hviid et al., "Antibodies to the N-terminal block 2 of *Plasmodium falciparum* merozoite surface protein 1 are associated with protection against clinical malaria," *Infection and Immunity*, vol. 72, no. 11, pp. 6492–6502, 2004.

[33] W. G. Metzger, D. M. N. Okenu, D. R. Cavanagh et al., "Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria," *Parasite Immunology*, vol. 25, no. 6, pp. 307–312, 2003.

[34] H. Xu, J. Wipasa, H. Yan et al., "The mechanism and significance of deletion of parasite-specific CD4+ T cells in malaria infection," *Journal of Experimental Medicine*, vol. 195, no. 7, pp. 881–892, 2002.

[35] S. S. Struik and E. M. Riley, "Does malaria suffer from lack of memory?" *Immunological Reviews*, vol. 201, pp. 268–290, 2004.

[36] A. P. Freitas Do Rosário, S. M. Muxel, S. M. Rodríguez-Málaga et al., "Gradual decline in malaria-specific memory T cell responses leads to failure to maintain long-term protective immunity to *Plasmodium chabaudi* AS despite persistence of B cell memory and circulating antibody," *Journal of Immunology*, vol. 181, no. 12, pp. 8344–8355, 2008.

[37] H. Nagaoka, G. Gonzalez-Aseguinolaza, M. Tsuji, and M. C. Nussenzweig, "Immunization and infection change the number of recombination activating gene (RAG)-expressing B cells in the periphery by altering immature lymphocyte production," *Journal of Experimental Medicine*, vol. 191, no. 12, pp. 2113–2120, 2000.

[38] E. W. Nduati, D. H. L. Ng, F. M. Ndungu, P. Gardner, B. C. Urban, and J. Langhorne, "Distinct kinetics of memory B-cell and plasma-cell responses in peripheral blood following a blood-stage *Plasmodium chabaudi* infection in mice," *PLoS ONE*, vol. 5, no. 11, Article ID e15007, 2010.

[39] M. M. Stevenson, M. F. Tam, and M. Nowotarski, "Role of interferon-γ and tumor necrosis factor in host resistance to *Plasmodium chabaudi* AS," *Immunology Letters*, vol. 25, no. 1–3, pp. 115–121, 1990.

[40] H. Helmby, G. Jönsson, and M. Troye-Blomberg, "Cellular changes and apoptosis in the spleens and peripheral blood of mice infected with blood-stage *Plasmodium chabaudi chabaudi* AS," *Infection and Immunity*, vol. 68, no. 3, pp. 1485–1490, 2000.

[41] K. N. Couper, R. S. Phillips, F. Brombacher, and J. Alexander, "Parasite-specific IgM plays a significant role in the protective immune response to asexual erythrocytic stage *Plasmodium chabaudi* AS infection," *Parasite Immunology*, vol. 27, no. 5, pp. 171–180, 2005.

[42] P. B. Falanga, M. R. D'Imperio Lima, A. Coutinho, and L. Pereira Da Silva, "Isotypic pattern of the polyclonal B cell response during primary infection by *Plasmodium chabaudi* and in immune-protected mice," *European Journal of Immunology*, vol. 17, no. 5, pp. 599–603, 1987.

[43] M. R. D'Imperio Lima, A. Bandeira, P. Falanga et al., "Clonal analysis of B lymphocyte responses to *Plasmodium chabaudi* infection of normal and immunoprotected mice," *International Immunology*, vol. 3, no. 12, pp. 1207–1216, 1991.

[44] Z. Su and M. M. Stevenson, "IL-12 is required for antibody-mediated protective immunity against blood-stage *Plasmodium chabaudi* AS malaria infection in mice," *Journal of Immunology*, vol. 168, no. 3, pp. 1348–1355, 2002.

[45] S. I. Castillo-Méndez, C. A. Zago, L. R. Sardinha, A. P. Freitas Do Rosário, J. M. Álvarez, and M. R. D'Imperio Lima, "Characterization of the spleen B-cell compartment at the early and late blood-stage *Plasmodium chabaudi* malaria," *Scandinavian Journal of Immunology*, vol. 66, no. 2-3, pp. 309–319, 2007.

[46] J. Wipasa, C. Suphavilai, L. C. Okell et al., "Long-lived antibody and B cell memory responses to the human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*," *PLoS Pathogens*, vol. 6, no. 2, Article ID e1000770, 2010.

[47] R. Stephens, F. R. Albano, S. Quin et al., "Malaria-specific transgenic CD4+ T cells protect immunodeficient mice from lethal infection and demonstrate requirement for a protective threshold of antibody production for parasite clearance," *Blood*, vol. 106, no. 5, pp. 1676–1684, 2005.

[48] M. R. D'Imperio Lima, J. M. Alvarez, G. C. Furtado, T. L. Kipnis, A. Coutinho, and P. Minóprio, "Ig-isotype patterns of primary and secondary B cell responses to *Plasmodium chabaudi chabaudi* correlate with IFN-gamma and IL-4 cytokine production with CD45RB expression by CD4+ spleen cells," *Scandinavian Journal of Immunology*, vol. 43, no. 3, pp. 263–270, 1996.

[49] F. M. Ndungu, E. T. Cadman, J. Coulcher et al., "Functional memory B cells and long-lived plasma cells are generated after a single *Plasmodium chabaudi* infection in mice," *PLoS Pathogens*, vol. 5, no. 12, Article ID e1000690, 2009.

[50] O. J. Akpogheneta, N. O. Duah, K. K. A. Tetteh et al., "Duration of naturally acquired antibody responses to blood-stage *Plasmodium falciparum* is age dependent and antigen specific," *Infection and Immunity*, vol. 76, no. 4, pp. 1748–1755, 2008.

[51] S. M. Kinyanjui, P. Bull, C. I. Newbold, and K. Marsh, "Kinetics of antibody responses to *Plasmodium falciparum*-infected erythrocyte variant surface antigens," *Journal of Infectious Diseases*, vol. 187, no. 4, pp. 667–674, 2003.

[52] M. Radwanska, P. Guirnalda, C. De Trez, B. Ryffel, S. Black, and S. Magez, "Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses," *PLoS Pathogens*, vol. 4, no. 5, Article ID e1000078, 2008.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 283416, 6 pages
doi:10.1155/2011/283416

Research Article

An ELISA to Detect Serum Antibodies to the Salivary Gland Toxin of *Ixodes holocyclus* Neumann in Dogs and Rodents

S. Hall-Mendelin,[1,2] P. O'Donoghue,[1] R. B. Atwell,[3] R. Lee,[1,4] and R. A. Hall[1]

[1] *School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, QLD 4072, Australia*
[2] *Public Health Virology, Queensland Health Forensic and Scientific Services, Coopers Plains, QLD 4108, Australia*
[3] *School of Veterinary Science, The University of Queensland, St. Lucia, QLD 4072, Australia*
[4] *Westmead Clinical School, The University of Sydney, NSW 2006, Australia*

Correspondence should be addressed to R. A. Hall, roy.hall@uq.edu.au

Received 3 January 2011; Accepted 13 March 2011

Academic Editor: Domenico Otranto

The *Ixodes holocyclus* tick causes paralysis in up to 10,000 companion and domestic animals each year in Australia. Treatment requires the removal of the parasite and the administration of a commercial tick antiserum that is prepared from hyperimmune dogs. Each batch of this serum is initially tested for toxin-neutralising potency in a mouse bioassay that is expensive, time consuming, and subjective. With the aim of developing a rapid *in vitro* assay to replace the bioassay, we used a partially purified antigen prepared from *I. holocyclus* salivary glands to develop an ELISA to detect toxin-reactive antibodies in hyperimmune dog sera. The optimised ELISA reliably detected antibodies reactive to *I. holocyclus* salivary gland antigens. Parallel testing of sera with a negative control antigen prepared from the salivary glands of the nontoxic tick *Rhipicephalus* (*Boophilus*) *microplus* provided further evidence that we were detecting toxin-specific antibodies in the assay. Using the ELISA, we could also detect antibodies induced in rats after experimental infestation with *I. holocyclus*. This assay shows promise as an alternative means of assessing the potency of batches of hyperimmune dog serum and to screen for toxin-reactive monoclonal antibodies produced from immunised rodents.

1. Introduction

Ixodid or hard ticks cause most toxicoses [1], affecting humans and animals around the world [2, 3]. The most severe form of toxicosis results in the paralysis of the infested host. Globally, just under 70 species of ticks have been described as being capable of inducing paralysis [2], the most important being *Ixodes holocyclus* in Australia, *Dermacentor andersoni*, *D. variabilis*, and *Argas* (*Persicargas*) *radiatus* in North America, *I. rubicundus* in South Africa, *Rhipicephalus evertsi evertsi* and *A.* (*P.*) *walkerae* in Ethiopia, and *A.* (*P.*) *radiatus* in the Nearctic region of North America [4].

In Australia, *I. holocyclus* can cause paralysis in a range of domestic animals and livestock [5], affecting up to 10,000 companion animals and up to 100,000 livestock per year [6]. It is considered highly toxic with one female able to kill a dog [1] or sheep [7].

I. holocyclus is found along the eastern seaboard of Australia and is most abundant from early spring to late summer. Paralysis is induced by a neurotoxin that is transmitted to the host in the saliva of a female *I. holocyclus* when the tick takes a blood meal. During feeding, toxicity in the salivary glands increases, peaking after 4-5 days of engorgement [8].

The treatment of paralysed hosts requires removal of the parasite and administration of a commercial tick antiserum (TAS) that is prepared from hyperimmune dogs [9]. To standardise the toxin-neutralising activity of each batch of TAS, a mouse bioassay has been traditionally used. However, the bioassay is expensive, time consuming, and subjective, relying on observations of paralytic signs in neonatal mice over a 24-hour period [10]. Developing a suitable *in vitro* immunoassay to quantify toxin-specific antibodies in commercial TAS could replace the *in vivo* test.

An *in vitro* assay may also facilitate timely diagnosis and inform on treatment options. Knowledge of the immune

status of dogs before and after treatment for tick paralysis will help to evaluate a minimal, effective dose of TAS, avoiding possible adverse reactions and making treatment more affordable.

In this paper, we describe the development of an enzyme-linked immunosorbent assay (ELISA) to measure antibody levels against tick salivary gland antigens in the serum of dogs and rodents after natural or laboratory tick infestation.

2. Materials and Methods

2.1. Preparation of Salivary Gland Antigens from Ticks. Ten adult unfed female *I. holocyclus* were allowed to feed on 10- to 11-week-old female Wistar rats following the methods of Stone et al. [11]. After 5 days, engorged ticks were carefully removed with tweezers, and salivary glands were excised and stored at −80°C. *Ixodes holocyclus* salivary glands were processed following the method of Stone et al. [10]. One hundred frozen salivary gland pairs were homogenised (Dounce, 7 mL) with 3 mL of sterile PBS and the homogenate clarified at 1500 g for 30 minutes at 4°C. The pellet was washed three times with 0.5 mL PBS and centrifuged as above. The pooled supernatant was sonicated to further disrupt remaining particulate matter with a Soniprep 150 MSE ultrasonicator using 30-second bursts followed by 2 minutes cooling on ice for a total of 10 minutes. The sonicated homogenate was pelleted at 109,000 g for 1 hour at 4°C, and the resulting supernatant was stored in aliquots at −80°C. Protein concentrations in antigen preparations were estimated with the BCA Protein Assay Kit as per protocol. All batches of *I. holocyclus* toxin were tested in the neonatal mouse bioassay to determine the level of paralysing activity [10].

2.2. Preparation of Control Antigen from the Cattle Tick Rhipicephalus (Boophilus) microplus. Salivary gland antigens from the cattle tick, *Rh.* (*B.*) *microplus,* which had fed for 5 days on cattle, were prepared in the same manner and used as a nontoxin control in all assays. These extracts were also tested in the bioassay, and no paralysis signs were observed in neonatal mice.

2.3. Dog Serum Samples. A standardised reference serum was prepared by pooling several batches of commercially available TAS prepared from ten hyperimmune (HI) dogs and confirmed for toxin-neutralising activity in the mouse bioassay. Ten nonreactive dog sera were obtained from Perth in Western Australia, an area where *I. holocyclus* does not naturally occur. Sera were also obtained from dogs that presented at Manly Road Veterinary Hospital, Brisbane, for treatment against tick paralysis. Samples were collected at the time of admission, prior to treatment, and again approximately 16 days later.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA). An indirect ELISA format was employed for the analysis of sera. Various parameters were systematically optimised by empirical trials. The basic protocol was as follows: tick salivary gland antigen was diluted in coating buffer (0.05 M carbonate/bicarbonate pH 9.6) and 50 μL added to wells of ELISA plates (FALCON flexible, 96 wells, U bottom, Becton Dickinson) at 4°C overnight. After washing (phosphate-buffered saline containing 0.05% Tween 20) the plates twice to remove unbound antigen, 100 μL of blocking buffer (0.05 M Tris, 0.001 M EDTA, 0.15 M NaCl, 0.2% w/v casein, 0.05% v/v Tween 20, pH 8.0) was applied to all wells for one hour at 37°C. After the removal of blocking buffer, 50 μL of dog sera diluted in blocking buffer was added per well for 1 h at 37°C. After 4 washes, rabbit antidog IgG conjugated to horse-radish peroxidase (whole molecule, HRPO, SIGMA) was diluted in blocking buffer, added (50 μL per well), and allowed to bind to captured dog IgG for a further hour at 37°C. After an additional 6 washes, bound conjugate was detected with 100 μL of ABTS substrate (2% of 2, 2′-Azinobis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt, SIGMA; 2% of 1.26% H_2O_2 in substrate buffer: 2.8% Na_2HPO_4 and 2.1% citric acid, pH 4.2). After a 1-h reaction at 37°C, the spectroscopic absorbance of each well was measured in an automated plate reader (MULTISKAN) at a wavelength of 405 nm.

2.5. Optimisation of ELISA to Detect Toxin-Reactive Antibodies in Rat Sera. A modified ELISA was used to test sera from rats experimentally infested with *I. holocyclus* ticks. Serum was collected from rats prior to and at various times after ticks had fed on the animals for five days. Pre- and post-tick infestation sera were then tested in the ELISA. In the absence of rat serum known to be reactive to tick toxin, the optimised protocol for dog sera was employed, with the exception that horseradish peroxidase-conjugated antirat IgG diluted 1 : 1000 was used for the second antibody step.

3. Results

3.1. Optimisation of ELISA Parameters. Initial analysis of HI dog sera revealed that high levels of nonspecific binding to the plastic in the wells of the ELISA plate occurred at dilutions below 1/50 (results not shown). Therefore, all dog sera were subsequently tested at dilutions of 1/50 or greater.

To determine the optimal dilution of *I. holocyclus* salivary gland antigen, pooled HI dog serum and antigen were titrated in a checkerboard format. Serum dilutions of 1/50–1/200 demonstrated maximum binding to *Ixodes* antigen at 6.25 μg/mL with optical densities (OD) between 1.5 and 2.3 (Figure 1(a)). Negligible binding was observed for each dilution of the negative control dog serum at all antigen concentrations examined (OD < 0.5). Therefore, 6.25 μg/mL was chosen as the antigen concentration for all subsequent assays.

HI and negative dog sera were also assayed against similar concentrations of salivary gland antigen prepared from the cattle tick, *Rh.* (*B.*) *microplus* (Figure 1(b)). All sera dilutions showed significantly reduced or negligible binding to this antigen compared to similar concentrations of antigen prepared from *Ixodes* salivary glands indicating that most of the ELISA-reactive antibody in the sera was specific to the *Ixodes* antigen. Some cross-reactivity was observed for the lowest dilutions (1/50 and 1/100) of HI dog (ODs between

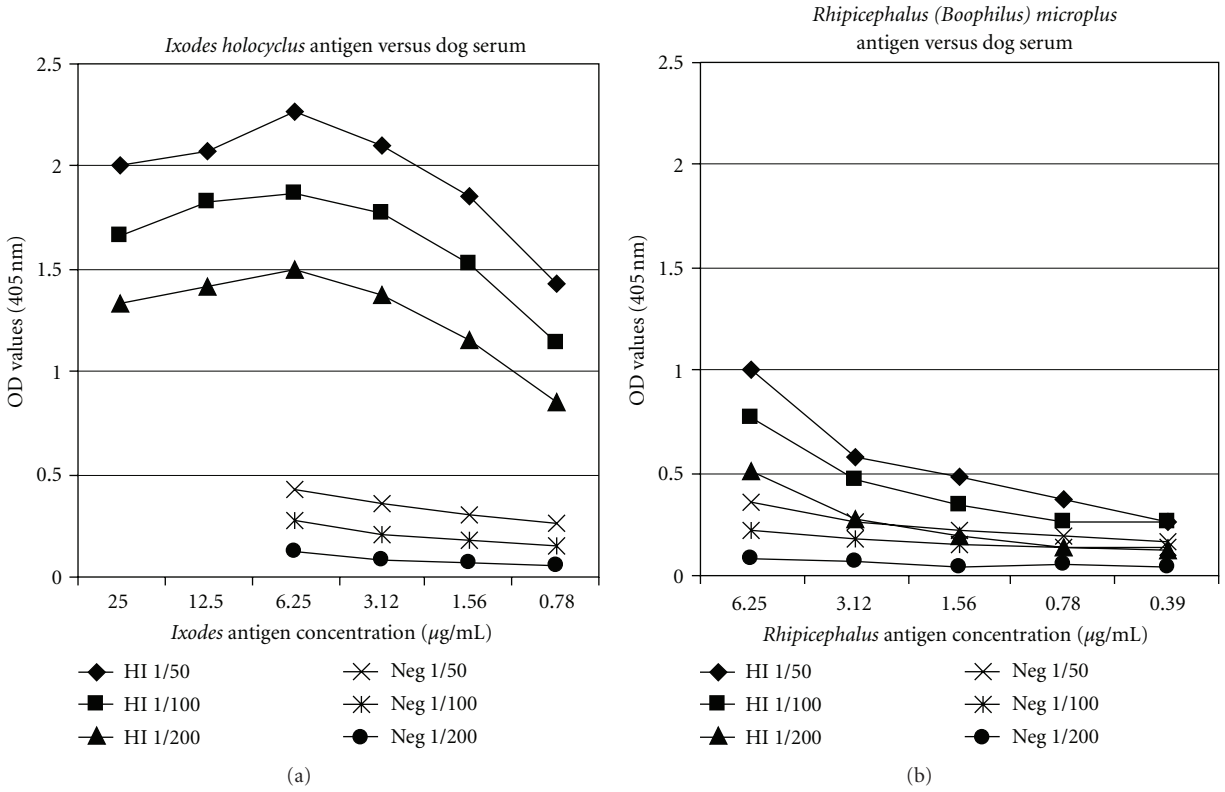


Figure 1: Optimisation of salivary gland antigen concentration in ELISA. Doubling dilutions (1/50–1/200) of pooled hyperimmune (HI) or negative (Neg) dog sera were incubated with varying concentrations of *Ixodes* (a) or *Rhipicephalus* (b) antigen in ELISA. Antigens were diluted in carbonate/bicarbonate coating buffer and adsorbed to the solid phase at 4°C overnight. All other steps were performed at 37°C for 1 h as described in Section 2.

0.5 and 1.0) with the *Rh.* (*B.*) *microplus* antigen at the two highest antigen concentrations (3.12 and 6.25 μg/mL) consistent with the recognition of salivary gland antigens common to both *I. holocyclus* and *Rh.* (*B.*) *microplus.* In summary, these results demonstrate that partially purified tick salivary gland antigens containing *I. holocyclus* toxin are specifically recognised by antibodies in hyperimmune dog sera in ELISA.

To determine whether antigen adsorbtion could be improved by various combinations of coating buffers, incubation temperatures, and times, *I. holocyclus* or *Rh.* (*B.*) *microplus* antigens were diluted in carbonate/bicarbonate buffer (pH 9.6) or PBS (pH 7.2) and incubated in ELISA plate wells at 4°C overnight or 37°C for 3 h. When ELISA plates were then tested against HI and negative dog sera titrated from 1/50 in blocking buffer, antigens adsorbed in coating buffer at 37°C resulted in significantly higher binding of both HI and negative control serum antibodies to both *I. holocyclus* and *Rh.* (*B.*) *microplus* antigens compared with adsorption at 4°C (results not shown). The best differential binding between HI and negative serum on *I. holocyclus* antigens and HI serum on *I. holocyclus* and *Rh.* (*B.*) *microplus* antigens was achieved by adsorbing antigens in carbonate coating buffer at 4°C (results not shown).

3.2. Establishing a Cutoff OD Value to Distinguish between Positive and Negative Reactions in Dog Sera. A panel of ten HI and ten negative sera at 1/50 was tested in the ELISA using the optimal conditions described above. The results are presented in a box and whisker diagram (Figure 2), illustrating the spread of data. The data set is divided into four equal parts, the quartiles, each representing 25% of the data. The lower quartile (q1) encompasses the lowest 25% of reactors and the upper quartile (q3) the highest 25% of reactors. The median is the middle value with 50% of the data below and above. Minimum (min) is the lowest value, and maximum (max) is the highest value of the data set.

The cutoff value for negative sera tested against *Ixodes* antigen was calculated as the mean of ten negative sera (0.525) plus twice the standard deviation (2×0.14) = 0.805.

3.3. Analysis of Sera from Dogs Naturally Infested with I. holocyclus and Suffering Tick Paralysis. Eight dogs (A to H) presented at the Manly Road Veterinary Hospital with signs of tick paralysis. On day 0 (upon presentation and prior to treatment) only sera from dogs C, D, and H reacted strongly with *Ixodes* antigen when tested in the ELISA (OD > 1.0) (Table 1). Only the day 0 sera from dog C showed significantly less reactivity with *Rhipicephalus* antigen, while the corresponding sample from dogs D and H reacted similarly with both antigens. Antibody levels to *Ixodes* antigen from the other five dogs fell below the cutoff value determined earlier. Testing of serum samples from day 16

(after administration of TAS) revealed a sharp increase of antibody levels to *Ixodes* antigen in dogs C and H, while a similar increase of antibody levels to *Rhipicephalus* antigen was also observed in dog C. Antibodies in sera from dog G did not react with *Ixodes* antigen on day of admission but rose just above the cutoff threshold on day 16, after treatment with TAS. Antibody levels from all other dogs did not rise above the levels from day 0. These results are summarised in Table 1.

3.4. Optimisation of ELISA for Testing Immune Rat Sera. Serum from seven rats taken prior to and 21 days after infestation with ticks was tested in an ELISA modified to detect rat immunoglobulin to tick toxin. All samples tested on *Ixodes* antigen in ELISA showed slightly increased antibody levels in postinfestation samples as compared to preinfestation serum, although OD levels remained well below values seen with HI dog sera. ODs for *Rhipicephalus* antigen did not increase in three rats while increased only marginally in the remaining four rats (see Table 2). These preliminary data indicated that we could detect, albeit weakly, antibodies to the toxin-associated antigen in rat sera after a single infestation with multiple ticks.

Further analysis of the seroconversion of a rat after sequential infestations with multiple ticks was also undertaken. Serum collected from this animal after two rounds of infestations over 248 days exhibited strong, specific reaction to *Ixodes* antigen when tested in ELISA compared to serum taken prior to infestation (see Figure 3).

Collectively, these results reveal that the ELISA can be used to detect serum antibodies specific for *Ixodes* antigen, induced by single or multiple exposures to *I. holocyclus* ticks.

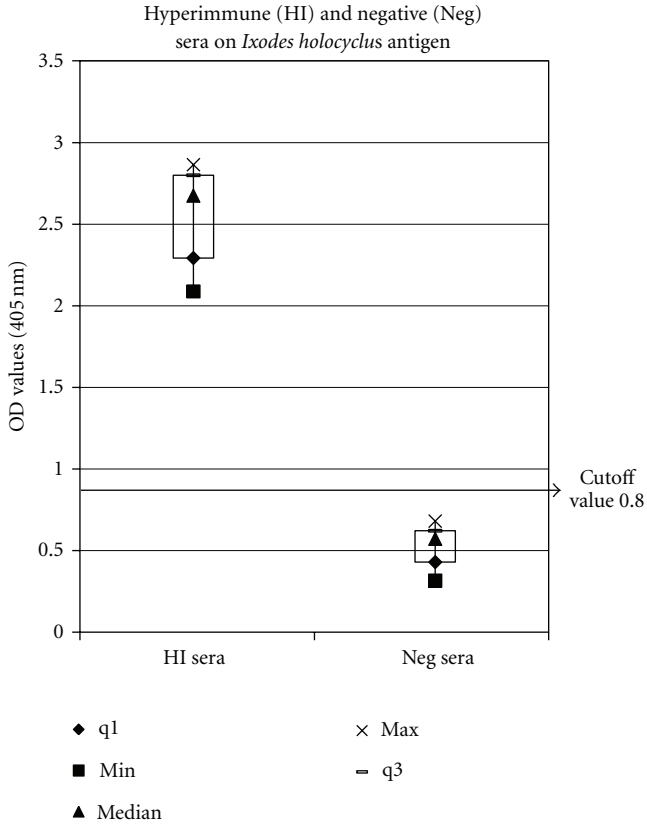


Figure 2: Reactivity of 10 HI and 10 negative dog serum samples tested in ELISA shown in a Box and Whisker diagram: q1 lower quartile, cuts off lowest 25% of data; q3 third quartile, cuts of highest 25% of data.

4. Discussion

A partially purified antigen preparation from the salivary glands of female *I. holocyclus* ticks was assessed for use as a diagnostic reagent in ELISA to measure tick toxin-specific antibodies in dog sera. The results from these assays indicated that sera from HI dogs reacted strongly to the *Ixodes* antigen, compared to negligible reactions of sera from dogs not exposed to *I. holocyclus* ticks. Furthermore, a control antigen prepared from the salivary glands of the cattle tick *Rh.* (*B.*) *microplus*, which does not produce paralysis toxin, showed relatively weak reactions for both HI (positive) and control (negative) sera. The reaction of some dog sera to antigen from *Rh.* (*B.*) *microplus* seen in ELISA was to be expected due to common antigens in the salivary glands in both tick species. Indeed, amino acid sequences between *Ixodes* and *Rhipicephalus* (*Boophilus*) ticks show a high degree of homology, >85% [12] indicating a close antigenic relationship. These data suggest that the ELISA detected antibodies specific to toxin-associated antigens and that *in vitro* analysis of commercially prepared TAS may be a viable alternative to the expensive and subjective mouse bioassay.

Previously, the Australian Pesticides and Veterinary Medicines Authority (APVMA) has declared that an *in vitro* immunoassay to evaluate commercial TAS would only be

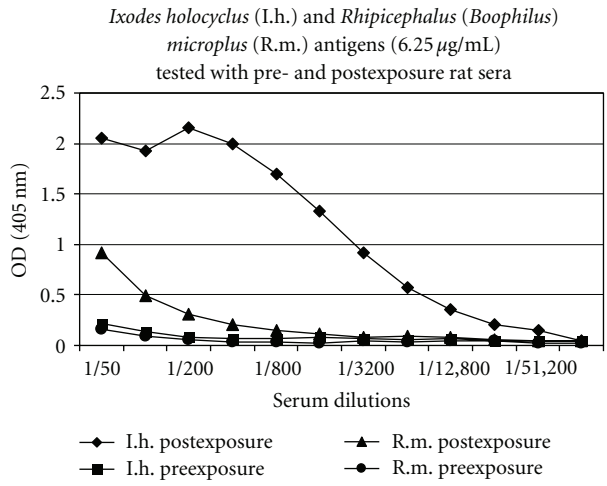


Figure 3: The reaction of rat sera to *Ixodes* antigen in ELISA after two exposures to *I. holocyclus* ticks. Preexposure (negative) and postexposure (positive) rat serum was titrated in doubling dilutions and tested against *Ixodes* and *Rhipicephalus* antigen in ELISA using the optimal conditions and protocol described earlier.

Table 1: Reactions of sera from dogs suffering from tick paralysis to *Ixodes holocyclus* (I.h) and *Rhipicephalus* (*Boophilus*) *microplus* (R.m) antigen in ELISA.

	Day 0[1]		Day 16[2]	
Dog ID	I.h	R.m	I.h	R.m
A	0.666[3]	0.518	n/a	n/a
B	0.536	0.477	0.515	0.443
C	1.227	0.706	2.223	1.358
D	1.902	1.13	1.769	0.993
E	0.41	0.348	0.43	0.359
F	0.538	0.335	0.536	0.363
G	0.565	0.318	0.828	0.362
H	1.047	1.05	2.068	1.201

[1] Sera were collected on day of admission prior to the administration of TAS.
[2] Sera were collected approximately 16 days after TAS was administered.
[3] Optical density (405 nm) of ELISA reaction of sera (1/50) to *Ixodes* (I.h) and *Rhipicephalus* (R.m) antigen.

Table 2: The reaction of sera from rats exposed to *Ixodes holocyclus* ticks with *Ixodes holocyclus* and *Rhipicephalus* (*Boophilus*) *microplus* antigens in ELISA.

Rat ID	$OD_{(405\,nm)}$ ELISA			
	Ixodes antigen		*Rhipicephalus antigen*	
	Preinfestation*	Postinfestation*	Preinfestation	Postinfestation
A	0.19 ± 0.04	0.29 ± 0.005	0.08 ± 0.002	0.07 ± 0.005
B	0.15 ± 0.02	0.31 ± 0.004	0.11 ± 0.003	0.15 ± 0.007
C	0.2 ± 0.01	0.25 ± 0.004	0.13 ± 0.06	0.05 ± 0.004
D	0.2 ± 0.05	0.4 ± 0.002	0.08 ± 0.002	0.16 ± 0.001
E	0.11 ± 0.03	0.2 ± 0.007	0.08 ± 0.006	0.08 ± 0.007
F	0.14 ± 0.02	0.25 ± 0.005	0.068 ± 0005	0.14 ± 0.003
G	0.17 ± 0.06	0.3 ± 0.005	0.1 ± 0.004	0.12 ± 0.003
Tick Naïve Rat	0.11 ± 0.03	0.09 ± 0.001	0.11 ± 0.004	0.07 ± 0.004

* Sera were collected from individual rats before and 21 days after tick infestation, diluted 1/50, and tested against *Ixodes* and *Rhipicephalus* antigens in ELISA using the optimised conditions and protocol described earlier.

approved if a highly specific antigen was used (B. Stone, pers comm.). Indeed, Morrison (unpublished data) [13] used a partially purified antigen extracted from *I. holocyclus* salivary glands in an ELISA and claimed good correlation with the bioassay. However, in the absence of a control antigen (a similar extract from toxin-free ticks), the author was unable to demonstrate the reaction to the antigen was toxinspecific.

In contrast to the strong reactivity of HI dog sera in the ELISA, an apparent lack of antibody responsiveness was observed in sera from dogs naturally infested with ticks and presenting with paralysis signs at a Brisbane veterinary clinic. These sera were tested in the ELISA to assess the level of toxin-specific antibodies at the onset of signs and approximately 16 days later. According to ELISA OD readings, three out of eight dogs showed significant antibodies to tick toxin antigen at the time of presentation, but only two of seven exhibited a significant rise in antitick antibody 16 days later. A fourth serum was negative to *Ixodes* antigen on presentation but seroconverted after treatment to just above threshold level. In the absence of additional information on the general health status of the dogs before and after treatment, including weight, breed, sex, age, history of previous tick exposure, and the dosage and batch of TAS used, interpretation of the data was limited. However, based on the observation that at least half (4/7) of the animals showed no increase in serum antibody levels to the *Ixodes* antigen after treatment with TAS, we may conclude that the increased ELISA response to this antigen detected in some animals was more likely due to an immune response to tick infestation rather than the passive transfer of antibodies through the TAS treatment.

Based on the low rate of seroconversion to toxin-specific antigens in dogs naturally exposed to *I. holocyclus*, even after treatment with TAS, it is unlikely that the assay will be a useful tool for clinicians to monitor the immune status of animals or the efficacy of treatment. This is consistent with findings of Stone and Wright [14] who observed slow and infrequent neutralising antibody responses (measured in a mouse bioassay) in dogs over 14 weeks of repeated tick infestation during the priming phase towards hyper-immunity. The lack of immune response is likely due to the equilibrium achieved between host immunity to the tick

and the tick's suppression of the host's immune system. The feeding tick modulates the host's haemostasis, inflammation, and immunity [15] to enable uninterrupted engorgement. This is supported by the fact that *I. holocyclus* ticks are not rejected (no weight loss) and dogs show very little cutaneous hypersensitivity despite repeated exposure [16].

In summary, an ELISA was established which differentiated strongly between HI dog sera and sera from dogs that had not been infested with *I. holocyclus* ticks. Since the *Ixodes* salivary gland antigen was only partially purified, the specificity of this ELISA was supported by parallel testing of the sera on an antigen prepared in the same way from nontoxic *Rhipicephalus* ticks. HI and nonreactive sera bound substantially weaker to the *Rhipicephalus* antigen, suggesting that reactions of HI sera to the *Ixodes* antigen were specific. Sera from dogs experimentally or naturally infested with a limited number of ticks, however, produce only weak reactions to the *Ixodes* antigen in ELISA, suggesting that antibody levels to low-frequency tick infestations are not detected in this assay.

In the absence of highly purified toxin antigen, the specificity of the assay may be enhanced by the use of tick toxin-reactive monoclonal antibodies to capture toxin-specific antigen to the solid phase in a modified ELISA format. To this end, the ELISA described above was successfully adapted for the detection of antibodies in rats exposed to tick infestation. Indeed, the detection of strong antibody responses in rats after two exposures to ticks indicates that this animal model will be useful for producing hybridomas to *I. holocyclus* toxin antigens and the ELISA an effective means for screening the resultant monoclonal antibodies.

Acknowledgments

This work was supported by funds from Merial Australia and Australian Research Council. Blood-fed *Rhipicephalus* ticks were kindly provided by Dr. C. Turni, of The Tick Fever Unit, QDPI, Wacol. Negative dog sera were a gift from Dr. Russell Hobbs, Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University. HI dog serum samples were a gift from the late Dr. Bernie Stone. Sera from dogs with paralysis after natural tick exposure were provided by Manly Road Veterinary Hospital.

References

[1] B. F. Stone and I. G. Wright, "Tick toxins and protective immunity," in *Tick Biology and Control. Proceedings of the International Conference*, G. B. Whitehead and J. D. Gibson, Eds., pp. 1–5, Grahamstown, South Africa, 1981.

[2] R. Gothe and A. W. H. Neitz, "Tick paralysis: pathogeneses and etiology," in *Advances in Disease Vector Research*, K. F. Harris, Ed., vol. 8, pp. 177–204, Springer, New York, NY, USA, 1991.

[3] B. J. Mans, R. Gothe, and A. W. H. Neitz, "Biochemical perspectives on paralysis and other forms of toxicoses caused by ticks," *Parasitology*, vol. 129, pp. S95–S111, 2004.

[4] B. F. Stone, "Toxicoses induced by ticks and reptiles in domestic animals," in *Natural Toxins. Animal, Plant, and Microbial*, J. B. Harris, Ed., pp. 56–71, Clarendon Press, Oxford, UK, 1986.

[5] S. Hall-Mendelin, S. B. Craig, R. A. Hall et al., "Tick paralysis in Australia caused by *Ixodes holocyclus* Neumann," *Annals of Tropical Medicine and Parasitology*, vol. 105, no. 2, pp. 95–106, 2011.

[6] B. F. Stone and J. H. Aylward, "Tick toxicoses and the causal toxins: tick paralysis," in *Progress in Venom and Toxin Research. Proceedings of the 1st Asia-Pacific Congress on Animal, Plant and Microbial Toxins*, P. Gopalakrishnakone and C. K. Tan, Eds., pp. 594–602, Singapore, 1987.

[7] C. A. Sloan, "Mortality in sheep due to Ixodes species," *Australian Veterinary Journal*, vol. 44, no. 11, p. 527, 1968.

[8] B. S. Goodrich and M. D. Murray, "Factors influencing the toxicity of salivary gland extracts of *Ixodes holocyclus* Neumann," *International Journal for Parasitology*, vol. 8, no. 4, pp. 313–320, 1978.

[9] R. B. Atwell and F. E. Campbell, "Reactions to tick antitoxin serum and the role of atropine in treatment of dogs and cats with tick paralysis caused by *Ixodes holocyclus*: a pilot survey," *Australian Veterinary Journal*, vol. 79, no. 6, pp. 394–397, 2001.

[10] B. F. Stone, M. R. Cowie, J. D. Kerr, and K. C. Binnington, "Improved toxin/antitoxin assays for studies on the Australian paralysis tick *Ixodes holocyclus*," *Australian Journal of Experimental Biology and Medical Science*, vol. 60, no. 3, pp. 309–318, 1982.

[11] B. F. Stone, B. M. Doube, K. C. Binnington, and B. V. Goodger, "Toxins of the Australian paralysis tick *Ixodes holocyclus*," *Recent Advances InAcarology*, vol. 1, pp. 347–356, 1979.

[12] Q. Fang, J. E. Keirans, and T. Mixson, "The use of the nuclear protein-encoding gene, RNA polymerase II, for tick molecular systematics," *Experimental and Applied Acarology*, vol. 28, no. 1–4, pp. 69–75, 2002.

[13] J. J. Morrison, *Experimental studies of mammalian envenomation: laboratory and clinical investigation of envenomation by the Australian rough-scaled snake (Tropidechis carinatus), certain other elapidae, and the Australian paralysis tick (Ixodes holocyclus)*, Ph.D. Thesis, The University of Queensland, Queensland, Australia, 1986.

[14] B. F. Stone and I. G. Wright, "Toxins of *Ixodes holocyclus* and immunity to paralysis," in *Ticks and Tick-Borne Diseases. Proceedings of the 56th annual conference of the Australian Veterinary Association*, L. A. Y. Johnston and M. G. Cooper, Eds., pp. 75–78, Townsville, Australia, 1980.

[15] J. M. C. Ribeiro and I. M. B. Francischetti, "Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives," *Annual Review of Entomology*, vol. 48, pp. 73–88, 2003.

[16] B. F. Stone, A. L. Neish, and I. G. Wright, "Tick (*Ixodes holocyclus*) paralysis in the dog—quantitative studies on immunity following artificial infestation with the tick," *Australian Veterinary Journal*, vol. 60, no. 3, pp. 65–68, 1983.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 901854, 14 pages
doi:10.1155/2011/901854

Research Article

Liver Metabolic Alterations and Changes in Host Intercompartmental Metabolic Correlation during Progression of Malaria

Arjun Sengupta,[1] Angika Basant,[2,3] Soumita Ghosh,[1] Shobhona Sharma,[2] and Haripalsingh M. Sonawat[1]

[1] *Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai, Maharashtra 400005, India*
[2] *Department of Biological Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai, Maharashtra 400005, India*
[3] *The Division of Biological Sciences, University of Chicago, 5812 S. Ellis Avenue, Chicago, IL 60637, USA*

Correspondence should be addressed to Haripalsingh M. Sonawat, hms@tifr.res.in

Received 1 December 2010; Revised 2 March 2011; Accepted 2 March 2011

Academic Editor: Joseph Schrevel

^{1}H NMR-based metabonomics was used to investigate the multimodal response of mice to malarial parasite infection by *P. berghei* ANKA. Liver metabolism was followed by NMR spectroscopy through the course of the disease in both male and female mice. Our results showed alterations in the level of several metabolites as a result of the infection. Metabolites like kynurenic acid, alanine, carnitine, and β-alanine showed significant alteration in the liver, suggesting altered kynurenic acid, glucose, fatty acid and amino acid pathways. Distinct sexual dimorphism was also observed in the global analysis of the liver metabolic profiles. Multiway principal component analysis (MPCA) was carried out on the liver, brain, and serum metabolic profile in order to explore the correlation of liver and brain metabolic profile to the metabolite profile of serum. Changes in such correlation profile also indicated distinct sexual dimorphism at the early stage of the disease. Indications are that the females are able to regulate their metabolism in the liver in such a way to maintain homeostasis in the blood. In males, however, choline in liver showed anticorrelation to choline content of serum indicating a higher phospholipid degradation process. The brain-serum correlation profile showed an altered energy metabolism in both the sexes. The differential organellar responses during disease progression have implications in malaria management.

1. Introduction

Plasmodium is the organism responsible for malaria. It affects 200–300 million people and leads to a million deaths annually [1], thereby posing a global threat. The clinical symptoms of the infection are manifested during the blood stage of the parasite life cycle in the human host [2]. In acute stages of the disease, more than one tissue types are known to be affected [2, 3]. It was shown that during this stage of the disease, the parasitized RBCs show sequestration thereby affecting the microvasculature of heart, kidney, liver, intestines, adipose tissues, and eyes [4–6]. This may result in localized metabolic stress. As the disease progresses towards severity, several complications arise, possibly due to the inflammatory immune response of the host which result in complications such as liver damage, renal failure, cerebral malaria, hypoglycemia and acidosis, some of which may lead to death [7–9]. Although, the transition into these conditions is poorly understood, all of them are associated with different metabolic complications. Thus, one approach towards understanding the disease progression is to understand the metabolic alterations that occur as the disease progresses towards severe stage. These alterations can be monitored at single tissue or biofluid level and/or at the level of the intertissue and/or tissue-biofluid correlation, where one focuses on more than one tissue/biofluid simultaneously.

^{1}H NMR spectroscopy of tissues and biofluids followed by multivariate statistical analyses in a systems biological approach has been widely used in understanding metabolic changes [10]. ^{1}H NMR spectra provide an unbiased profile of metabolites present in the tissue or biofluids thereby providing a good platform for the study of different conditions like breast cancer, diabetes, coronary artery disease, and high blood pressure [11–14]. Metabolic response towards parasitic infections has also been reported in the mouse models of *Trypanosome* and *Schistosome* infection [15, 16]. Recently, our group has shown relevant metabolic links and distinct sexual dimorphism with the progression of malaria in the mouse model by NMR spectroscopy and multivariate statistical analysis on urine, sera, and brain [17].

^{1}H NMR spectra from a biofluid like serum or urine at a single time point provide the snapshot of the molecular phenotype or metabotype of the organism at a global level. This, in principle, arises from the complex exchanges of the metabolites among different compartments. Therefore, the understanding of metabolic processes of multicellular organisms at a global level requires the study of metabolic correlations among the tissue and biofluid metabolic profiles. Sophisticated chemometric techniques have been developed to address such problems. Montoliu et al. compared different unsupervised chemometric techniques in order to study intercompartmental metabolic analysis [18].

In this contribution, we report a NMR-based metabolite profiling followed by the multivariate statistical analysis strategy to delineate a distinct sexual dimorphism in the liver metabolic profile in the mouse model (BALB/c mouse infected with *Plasmodium berghei* ANKA) of malaria parasite infection. Furthermore, employing an unsupervised chemometric strategy (multiway principal component analysis-MPCA), we show the correlation of brain and liver metabolic profile with that of serum metabolic profile. The implications of these results are also discussed.

2. Materials and Methods

2.1. Animal Experiments. The animals in the study were treated in accordance with the prescribed guidelines of the institutional animal ethics committee.

2.2. Collection and Extract Preparation of the Sera, Brain and Liver Samples. The experiments were carried out in a similar fashion as described elsewhere [17]. Briefly, 24 inbred BALB/c mice (12 males and 12 females) aged 6 to 8 weeks were used for the study. Eight male and female mice were injected intraperitonally with 10^6 *P. berghei* ANKA-infected erythrocytes. The remaining eight animals served as controls. Four infected males, four infected females and four control mice (two males and two females) were sacrificed on day 5 after infection. The rest of them were sacrificed on day 13 after infection. The animals were immediately dissected to extract the liver out. The liver samples were snap frozen and kept frozen until the preparation of perchloric acid extract as described earlier [17]. The sera and brain sample collection and extraction were done by the method described in [17].

2.3. NMR Spectroscopy of Tissue and Biofluid Extracts. ^{1}H NMR spectra were acquired on 700 MHz spectrometer (Bruker Biospin, Germany) equipped with broad band inverse probe using 2-dimethyl-2-silapentane-5-sulfonic acid (DSS) as an internal standard and the D_2O as the field frequency lock at 300 K. The pulse sequence used included an excitation sculpting routine for the suppression of the water signal. 1024 transients were collected into 32,768 data points using a spectral width of 12.01 ppm resulting in an acquisition time of 1.94 s. A relaxation delay of 1 s was used between consecutive pulses. The FIDs so obtained were subjected to an exponential multiplication leading to an additional line broadening of 0.2 Hz. A sine bell apodization function was used followed by the Fourier transformation. The spectra were phase and baseline corrected manually and used for further data reduction.

2D COSY spectra were obtained for the identification of the metabolites in the following manner. In the direct dimension, 64 transients were collected with 256 increments in the indirect dimension. A QSINE function was used for processing with 2048 and 1024 data points in direct and indirect dimension, respectively.

2.4. Data Reduction. Two different data reduction procedures were followed. For the purpose of single tissue analysis (liver, for our case), the spectral region 9.5 ppm to 0.5 ppm was segmented in frequency regions of 0.04 ppm, and each bin was integrated using MestReC 4.7.0. The region corresponding to water and urea (4.5 ppm to 6.5 ppm) was excluded to avoid artifacts due to the water suppression and highly variable urea. Certain regions of the spectra are known to have high concentration metabolites with high variance that may mask the important features in the peaks with lower concentration. These regions were also excluded as described earlier [17]. The resulting integrals were normalized to total intensity and Pareto scaled to generate the working data matrix.

For the purpose of the intercompartmental correlation analysis, the binning procedure was done using Amix 5.0. Here, a binning width of 0.003 ppm was used, and only the water region was excluded (4.5 ppm to 5.1 ppm). The bins were integrated and normalized with respect to the total integral to generate the working data matrix.

2.5. Statistical Analyses

2.5.1. Multivariate Analysis on Liver Samples. The liver spectra were subjected to the principal component analysis (PCA) and orthogonal partial least square-discriminant analysis (OPLS-DA), which were performed using Simca-P 12.0 (Umetrics, Sweden). Briefly, PCA is an unsupervised method which is used for revealing any inherent trend or pattern in the data set whereas, OPLS-DA is a supervised method. Here, the class entity towards the sample set is provided *a priori*, thereby showing the class-specific distinction. Q^2(cum) is the diagnostic parameter for the OPLS-DA model which represents the separation of the classes assigned. The models were set up in the following ways: (1) using data from uninfected males and day 5

post-infection males, (2) using data from uninfected males and day 13 post-infection males, (3) same as (1) for females, and (4) same as (3) for females. Initially, PCA was employed (data not shown) in order to find out any hidden trend in the data and to find outliers. This was followed by OPLS-DA. The visualization was aided by two-dimensional scores plot that shows the sample clustering in a two-dimensional space. The variables contributing towards the clustering in the scores plot were extracted using loadings S-plot and VIP plot. The metabolites were identified using Human Metabolome database (HMDB) along with the two-dimensional NMR spectral profiles.

2.5.2. Peak Integration. After extracting the metabolites from the multivariate analytical techniques (PCA and OPLS-DA), individual peaks from the metabolites were selected in the one-dimensional NMR spectra, and they were integrated using Topspin 2.1. The crowding in the NMR spectral profile sometimes resulted in overlap of the peaks. In those cases, the metabolites are quoted together. The peak intensities of individual metabolites were calculated with respect to the intensity of internal standard DSS and normalized to the total tissue weight. Significance test for comparison were performed using Student's t-test. Early- and late-stage infection time points were compared with uninfected controls of the same sex. If the relative peak intensities of a metabolite were comparable ($P > .15$) between male and female uninfected samples, then a t-test was conducted to compare males and females at each post-infection time point. If the levels of the metabolite were different ($P < .15$) between uninfected males and females, then from each post-infection data point the value of the corresponding control average was subtracted. An average of these normalized values was taken and males and females were now compared for each post-infection time point to determine if the deviation from corresponding controls was significantly different between them.

2.5.3. Intercompartmental Metabolic Correlation Analysis. Multiway PCA (MPCA) as described elsewhere [18] was used for the intercompartmental correlation analysis. Initially a Multivariate Curve Resolution-Alternating Least Square algorithm (MCR-ALS) was employed to check the relative contribution of the two relevant compartments. Broadly, two kinds of intercompartmental analysis were sought. (1) liver and serum and (2) brain and serum. In each of these categories, three different models were made for males and females, (a) controls, (b) day 5 after infection, and (c) day 13 after infection. This helps in the visualization of how the intercompartmental correlation changes as the disease progresses. For this purpose, a 3D matrix was prepared from the spectral integrals mentioned earlier using Matlab 7.0.1. In this matrix, the rows are the samples/animals, the columns are the spectral variables, and the third mode is the tissues/biofluids. This matrix was used for MPCA in Solo 5.8 (Eigenvector Inc.). The mathematical details of the method can be found elsewhere [18].

3. Results

Results for the serum and brain analysis have already been discussed elsewhere [17]. Here, we report the alteration of metabolic profile of liver and the intercompartmental correlation of brain and liver with sera.

3.1. Changes in Liver Metabolic Profile with Progression of the Disease. The liver extracts for the males showed no perturbation when the early infection time points were compared with uninfected animals (Q^2(cum) = −0.882). However, in the early infection time points, females showed a large change from that of uninfected controls (Q^2(cum) = 0.988). When the late-stage animals were compared with the uninfected controls, drastic changes were observed irrespective of the sex (Q^2(cum for males = 0.975 and Q^2(cum) for females = 0.993). The representative scores plot of the respective OPLS-DA models is shown in the Figure 1. The VIP plots (See Supplementary Information 1 available at doi:10.1155/2011/901854) and loadings S-plot (supplementary information 2) were further investigated in order to extract the most significant spectral variables/bins. These bins were further analyzed by the help of HMDB and two-dimensional NMR spectral profiles to generate the significant metabolites that contribute towards the pattern seen in the OPLS-DA models. Table 1 shows a list of the metabolites perturbed in the liver as the disease progresses in both the sexes. However, as the early infection stage of the males showed insignificant variation (Q^2(cum)=−0.882, Figure 1(a)) from the control animals, hence, metabolites corresponding to that stage are not investigated. Further, the specific peaks from the one-dimensional NMR profiles were integrated with respect to the total intensity of the internal standard (DSS). Many of the metabolites identified from the OPLS-DA model showed statistically significant difference as the disease progresses (Figure 2). Moreover, analysis was done to compare some of the metabolites in the two post-infection stages which were different in the uninfected males and females to check whether they deviate significantly from the same sex control. These are shown in the Figure 3. OPLS-DA analysis of liver spectra showed some of the metabolites (Table 1) which were not observed in other tissues or biofluids [17]. However, although the OPLS-DA analysis showed a large difference in the early infection stage of females and uninfected controls (Table 1), some of the metabolites that were expected to be increased in the infected animals showed a large deviation in the VIP (supplementary information 1). Therefore, OPLS-DA was unable to predict the dramatic change in the early infected females with certainty; hence, the early-stage changes in the female liver remain to be characterized.

In the late stage, the liver extracts showed an increase in 2-hydroxy-2-methylbutyrate and beta-alanine (Figures 2(a) and 2(e)) and a decrease in an unidentified compound at 3.82 ppm (Figure 2(f)) in both males and females. Along with these, females showed increase in kynurenic acid in the late stage (Figure 2(h)). Increase in two unidentified peaks at 2.34 and 3.86 ppm was also shown by the females (Figures 2(b) and 2(g)).

Table 1: Metabolites perturbed in the mouse liver during infection with *P. berghei* ANKA. Italicized metabolites could not be confirmed. Cystine, serine and phosphoserine could not be confirmed because their crosspeaks occur in very crowded regions of the spectrum. Presence of fatty acids cannot be confirmed due to low concentrations.

Increased compound	Chemical shift	VIP value	Loading	Decreased compound	Chemical shift	VIP value	Loading
Females early stage							
N-methyl-a-aminobutyric acid, Ribonolactone	3.82	3.01	0.242	Beta-alanine	3.18	5.66	−0.455
Dimethylglycine	3.7	2.85	0.229	O-phosphoethanolamine	4.02	2.5	−0.201
Phosphocreatine, Creatine, Betaine	3.9	2.72	0.219	Unidentified	3.66	2.12	−0.171
Unidentified	3.46	2.58	0.207	Sarcosine	3.62	2.84	−0.228
Alanine, methylacetoacetic acid, acetylcholine	3.74	1.91		Choline, Creatinine	4.06	2.37	−0.191
Choline,	3.5	1.5	0.153				
3-hydroxyisovaleric acid, 4-pyridoxic acid	2.34	1.62	0.130				
Female late stage							
2-hydroxy-2-methylbutyric acid	0.94	3.01	0.242	Dimethylglycine	3.7	3.34	−0.268
2-ethylacrylic acid	0.98	2.41	0.194	N-methyl-a-aminobutyric acid, ribonolactone	3.82	3.34	−0.268
Diethyl-thiophosphate, O-phosphoethanolamine, Phosphoserine	3.98	2.38	0.191	Unconfirmed	3.46	3.28	−0.263
Beta-alanine	3.18	2.21	0.178	N-methyl-a-aminobutyric acid,3-mercaptopyruvic acid	3.86	3.05	−0.245
Dimethyl-sulphide, Acetyl-phosphate, Amino-acetone, cis-2-methylaconitate	2.10	2.15	0.173	Alanine, methylacetoacetic acid, acetylcholine	3.74	2.73	−0.219
Putrescine, L-leucine, 2-hydroxy-2-methylbutyric acid	1.7	2.03	0.163	Lactate,long chain fatty acids	1.3	2.18	−0.175
3-hydroxyisovaleric acid, 4-pyridoxic acid	2.34	1.87	0.150	Glycine	3.54	2.13	−0.171
2-ethylacrylic acid, stearic acid	1.02	1.82	0.146	lactic acid, N-acetyl-L-alanine	4.1	1.74	−0.140
Males late stage							
Beta-alanine, Cystine	3.18	3.26	0.262	N-methyl-a-aminobutyric acid,Ribonolactone	3.82	3.52	−.282
2-hydroxy-2-methyl-butyric acid	0.94	3.00	0.241	N-methyl-a-aminobutyric acid,3-mercaptopyruvic acid	3.86	3.38	−.271
2-ethylacrylic acid	0.98	2.44	0.196	Serine, Dimethylglycine	3.7	3.13	−.251
Dimethyl-sulphide, Acetyl-phosphate, Amino-acetone, cis-2-methylaconitate	2.1	1.98	0.159	Alanine, methylacetoacetic acid, acetylcholine	3.74	2.99	−.20
Putrescine, 2-hydroxy-2-methylbutyric acid, L-leucine	1.7	1.95	0.156	Phosphocreatine, Creatine, Betaine	3.9	2.44	−.196
2-ethylacrylic acid, stearic acid	1.02	1.77	0.142	cis-aconitic acid	3.46	2.33	−.187
Long-chain fatty acids	0.9	1.73	0.139	lactic acid, N-acetyl-L-alanine	4.1	1.98	−.160
O-phosphoethanolamine, phosphoserine	3.98	1.73	0.139	Alanine, Acetylcholine, guanidinoacetic acid	3.78	1.71	−.136

In spite of the poor difference shown in the OPLS-DA between the male control and early-stage infected animals (Figure 1), some of the metabolites showed significant changes. These include asparagine, DMG, and creatine (Figures 2(c) and 2(d)), all of which are significantly decreased from the control animals in the early stage.

Comparisons of the individual metabolite level of the animals were also made with that of the controls (Figure 3), and several interesting results were obtained. This was done for the fact that several metabolites in the liver showed significant difference in their levels between male and female control animals (data not shown). Therefore, we investigated whether the post-infection alterations are different between males and females. Several of the changes observed in such analysis were often only in the early-stage. Females showed a slight increase from the corresponding controls, while males showed a decrease in the carnitine level at this stage (Figure 3(b)). Kynurenic acid levels in females did

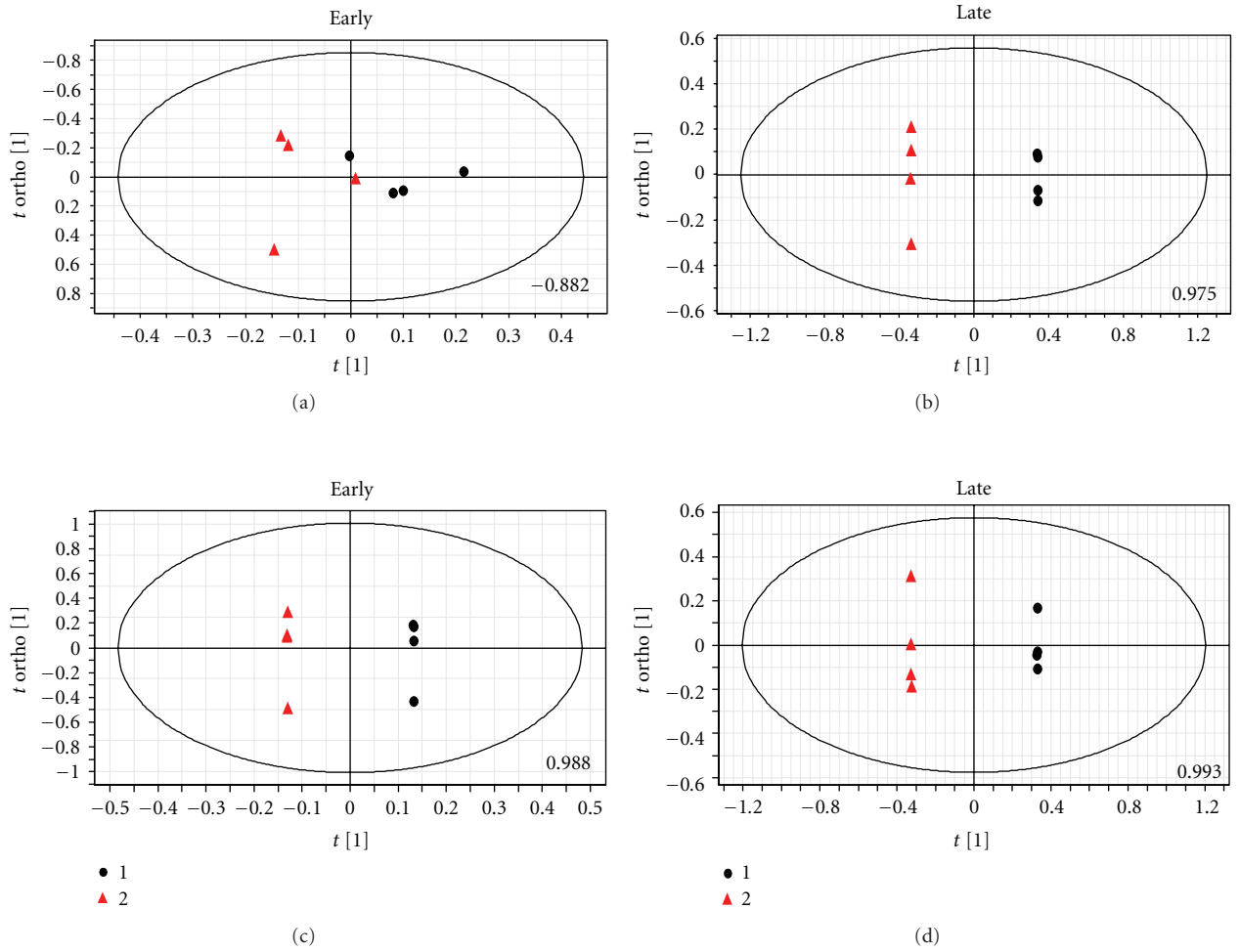


Figure 1: OPLS-DA scores plot based on the ^{1}H NMR metabolic profile of the liver. The experiments consisted of 12 male and 12 female mice. Eight male and 8 female mice were injected with *P. berghei* ANKA. On day 5, 4 infected males, 4 infected females, 2 control males, and 2 control females were sacrificed, and the liver is extracted with perchloric acid (early-stage of infection). Again on day 13 (late-stage infection). A/B, early- and late-stage infection males are compared with male controls; C/D, early- and late-stage infection females are compared with female controls. In each plot, the Q^2(cum) is shown at the bottom. The red triangle—profile of the infected animals and the black dots—the control animals.

not change much from their controls whereas the males showed a decrease (Figure 3(f)). Asparagine, DMG and O-phosphoethanolamine levels were seen to be reduced significantly more in the early stage infected male liver samples as compared to females (Figures 3(c) and 3(e)). Some distinct sexual dimorphism in the alteration of the metabolite levels was observed. For example, an unidentified peak at 2.34 ppm showed decrease in the males while slight increase in the females compared to the corresponding controls (Figure 3(a)). Similar trend was observed for carnitine (Figure 3(b)), serine and DMG (Figure 3(d)).

Interesting here to note that is although the global metabolic profile of the male controls does not differ much from the male early infection animals, some of the individual metabolites show a significant alteration (Figures 2(c), 2(d), and 3). However, these alterations are possibly not enough to have an impact on the differential global metabolic profile (Figures 1(a) and 1(b)).

3.2. Intercompartmental Correlation Analysis by Multiway PCA (MPCA). MPCA has been established as useful tool for correlation analysis between tissues and circulating biofluids [18]. In this paper, we have used the technique to delineate the effect of disease progression on the correlation of metabolites across tissues/biofluids. We used earlier data of serum and brain [17] and the liver data described here to investigate the correlations of metabolites in brain and liver to that of serum with the disease progression in both the sexes. Earlier studies made by our group [17] showed that the brain metabolic profile is significantly altered in both the sexes. Although this model of malaria is not a cerebral variety, it is important to understand how the brain metabolic profile varies with the circulating biofluid. Initially, MCR-ALS algorithm (data not shown) was employed that showed appreciable contribution of one compartment on the spectrotype of the other. Earlier studies also found appreciable contribution of the blood serum

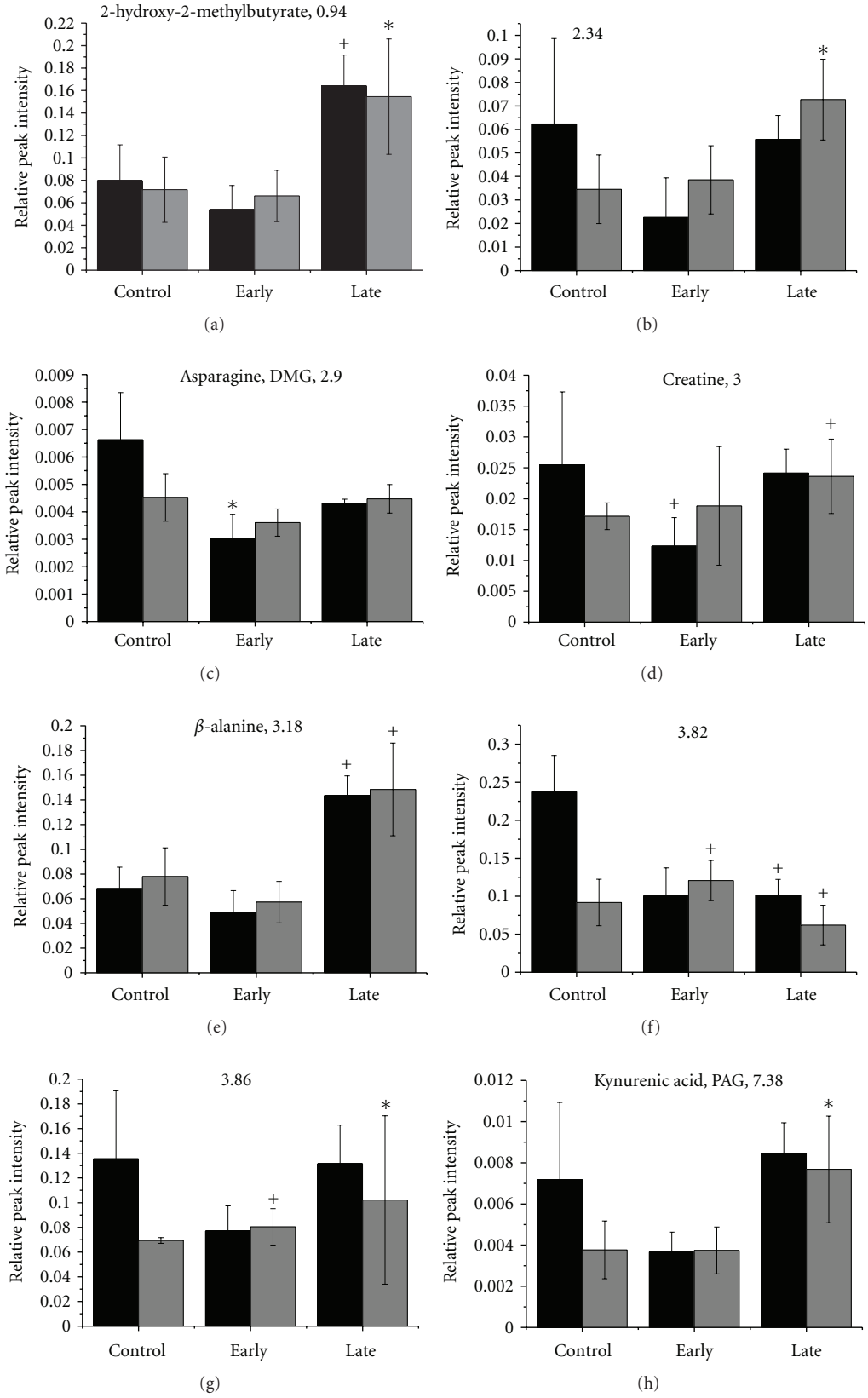


Figure 2: Perturbed metabolite levels in mouse liver at early- and late-stage malarial infection. All peak intensities have been calculated relative to the peak height of 0.132 mg/ml DSS present in every sample. Panels showing two metabolites represent overlapping ^{1}H NMR resonances at the chemical shift mentioned. Where only chemical shifts are mentioned, identity of metabolites could not be confirmed. Black = males and grey = females. * indicates $P < .05$, $^+$ indicates $P < .08$ in comparison to the uninfected controls of the same sex.

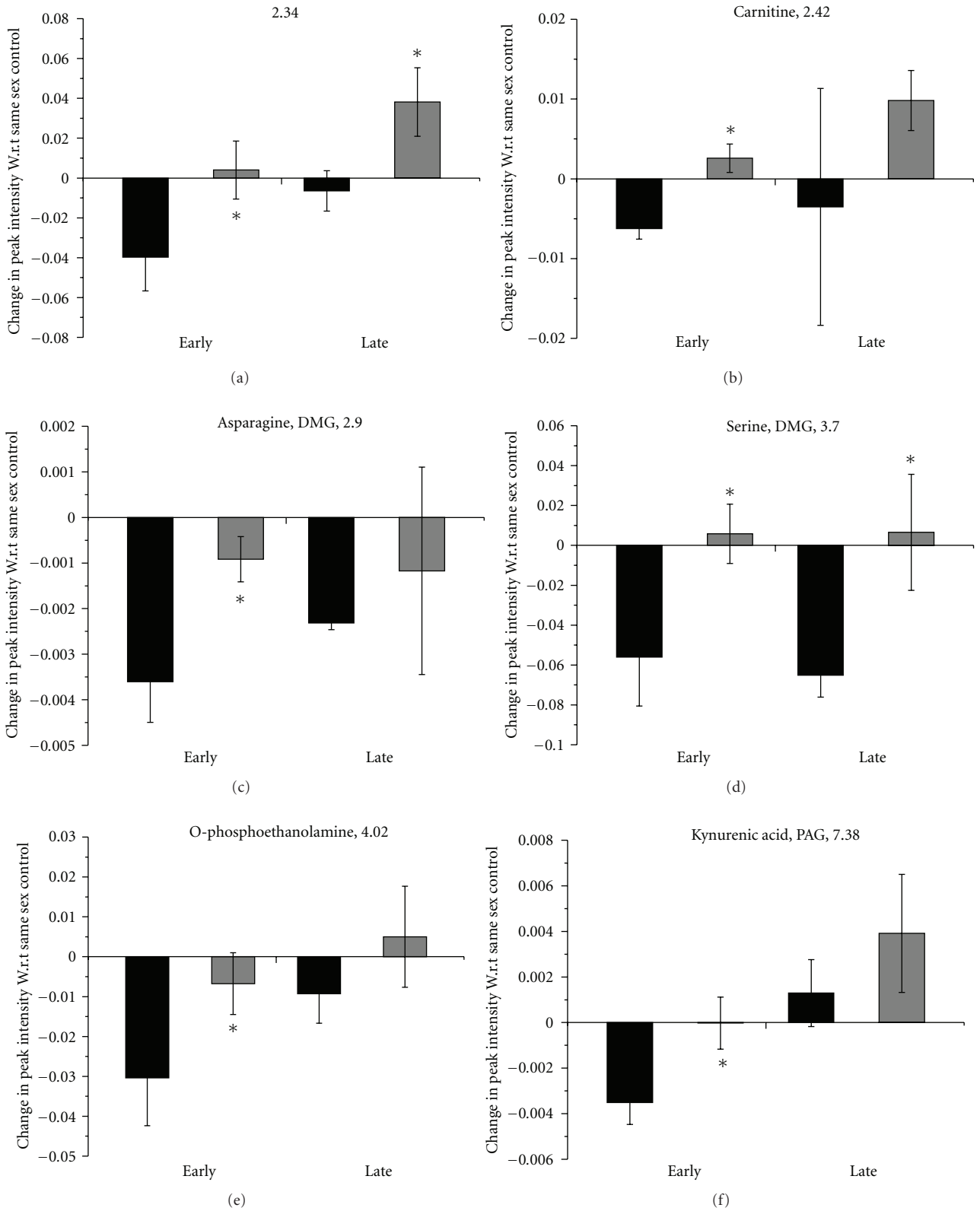


Figure 3: Perturbed metabolite levels in mouse liver at early- and late-stage malarial infection: comparison of effects in male and female animals. The average peak intensity for the metabolite in uninfected samples of the same sex was subtracted from individual peak intensities of infected animals at each stage of infection. The average change in intensity with respect to the same sex control is plotted here. * indicates $P < .05$, $^{+}$ indicates $P < .08$ in comparison to the males at each stage. Black = male, and grey = female. Panels showing two metabolites represent overlapping ^{1}H NMR resonances at the chemical shift mentioned. Where only chemical shifts are mentioned, identity of metabolites could not be confirmed.

Table 2: MPCA model statistics in terms of total explained variances by two PCs calculated in percentage.

Model	Total variation explained (%)
Liver-serum correlation, female	
Control	93.17
Early infection	98.15
Late infection	99.25
Liver- serum correlation, male	
Control	96.40
Early infection	99.02
Late infection	98.08
Brain- serum correlation, female	
Control	94.55
Early infection	98.81
Late infection	99.25
Brain- serum correlation, male	
Control	96.72
Early infection	98.02
Late infection	98.58

spectrotype to the liver [18]. Further, MPCA models were built using the NMR metabolite profiles of the sera and the relevant tissue from a set of animals (e.g., 4 female controls, sera, and brain NMR metabolite profile). 12 such models were generated (control, early- and late-stage infection for males and females) for both types of correlations (brain-sera and liver-sera) to be addressed. The first two principal components (PCs) were calculated for each of them. Table 2 shows the amount of variation explained by the models. All of them explained >90% of cumulative variance. The loadings in PC2 described the correlation of the NMR spectral peaks across the relevant tissue (either liver or brain) and serum.

In female control animals, high concentration of branched chain amino acids (BCAA) (1) leucine, isoleucine, valine, choline (2) and a low concentration of glucose (3) in the liver were associated with low lactate (4), glucose (3) and high concentration of lysine (5) in serum (Figure 4(a)). There was a peak at 3.20 ppm (close to choline peak) which could not be identified. The females at early-stage infection showed essentially a similar serum profile to that of control animals, with an exception of a high dimethylamine (DMA) (9) in the serum (Figure 4(b)). However, the liver profile of these animals showed high branched chain amino acids (1), alanine (6), trimethylamine-N-oxide (TMAO) (7), glycine (8) and lactate (4) (Figure 4(b)). In the late-stage infection of the females, the liver showed a high choline (2) and glucose (3) which were associated with low lactate (4) and high glucose (3) level in the serum (Figure 4(c)).

Male control animals showed a different profile than that of the females. In the liver of the male control animals isoleucine (1 as BCAA), leucine (1 as BCAA), lactate (4), choline (2) and, TMAO (7) were low and glucose (3) was high. These were correlated to a low lactate (4) and high DMA (9) in the serum (Figure 4(d)). When the early-stage infection for the male animals was considered, the liver profile showed increase in BCAA (1), alanine (6), choline (2), and lactate (4) compared to the control animals, with the contribution of TMAO (7) becoming insignificant. The serum profile for this group of animals showed a high DMA (9) and low choline (2) level with respect to the liver profile (Figure 4(e)). The late-stage infection for the males was also relatively simple with low glucose (3) in liver being associated to high glucose (3), lactate (4) and low branched chain amino acids (1) in the serum (Figure 4(f)).

Our earlier studies (15) showed a significant deviation of the brain metabolic profile in the early-stage infection as well as the late-stage infection from the control animals. Thus, it would be interesting to see whether the serum-brain intercompartmental correlation is also affected with the disease progression. Therefore, the correlation of the brain metabolic profile with that of serum was assessed in order to delineate the crosstalk between these two biological compartments. The female control animals showed a high lactate (4), N-acetyl-aspartate (NAA, 10), sarcosine (11), and low choline (2) in the brain that correlated with high lysine (5) and low lactate (4) and glucose (3) in the serum (Figure 5(a)). In the early infection, the brain profile showed high lactate (4), DMA (9), creatine (12), and betaine (13). However, the serum profile remained similar to that of controls, although it showed a high DMA (9) (Figure 5(b)). In the late-stage, the brain metabolic profile showed high values of lactate (4), NAA (10), DMA (9), creatine (12), and choline (2). These were found to be significantly correlated with the high glucose (3) and low lactate (4) in the serum (Figure 5(c)).

The male metabolic intercompartmental analysis was also performed. The male controls, as expected, were different from that of females. They showed a low lactate (4), NAA (10), DMA (9), choline (2), and creatine (12) in the brain profile associated to a low lactate (4) and high DMA (9) in the serum (Figure 5(d)). The males showed change in the serum profile only in terms of a high lactate and low choline level, and the rest of the profile remained quite similar at the early-stage infection. At this stage, they showed a low creatine (12) and high DMA (9) in the brain (Figure 5(e)). The late infection stage showed a low lactate (4), high creatine (12), and DMA (9) in the brain and high lactate (4), glucose (3) and low BCAA in the serum (Figure 5(f)).

Figure 4 (liver-serum correlation) and Figure 5 (brain-serum correlation) show a comprehensive visualization of all the increased and decreased metabolites in different compartments during the various stages of the disease. Important to note here is that the set of metabolites that were found to be significant from the OPLS-DA (Table 1, Figures 2 and 3 for liver metabolites and [17] for brain metabolites) study are different from those found from MPCA (Figures 4 and 5). It is important to realize at this point that OPLS-DA showed a set of metabolites that were significantly altered among the infected and control sets in a single tissue or biofluid. However, MPCA resulted in a set of metabolites which are significant in terms of the intercompartmental correlation.

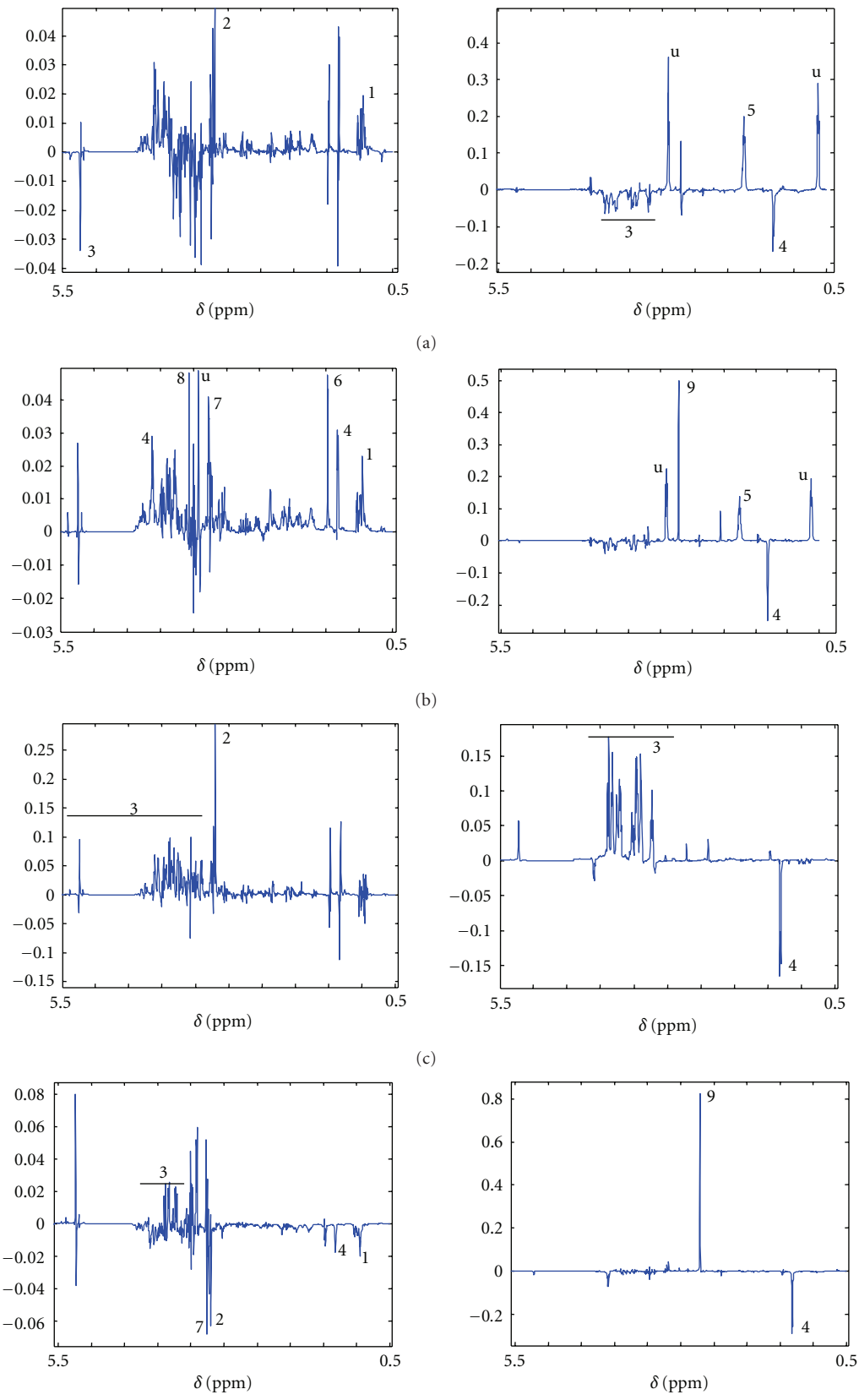


Figure 4: Continued.

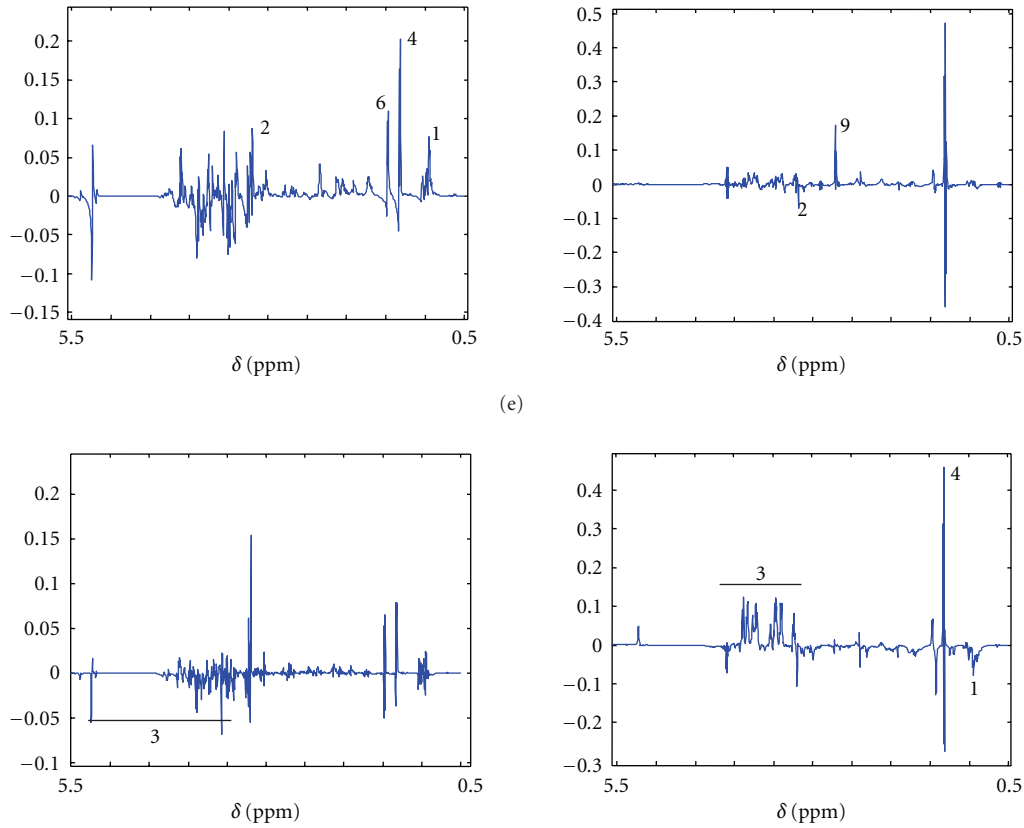


Figure 4: The liver and serum correlation profile depicted by MPCA PC2 loadings plot. The aliphatic region of the spectra is shown. (a–c) are females and (d–f) are the males. In each set, the left figure is the liver profile and the right one is the serum profile. The y-axis in each panel represents the loading axis. The compartmental loadings are obtained by cropping the model loading. (a/d): control, (b/e): early infection, and (c/f): late infection. Symbols: 1: branched chain amino acids, 2: choline, 3: glucose, 4: lactate, 5: lysine, 6: alanine, 7: trimethylamine oxide (TMAO), 8: glycine, 9: dimethylamine, and u: unidentified peak.

4. Discussion

In this paper, we report our results on the alterations of the liver metabolism during the progress of malaria as well as the change in the correlation of the metabolic profile of the liver and brain with that of blood serum as the disease progresses. Murine model (BALB/c mice infected with *P. berghei* ANKA) using animals from both the sexes used for this purpose. Earlier results from our group [17] suggested that the host response towards the infection exhibits sexual dimorphism in the urine and serum metabolic profile. The alteration of the liver metabolism during the disease progression supports this fact. The liver profile in the female mice was altered in the early stage of the disease as compared to the males (Figure 1). Liver being the regulatory organ for metabolic activities, a drastic change in its metabolic profile in the early stage of the disease raises the possibility that the female hosts are able to respond to the infection by maintaining the homeostasis within them. This is supported by our earlier result that the female hosts show no early changes of the metabolic profile of the serum [17], while the males do. At the late stage, drastic changes in the metabolic alteration are observed irrespective of the sex of the host (Figure 1), which is anticipated because of the fact that the animals are very ill at this time point.

Our earlier findings [17] indicated several alterations in the metabolic pathways during the progression of the disease. Among them, glucose metabolism, amino acid metabolism, kynurenine pathway, uracil degradation, and fatty acid metabolism were found to be significant. These observations were based on the alteration on the urine, sera, and brain metabolic profile. Our data on the liver metabolism reported here is in good agreement with that.

Glucose metabolism is known to be perturbed during malaria parasite infection largely due to the enhanced rate of glycolysis [19–21]. Lactic acidosis is an important pathophysiological feature of malaria. Our earlier results in the serum and brain metabolic profile showed a sexual dimorphism in lactate levels of those tissues. However, the comparison made with the liver metabolic profile does not show a pronounced increase in the lactate level. Instead, in the late-stage the lactate in the liver is shown to be decreased in males as well as females (Table 1). Enhancement of rate of glycolysis leads to an increased formation of pyruvate which may be transaminated to alanine. Thus, the change

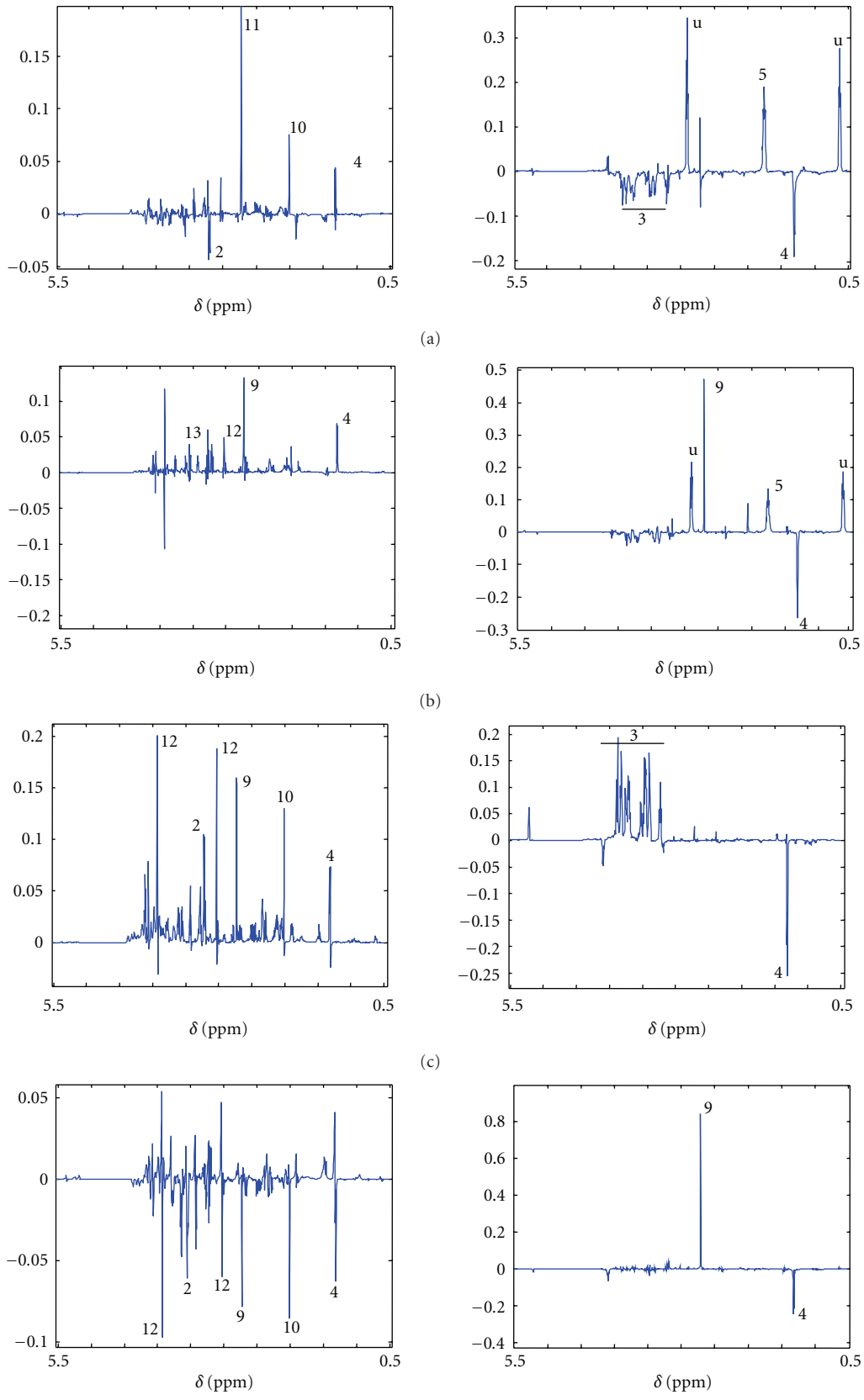


Figure 5: Continued.

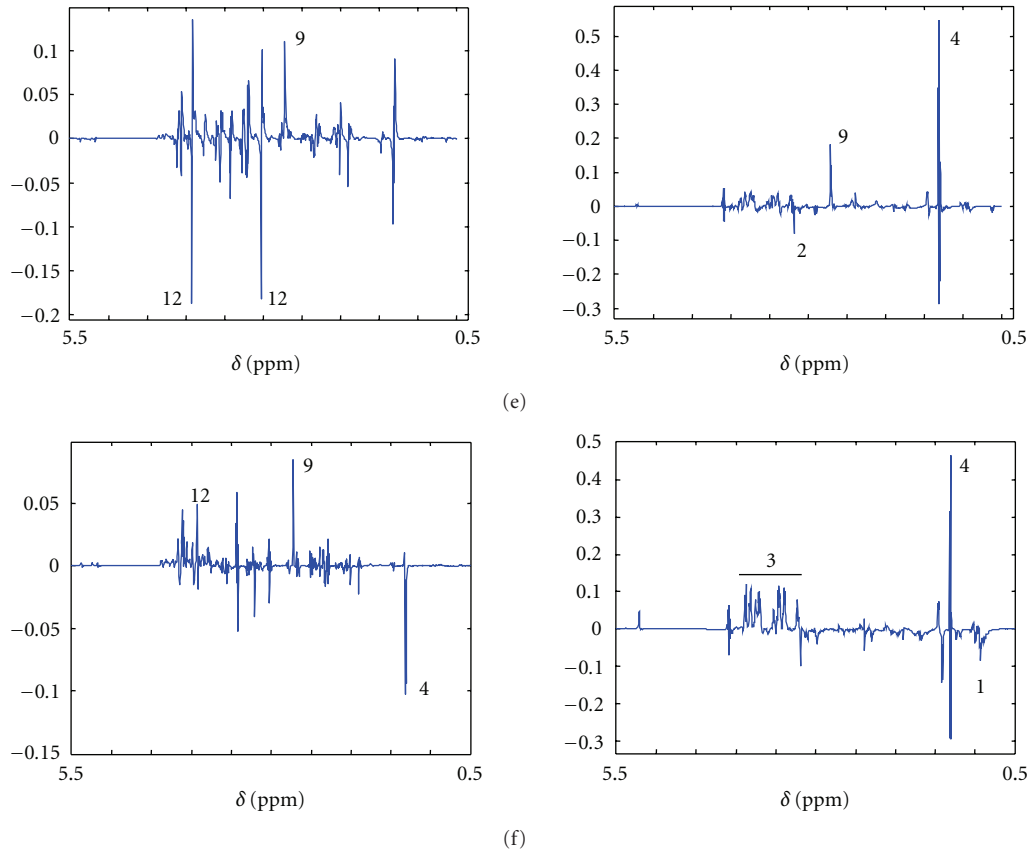


Figure 5: The brain and serum correlation profile depicted by MPCA PC2 loadings plot. The aliphatic region of the spectra is shown. The panels (a–c) are the females and (d–f) are the males. In each set, the left figure is the brain profile and the right is the serum profile. The y-axis in each panel represents the loading axis. The compartmental loadings are obtained by cropping the model loading. (a/d): control, (b/e): early infection, and (c/f): late-infection. Symbols: 10: N-acetylaspartate, 11: sarcosine, 12: creatine, 13: betaine, u: unidentified peak.

in the level of alanine is important in this regard. Alanine did not show the trend like lactate (Table 1). In the early-stage female animals, alanine is increased compared to the controls. The change in the alanine level is an indicator of the alteration of circulating amino acid levels. [17]. Liver being the metabolic regulatory organ, alteration in the level of the amino acids in the liver is expected. Along with alanine, the level of serine is also seen to be altered in the liver extracts. In the males, it is decreased, and in the females, the level is increased marginally when compared to the corresponding control thereby showing a sexual dimorphism (Figure 3(d)). Asparagine level also showed a significantly greater decrease in the males compared to the females with respect to the corresponding controls (Figure 3(c)). *Plasmodium sp.* are known to incorporate disproportionately a large amount of asparagine in their proteome in the ubiquitous low-complexity region [22]. Along with the amino acid mentioned, leucine levels was also increased in both males and females in the late stage (Table 1). However, alteration in the branched chain amino acids and aromatic amino acids were reported in context of *Trypanosome* infection also [23, 24].

Our earlier findings [17] showed a perturbation in the kynurenine pathway. We were the first to observe the increased excretion of kynurenic acid (KA) and quinolinic acid in the male infected mice. In the sera and brain, they showed a pronounced sexual dimorphism. When liver extracts are considered, KA exhibited a sexual dimorphism in the early-stage liver that its level in the infected male liver extracts decreased, while those in female did not (Figure 2(h)). The kynurenine pathway is activated by the immunological factor IFN-γ; therefore; it is systematically upregulated during the activation of the immune response under multiple disease condition [25]. Therefore, an interesting question that remains to be asked is whether the sexual dimorphism that we see in the pathway is a cause for the differential immunological activation between males and females.

Several components of fatty acid metabolic pathway are indicated to be perturbed in the liver extracts. A rise in fatty acid concentration in the male and female liver is indicated by the 0.9 ppm peak (Table 1). Oxidation of these fatty acids would entail generation of carnitine which we reported to be decreased in the urine of female mice [17]. This suggests a selectively higher retention of this metabolite for the cellular β-oxidation. The liver levels of carnitine also showed a sexual dimorphism in the early stage (Figure 3(b)). Whereas the males showed a slight decrease, the females demonstrated

a slight increase from the corresponding controls. Along with this, choline was indicated to be increased in the early-stage infected females (Table 1). O-phosphoethanolamine was detected in the liver sample to show a sexual dimorphism in the late stage (Figure 3(e)) along with 2-hydroxy-2-methylbutyrate which was increased significantly in both the sexes in the late stage (Figure 2(a)).

The crosstalk (correlation) of metabolites across organs and biofluids is an important parameter in their changing dynamical interaction during disease progression. For this purpose, we analyzed the correlation of the liver and brain metabolic profile with that of blood serum. Previously, we delineated the effect of disease progression on the brain metabolic profile individually [17]. Important to note is that at the late stage of infection, the correlation profiles look drastically different than that of the controls (Figures 4(c), 4(f), 5(c), and 5(f)). This is expected because of the enhanced severity of the disease. Therefore, we would concentrate more on the early-stage correlation profile. As noted earlier the liver serum correlation of the female animal is distinct from that of the males in the control animals (Figures 4(a) and 4(d)). Initial study on the liver profile of the control animals showed no distinction between the two sexes (data not shown), and similar results were observed for the serum profile [17]. Thus, it is evident that the dynamic intercompartmental correlation is maintained differently in the two sexes which result in similar global metabolic profile of the individual compartment. For example, in both of them, the amino acids (1) in the liver are anticorrelated to the glucose (3) in the liver. This possibly refers to the gluconeogenetic pathway. However, the females and males show different modes of correlation. Males showed a low amino acid concentration to be correlated to a high glucose concentration (Figure 4(d)) whereas females showed the opposite (Figure 4(a)). In addition, males showed a low serum lactate (4) to be correlated with high liver glucose (3) (Figure 4(d)), and females showed a low serum lactate (4) to be correlated with low liver glucose (3). This suggests a complex glycolytic-gluconeogenetic relationship which keeps the global metabolic profile of the individual tissue and/or biofluid more or less similar between the two sexes.

The data presented here also suggests that this correlation is compromised during the course of the disease in some way or another. For the females in the early stage, the serum compartment of the correlation profile looks more or less the same except for an increased contribution from the DMA (9), whereas there are notable changes in the liver segments of the profile (Figures 4(a) and 4(b)). However, in the males, both serum and liver segment showed notable changes (Figures 4(d) and 4(e)). Therefore, we observe a sexual dimorphism even in the intercompartmental correlation status when the liver and serum are considered. This may implicate that the amount of circulating metabolites in the females remain under homeostasis, while their metabolism in the liver is altered drastically. This supports the hypothesis that there is significant amount of change in the early-stage female liver in order to maintain the homeostasis in the circulating fluid in response to the pathogen. In the female early-stage infection an increased alanine (6), TMAO (7), glycine (8), and lactate (4) in the liver were correlated with an increased DMA (9) in the serum. DMA is a metabolic product of TMAO, so an increased DMA in the serum can be correlated with an increased TMAO in the liver. TMAO has been reported to be associated with kidney disease [26] and is used by the body as an osmolyte to counter-act the accumulation of urea due to renal failure. This refers to the fact that the females can or at least try to maintain other organs under functional state at the early stage of the disease. As noted earlier, lactate did not show a significant contribution towards the differentiation of infected and control animals; however, in the correlation analysis, we observe an inverse relation of the liver and serum lactate for the early-stage females (Figure 4(b)). Here, a relative buildup in the liver lactate is associated with the depletion of the serum lactate. Lactate is a gluconeogenic precursor in the liver. It is possible that the heavy utilization of glucose in the blood is counteracted by the host by enhancing the rate of gluconeogenesis in the liver. However, if that is the case, then one would expect an opposite relation between the liver and serum lactate to that we see here. Gluconeogenesis is enhanced in the severe malaria patients [27]. This study also reported an enhancement of serum lactate. Our result, therefore, suggests a more intricate functional and spatiotemporal relationship between the liver and serum lactate. For example, it might be possible that an alteration in the Cori cycle pathway changes the uptake of lactate from serum into liver. However, the details of this process remain to be investigated. In males, however, both the liver and serum profile changes in the early stage of infection (Figure 4(e)), referring to the fact that the males are unable to maintain the blood homeostasis. Moreover, the liver metabolism of these animals is altered drastically. Here, the choline of liver is found to be anticorrelated to the choline of serum. Precisely, an increased choline in the liver is associated with a decreased choline in the serum. This may be due to the fact that the intraerythrocytic parasite causes a 5-fold increase in the phospholipid content of the parasitized cell [28], which may eventually lead to a phospholipid degradation in the liver of the host, thereby generating more of the phosphocholine components.

As far as the brain to serum correlation analysis is concerned, for the females, the contribution from the serum towards the brain-serum crosstalk does not alter much; however, the brain profile did change notably (Figures 5(a) and 5(b)). For the males, both the brain and serum profile was altered (Figures 5(d) and 5(e)), although the early-stage brain profile looks similar to that of control animals. Earlier report showed that the brain profile in the early-stage males and females did not change significantly [17]. However, this data suggests that the brain profile is maintained by the aid of a complex relationship with the serum. In the females, as noted earlier, the homeostasis of the serum is maintained, while for the males, the brain profile is maintained at the cost of serum homeostasis. In females, the brain showed a relative increase in the lactate and creatine, whereas the males showed a relative decrease of creatine. This indicates that the energy metabolism of the brain is altered in the early stage of the infection in both sexes. However, the alteration in the

energy metabolism probably bears different meanings in the two sexes. In the males, this is an effect of the maintenance of brain homeostasis at the cost of serum homeostasis, whereas, for the females it is the other way around.

This paper presents the initial understanding of the complex interplay of the various biological compartments during the progression of malaria in mice. This, therefore, opens up the possibility of a greater insight into the changes in the metabolic pathways as a response to malarial parasite infection.

Acknowledgments

The authors thank the National Facility for High Field NMR and the Animal House Facility, Tata Institute of Fundamental Research. A. Sengupta thanks the Council of Scientific and Industrial Research, Government of India for providing SPM fellowship. A. Sengupta and Angika Basant contributed equally to the paper.

References

[1] T. E. Wim Van Lerberghe, R. Kumanan, and M. Abdelhay, *World Malaria Report*, World Health Organization, 2008.

[2] L. H. Miller, D. I. Baruch, K. Marsh, and O. K. Doumbo, "The pathogenic basis of malaria," *Nature*, vol. 415, no. 6872, pp. 673–679, 2002.

[3] M. F. Penet, F. Kober, S. Confort-Gouny et al., "Magnetic resonance spectroscopy reveals an impaired brain metabolic profile in mice resistant to cerebral malaria infected with Plasmodium berghei ANKA," *Journal of Biological Chemistry*, vol. 282, no. 19, pp. 14505–14514, 2007.

[4] A. M. Dondorp, P. A. Kager, J. Vreeken, and N. J. White, "Abnormal blood flow and red blood cell deformability in severe malaria," *Parasitology Today*, vol. 16, no. 6, pp. 228–232, 2000.

[5] G. G. MacPherson, M. J. Warrell, N. J. White, S. Looareesuwan, and D. A. Warrell, "Human cerebral malaria. A quantitative ultrastructural analysis of parasitized erythrocyte sequestration," *American Journal of Pathology*, vol. 119, no. 3, pp. 385–401, 1985.

[6] A. M. Dondorp, E. Pongponratn, and N. J. White, "Reduced microcirculatory flow in severe falciparum malaria: pathophysiology and electron-microscopic pathology," *Acta Tropica*, vol. 89, no. 3, pp. 309–317, 2004.

[7] K. Haldar, S. C. Murphy, D. A. Milner, and T. E. Taylor, "Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease," *Annual Review of Pathology*, vol. 2, pp. 217–249, 2007.

[8] T. Planche and S. Krishna, "Severe malaria: metabolic complications," *Current Molecular Medicine*, vol. 6, no. 2, pp. 141–153, 2006.

[9] A. Trampuz, M. Jereb, I. Muzlovic, and R. M. Prabhu, "Clinical review: severe malaria," *Critical Care*, vol. 7, no. 4, pp. 315–323, 2003.

[10] J. K. Nicholson, J. Connelly, J. C. Lindon, and E. Holmes, "Metabonomics: a platform for studying drug toxicity and gene function," *Nature Reviews Drug Discovery*, vol. 1, no. 2, pp. 153–161, 2002.

[11] I. Barba, G. de León, E. Martín et al., "Nuclear magnetic resonance-based metabolomics predicts exercise-induced ischemia in patients with suspected coronary artery disease," *Magnetic Resonance in Medicine*, vol. 60, no. 1, pp. 27–32, 2008.

[12] T. L. Whitehead and T. Kieber-Emmons, "Applying in vitro NMR spectroscopy and ^{1}H NMR metabonomics to breast cancer characterization and detection," *Progress in Nuclear Magnetic Resonance Spectroscopy*, vol. 47, no. 3-4, pp. 165–174, 2005.

[13] V. P. Mäkinen, P. Soininen, C. Forsblom et al., "^{1}H NMR metabonomics approach to the disease continuum of diabetic complications and premature death," *Molecular Systems Biology*, vol. 4, article 167, 2008.

[14] E. Holmes, R. L. Loo, J. Stamler et al., "Human metabolic phenotype diversity and its association with diet and blood pressure," *Nature*, vol. 453, no. 7193, pp. 396–400, 2008.

[15] Y. Wang, E. Holmes, J. K. Nicholson et al., "Metabonomic investigation in mice infected with *Schistosoma mansoni*: an approach for biomarker Identification," *Prot Nat Acad Sci USA*, vol. 101, no. 34, pp. 12676–12681, 2004.

[16] Y. Wang, J. Utzinger, J. Saric et al., "Global metabolic response of mice to trypanosoma brucei brucei infection," *Prot Nat Acad Sci USA*, vol. 105, no. 16, pp. 6127–6132, 2008.

[17] A. Basant, M. Rege, S. Sharma, and H. M. Sonawat, "Alterations in urine, serum and brain metabolomic profiles exhibit sexual dimorphism during malaria disease progression," *Malaria Journal*, p. 110, 2010.

[18] I. Montoliu, F. P. J. Martin, S. Collino, S. Rezzi, and S. Kochhar, "Multivariate modeling strategy for intercompartmental analysis of tissue and plasma ^{1}H NMR spectrotypes," *Journal of Proteome Research*, vol. 8, no. 5, pp. 2397–2406, 2009.

[19] E. F. Roth, M. C. Calvin, I. Max-Audit, J. Rosa, and R. Rosa, "The enzymes of the glycolytic pathway in erythrocytes infected with Plasmodium falciparum malaria parasites," *Blood*, vol. 72, no. 6, pp. 1922–1925, 1988.

[20] W. C. Kruckeberg, B. J. Sander, and D. C. Sullivan, "Plasmodium berghei: glycolytic enzymes of the infected mouse erythrocyte," *Experimental Parasitology*, vol. 51, no. 3, pp. 438–443, 1981.

[21] M. Mehta, H. M. Sonawat, and S. Sharma, "Malaria parasite-infected erythrocytes inhibit glucose utilization in uninfected red cells," *FEBS Letters*, vol. 579, no. 27, pp. 6151–6158, 2005.

[22] L. Aravind, L. M. Iyer, T. E. Wellems, and L. H. Miller, "Plasmodium biology: genomic gleanings," *Cell*, vol. 115, no. 7, pp. 771–785, 2003.

[23] Y. Wang, J. Utzinger, J. Saric et al., "Global metabolic responses of mice to Trypanosoma brucei brucei infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 16, pp. 6127–6132, 2008.

[24] A. El Sawalhy, J. R. Seed, J. E. Hall, and H. El Attar, "Increased excretion of aromatic amino acid catabolites in animals infected with Trypanosoma brucei evansi," *Journal of Parasitology*, vol. 84, no. 3, pp. 469–473, 1998.

[25] Y. Chen and G. J. Guillemin, "Kynurenine pathway metabolites in humans: disease and healthy states," *International Journal of Tryptophan Research*, vol. 2, pp. 1–19, 2009.

[26] M. A. Bain, R. Faull, R. W. Milne, and A. M. Evans, "Oral L-carnitine: metabolite formation and hemodialysis," *Current Drug Metabolism*, vol. 7, no. 7, pp. 811–816, 2006.

[27] H. Van Thien, M. T. Ackermans, E. Dekker et al., "Glucose production and gluconeogenesis in adults with cerebral malaria," *QJM*, vol. 94, no. 12, pp. 709–715, 2001.

[28] G. G. Holz, "Lipids and the malarial parasite," *Bulletin of the World Health Organization*, vol. 55, no. 2-3, pp. 237–248, 1977.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 178748, 4 pages
doi:10.1155/2011/178748

Research Article

Angiostrongylus vasorum: Experimental Infection and Larval Development in *Omalonyx matheroni*

L. R. Mozzer,[1] L. C. Montresor,[2] T. H. D. A. Vidigal,[2] and W. S. Lima[1]

[1] *Departamento de Parasitologia, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos 6627, Caixa Postal 486, 31270-901, Belo Horizonte, MG, Brazil*
[2] *Departamento de Zoologia, Universidade Federal de Minas Gerais, Avenida Presidente Antônio Carlos 6627, Caixa Postal 486, 31270-901, Belo Horizonte, MG, Brazil*

Correspondence should be addressed to W. S. Lima, wlima@icb.ufmg.br

Received 2 December 2010; Revised 12 March 2011; Accepted 13 April 2011

Academic Editor: Maria V. Johansen

The susceptibility and suitability of *Omalonyx matheroni* as an intermediate host of *Angiostrongylus vasorum* and the characteristics of larval recovery and development were investigated. Mollusks were infected, and from the 3rd to the 25th day after infection, larvae were recovered from groups of 50 individuals. The first observation of L2 was on the 5th day, and the first observation of L3 was on the 10th day. From the 22nd day on, all larvae were at the L3 stadium. Larval recovery varied from 78.2% to 95.2%. We found larval development to be faster in *O. matheroni* than in *Biomphalaria glabrata*. Our findings indicate that this mollusk is highly susceptible to *A. vasorum*. Infective L3 were orally inoculated into a dog, and the prepatent period was 39 days. This is the first study to focus on *O. matheroni* as an intermediate host of *A. vasorum*.

1. Introduction

The nematode *Angiostrongylus vasorum* is a parasite of wild and domestic canids. Adult worms are found in the right ventricle, pulmonary artery, and its branches, where sexual reproduction and oviposition take place. The first-stage larvae (L1) hatch in the alveoli, migrate up the bronchial tree, and are swallowed and then excreted into the environment along with the host feces. Infection frequently leads to pneumonia, loss of racing performance, coughing, and anemia [1]. Severely infected dogs may develop cardiac insufficiency and pulmonary fibrosis, followed by weight loss, hemorrhagic diatheses, and death [2, 3]. Several terrestrial and aquatic mollusks may act as intermediate hosts [4–7]. The genus *Omalonyx* (Pulmonata: Stylommatophora) belongs to the family Succineidae, which is composed of hermaphroditic terrestrial pulmonates that are morphologically diverse. *Omalonyx* sp. have a reduced flat shell and slug-like body, and they can be found in humid soil and in macrophytes [8–10]. They have a broad geographical distribution east of the Andes in South America and in the Lesser Antilles Islands [9], including localities where *A. vasorum* is known to occur [11, 12]. These mollusks are important intermediate hosts of the trematode *Leucochloridium* [13–15] and are able to support the life cycle of *Angiostrongylus costaricensis* in the laboratory [12]. There is no record of *Angiostrongylus* naturally infecting *Omalonyx*. This investigation aimed to evaluate the susceptibility and suitability of *Omalonyx matheroni* as an intermediate host of *A. vasorum* and to analyze the parasite's larval development from L1 to L3. Studies on the development of *A. vasorum* in different hosts contribute to the understanding of the parasite's biology and of the host-parasite relationship.

2. Methods

2.1. Mollusks. young individuals (from 25 to 30 days old) of *O. matheroni* ($n = 1150$) measuring from 9 to 14 mm in length, raised under laboratory conditions, and from parental specimens from Pampulha Lake in Belo Horizonte, Minas Gerais State, Brazil were employed in this trial.

Table 1: Larval recovery from groups of 50 *Omalonyx matheroni* experimentally infected with 12500 L1 of *Angiostrongylus vasorum* (250 L1 per mollusk).

DPI	Number of larvae that entered the hosts	Percentage of larvae that entered the hosts (%)	Number of larvae recovered	Percentage of larvae recovered (%)	Number of L1 recovered	Number of L2 recovered	Number of L3 recovered
3	11997	95.9	10009	83.4	10009	0	0
4	11900	95.2	9300	78.2	9300	0	0
5	12038	96.3	10113	84.0	9364	749	0
6	11937	95.5	9750	81.7	5675	4075	0
7	12149	97.2	10154	83.6	6417	3737	0
8	12225	97.8	11635	95.2	7446	4189	0
9	11987	95.9	9942	82.9	5349	4593	0
10	12187	97.5	10207	83.8	4001	5675	531
11	12006	96.1	10012	83.4	1782	7028	1202
12	12033	96.3	10024	83.3	1484	6435	2105
13	12076	96.6	10131	83.9	932	6768	2431
14	11993	95.9	9974	83.2	0	6413	3561
15	12207	97.7	11018	90.3	0	5246	5772
16	11972	95.8	9891	82.6	0	3858	6033
17	11984	95.9	9923	82.8	0	2322	7601
18	11905	95.2	9539	80.1	0	1784	7755
19	11954	95.6	9840	82.3	0	1081	8759
20	12075	96.6	10116	83.8	0	364	9752
21	11979	95.8	9902	82.7	0	246	9656
22	11910	95.3	9573	80.4	0	0	9573
23	11918	95.3	9727	81.6	0	0	9727
24	11995	95.9	10008	83.4	0	0	10008
25	11911	95.3	9620	80.8	0	0	9620

2.2. Parasites. *A. vasorum* L1 were obtained from the cycle maintained in the laboratory using successive passages in snails (*Biomphalaria glabrata*) and dogs (*Canis familiaris*). This strain was isolated from a dog in Caratinga, Minas Gerais State [11].

2.3. Mollusk Infection. The feces of infected dogs was collected, and L1 were recovered by the modified Baermann apparatus [16]. After 24 hours of fasting, mollusks were individually placed in polystyrene culture test plates with 24 wells of 15 mm diameter (TPP—Techno Plastic Products, Switzerland) and fed with 250 L1 on a fragment of lettuce (approximately 15 mm diameter). After 8 hours, they were transferred to a plastic container (20 × 12 cm) with 250 mL of dechlorinated tap water and wood pieces. Groups of 10 individuals were kept in these containers during this trial. They were maintained at room temperature (25 to 27°C) and were fed on lettuce. Larvae that stayed in each test plate were counted and subtracted from the total amount offered to each individual. It is assumed that this is the number of larvae that entered each individual and calculate, for groups of 50 individuals: the number of larvae that entered the hosts, the percentage of larvae that entered the hosts, and the percentage of larvae recovered (Table 1).

2.4. Larval Development. From the 3rd to the 25th day after infection, larvae (L1, L2 and L3) were recovered from groups of 50 mollusks in a Baermann apparatus and fixed in Railliet-Henry at 60°C for quantification and identification of the larval stage [7]. Larval stage was identified based on published descriptions [7, 11, 17].

2.5. Dog Infection. To verify whether L3 from *O. matheroni* (21 days after infection) were infective, 1000 larvae were orally inoculated into a male mongrel dog weighing 10 kg born in the breeding facilities of the Universidade Federal de Minas Gerais, under the management systems on animal well-being and according to the ethics committee of the university (CETEA/UFMG). After the 20th day of infection, feces was collected daily for parasitological investigation of the presence of larvae.

3. Results

3.1. Mollusk Infection. *O. matheroni* was susceptible to the infection. Larvae (L1, L2 and L3) were recovered from the 3rd to the 25th day after infection. After 8 hours of contact, 95.2 to 97.8% of the larvae had penetrated the mollusks (Table 1).

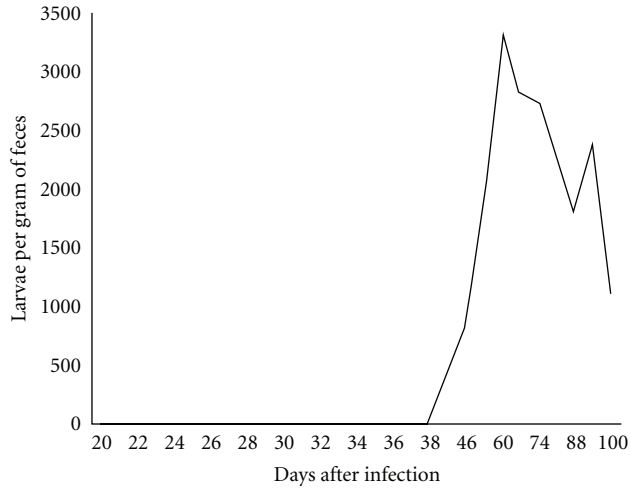


Figure 1: Number of larvae per gram of feces recovered from an experimentally infected (1000 L3) *Canis familiaris*.

3.2. Larval Development. The amount of larvae recovered each day is presented in Table 1. Mean L3 recovery is reported as the number of L3 recovered divided by the number of L1 that penetrated the host. These proportions were between 78.2% and 95.2%.

3.3. Dog Infection. Larvae were first detected in the feces in the 39th day (512 per g of feces) and increased until the 60th day (3320 per g of feces). This increase was followed by a gradual decrease that reached 1120 larvae per gram of feces on the 100th day (Figure 1). During this 100-day period, the amount of larval release varied, but larvae were never absent.

4. Discussion

Nematodes of the genus *Angiostrongylus*, including the species *A. vasorum,* can infect a wide spectrum of intermediate hosts of the class Gastropoda [18]. This system thus represents an interesting experimental model for the study of the host-parasite relationship. The susceptibility of a mollusk to a protostrongylid has been defined in terms of L1 penetration capability, the possibility of L3 development and time required to complete larval development [19, 20]. The present investigation demonstrates the susceptibility and suitability of *O. matheroni* as an intermediate host of *A. vasorum*. The percentage of L3 recovery in *O. matheroni* varied from 78.2% to 95.2%. The high percentage of larval recovery confirms our findings and indicates that this mollusk is highly susceptible to *A. vasorum*. Infective L3 recovered from these mollusks developed into fertile adults. L1 were observed in the feces of the infected dog.

Several factors influence the larval development of protostrongylids in the intermediate host such as environmental conditions (i.e., temperature) and biological conditions (i.e., hosts species and age) [21–24]. Geritcher [21] emphasized that among the environmental factors affecting the development of protostrongylid larvae in snails, the most important is temperature [21]. Low temperature (18 to 20°C) increases the time of development of the larvae, whereas high temperatures accelerate their development (25 to 28°C), as observed for the genus *Angiostrongylus* [17, 25, 26]. In this work, we observed that larval development of *A. vasorum* is faster in *O. matheroni* than in other known intermediate hosts [17, 27]. This conclusion is based on comparisons with data that is available in the literature. Experimental infection of several species of terrestrial mollusks (maintained at 18 to 23°C) allowed the first observations of L3 on the 16th and 17th day after infection [27]. Such low temperatures increase the time of larval development, and we are focusing our discussion on works that were performed at higher temperatures (25 to 28°C). In a trial where *B. glabrata* was maintained at 25 to 27°C, L2 were recovered between the 7th and 8th day after infection and L3 on the 14th and 15th [17]. Our results for *O. matheroni* demonstrated that L2 can be observed for the first time on the 5th day after infection and L3 can be observed for the first time on the 10th day. Furthermore, after 21 days, almost all larvae recovered were L3. The exploitation of hosts' immune response by the parasite was discussed by Damian [28], and the encapsulation of *A. costaricensis* in veronicellidae slugs has been considered an example of such a process [29].

Larvae were observed in the feces of the experimentally infected dog 39 days after infection. These results corroborate those of Bessa et al. [7], Oliveira-Júnior et al. [30], and Barçante et al. [16], who observed a prepatent period varying from 28 to 108 days afterinfection.

In view of the high reproductive rates of *O. matheroni* and the feasibility of laboratory rearing (accelerated larval development, efficient larval recovery, and larval viability), we consider such mollusks very useful for the maintenance of the *A. vasorum* cycle in the laboratory. Moreover, this mollusk is also an interesting experimental model for studies on the host-parasite relationship of *A. vasorum* and its intermediate hosts.

References

[1] G. W. Jones, C. Neal, and G. R. Turner, "*Angiostrongylus vasorum* infection in dogs in Cornwall," *Veterinary Record*, vol. 106, no. 4, p. 83, 1980.

[2] K. Dodd, "*Angiostrongylus vasorum* (Baillet, 1866) infestation in a greyhound kennels," *Veterinary Record*, vol. 92, no. 8, pp. 195–197, 1973.

[3] C. W. Lombard, "Normal values of the canine M-mode echocardiogram," *American Journal of Veterinary Research*, vol. 45, no. 10, pp. 2015–2018, 1984.

[4] L. Rosen, L. R. Ash, and G. D. Wallace, "Life history of the canine lungworm *Angiostrongylus vasorum* (Baillet)," *American Journal of Veterinary Research*, vol. 31, no. 1, pp. 131–143, 1970.

[5] J. Guilhon and B. Cens, "*Angiostrongylus vasorum* (Baillet, 1866) Etude biologique et morfologique," *Annales de Parasitologie Humaine et Comparee*, vol. 48, no. 4, pp. 567–596, 1973.

[6] R. Sauerländer and J. Eckert, "The african giant snail (*Achatina fulica*) as experimental intermediate host of *Angiostrongylus*

vasorum (Nematoda)," *Zeitschrift für Parasitenkunde*, vol. 44, no. 1, pp. 59–72, 1974.

[7] E. C. A. Bessa, W. S. Lima, E. Daemon, M. C. Cury, and J. L. B. Araújo, "Desenvolvimento biológico de *Angiostrongylus vasorum* (Baillet) Kamensky (Nematoda: Angiostrongylida) em *Subulina octona* Bruguere (Mollusca: Subulinidae) em condições de laboratório," *Revista Brasileira de Zoologia*, vol. 17, pp. 29–41, 2000.

[8] C. M. Patterson, "Taxonomic studies of the land snail family Succineidae," *Malacological Review*, vol. 4, pp. 131–202, 1971.

[9] S. Tillier, "South American and Juan Fernandez succineid slugs (Pulmonata)," *Journal of Molluscan Studies*, vol. 47, pp. 125–146, 1981.

[10] M. G. Barker, "Gastropods on land: phylogeny, diversity and adaptative morphology," in *The Biology of Terrestrial Mollusks*, M. G. Barker, Ed., pp. 1–146, CABI, New York, NY, USA, 2001.

[11] W. dos Santos Lima, H. M. de Araujo Costa, M. P. Guimarães, and A. C. Leite, "*Angiostrongylus vasorum* (Baillet, 1866) vematoda: protostrongylidae, in dogs of minas gerais, Brazil," *Memorias do Instituto Oswaldo Cruz*, vol. 80, no. 2, pp. 233–235, 1985.

[12] L. C. Montresor, T. H. D. A. Vidigal, C. L. G. F. Mendonça et al., "*Angiostrongylus costaricensis* (Nematoda: Protostrongylidae): migration route in experimental infection of *Omalonyx* sp. (Gastropoda: Succineidae)," *Parasitology Research*, vol. 103, no. 6, pp. 1339–1346, 2008.

[13] A. Lutz, "Observações sobre o gênero *Urogonimus* e uma nova forma de *Lecochloridium* em novo hospedador," *Memórias do Instituto Oswaldo Cruz*, vol. 13, pp. 136–140, 1921.

[14] L. Travassos, "Fauna helminthológica de Mato Grosso. Trematódeos—Parte I," *Memórias do Instituto Oswaldo Cruz*, vol. 21, pp. 309–741, 1928.

[15] S. P. Casey, T. A. Bakke, P. D. Harris, and J. Cable, "Use of ITS rDNA for discrimination of European green- and brown-banded sporocysts within the genus *Leucochloridium Carus*, 1835 (Digenea: Leucochloriidae)," *Systematic Parasitology*, vol. 56, no. 3, pp. 163–168, 2003.

[16] J. M. P. Barçante, T. A. Barçante, S. R. C. Dias, L. Q. Vieira, W. S. Lima, and D. Negrão-Corrêa, "A method to obtain axenic *Angiostrongylus vasorum* first-stage larvae from dog feces," *Parasitology Research*, vol. 89, no. 2, pp. 89–93, 2003.

[17] J. Guilhon and A. de Gaalon, "Evolution larvaire d'un nématode parasite de l'appareil circulatoire du chien dans l'organisme de mollusques dulçaquicoles," *Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences*, vol. 268, no. 3, pp. 612–615, 1969.

[18] P. S. Grewal, S. K. Grewal, L. Tan, and B. J. Adams, "Parasitism of molluscs by nematodes: types of associations and evolutionary trends," *Journal of Nematology*, vol. 35, no. 2, pp. 146–156, 2003.

[19] M. P. Morrondo and M. Y. Manga, "Experimental study on the susceptibility of five Helicidae species to larvae of Protostrongylinae," *Malacologia*, vol. 22, pp. 23–28, 1982.

[20] C. López, R. Panadero, A. Paz et al., "Larval development of Aelurostrongylus abstrusus (Nematoda, Angiostrongylidae) in experimentally infected Cernuella (Cernuella) virgata (Mollusca, Helicidae)," *Parasitology Research*, vol. 95, no. 1, pp. 13–16, 2005.

[21] C. B. Gerichter, "Observations on the life history of lung nematodes using snails as intermediate hosts," *American Journal of Veterinary Research*, vol. 9, pp. 109–112, 1948.

[22] J. Cabaret, "Age susceptibility of molluscan intermediate hosts to protostrongylid nematodes," *American Society of Parasitologists*, vol. 73, pp. 857–858, 1987.

[23] O. Halvorsen and A. Skorping, "The influence of temperature on growth and development of the nematode *Elaphostrongylus rangiferi* in the gastropods *Arianta arbustorum* and *Euconulus fulvus*," *Oikos*, vol. 38, no. 3, pp. 285–290, 1982.

[24] T. Ferdushy, C. M. O. Kapel, P. Webster, M. N. S. Al-Sabi, and J. R. Grønvold, "The effect of temperature and host age on the infectivity and development of *Angiostrongylus vasorum* in the slug *Arion lusitanicus*," *Parasitology Research*, vol. 107, no. 1, pp. 147–151, 2010.

[25] S. Lv, X. N. Zhou, Y. I. Zhang et al., "The effect of temperature on the development of *Angiostrongylus cantonensis* (Chen 1935) in *Pomacea canaliculata* (Lamarck 1822)," *Parasitology Research*, vol. 99, no. 5, pp. 583–587, 2006.

[26] S. J. Kutz, E. P. Hoberg, and L. Polley, "*Umingmakstrongylus pallikuukensis* (nematoda: Protostrongylidae) in gastropods: larval morphology, morphometrics, and development rates," *Journal of Parasitology*, vol. 87, no. 3, pp. 527–535, 2001.

[27] J. Guilhon and A. Afghahi, "Larval development of *Angiostrongylus vasorum* (Baillet, 1866) in the body of various species of terrestrial mollusks," *Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences*, vol. 268, no. 2, pp. 434–436, 1969.

[28] R. T. Damian, "Immunological aspects of host-schistosome relationships," *Memorias do Instituto Oswaldo Cruz*, vol. 82, pp. 13–16, 1987.

[29] H. L. Lenzi, R. G. Pacheco, M. Pelajo-Machado, M. S. Panasco, W. S. Romanha, and J. A. Lenzi, "Immunological System and *Schistosoma mansoni*: co-evolutionary Immunobiology. What is the eosinophil role in parasite-host relationship?" *Memorias do Instituto Oswaldo Cruz*, vol. 92, pp. 19–32, 1997.

[30] S. D. Oliveira-Júnior, J. M. P. Barçante, T. A. Barçante, S. R. C. Dias, and W. S. Lima, "Larval output of infected and re-infected dogs with *Angiostrongylus vasorum* (Baillet, 1866) Kamensky, 1905," *Veterinary Parasitology*, vol. 141, no. 1-2, pp. 101–106, 2006.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 682039, 3 pages
doi:10.1155/2011/682039

Case Report

A Dog with Pseudo-Addison Disease Associated with *Trichuris vulpis* Infection

Luigi Venco,[1] Valentina Valenti,[1] Marco Genchi,[2] and Giulio Grandi[3]

[1] *Veterinary Hospital "Città di Pavia", Viale Cremona 179, 27100 Pavia, Italy*
[2] *Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, University of Milan, Veterinary School, Via Celoria 10, 20133 Milano, Italy*
[3] *Dipartimento di Salute Animale, University of Parma, Veterinary School, Via del Taglio 10, 43100 Parma, Italy*

Correspondence should be addressed to Luigi Venco, luigivenco@libero.it

Received 26 November 2010; Accepted 19 January 2011

Academic Editor: M. J. Stear

A female Rottweiler dog was presented with a history of intermittent vomiting and diarrhoea, dysorexia, weakness, and weight loss. Haemocytometry and biochemistry values were within normal ranges except for electrolyte analyses, that demonstrated hyponatremia and hyperkalemia with a decreased sodium/potassium ratio. A diagnosis of hypoadrenocorticism was suspected. Basal and post-ACTH stimulation cortisolemia were within the normal values. Electrocardiography was normal, and thoracic radiography showed no significant modifications. On abdominal ultrasonography, adrenal glands appeared normal, while the bowel was distended, and several thin linear hyperechoic objects floating in the lumen were observed. Two adult female whipworms (*Trichuris vulpis*) were collected following bowel irrigation. Anthelmintic treatment against the parasite was curative.

1. Introduction

Trichuris vulpis, commonly known as whipworm, is characterized by a direct life cycle and by the extremely resistant lemon-shaped eggs that can remain infective in the environment for several years. Usually, *T. vulpis* infections are asymptomatic, but the presence of high worm burdens in the large intestine may cause the occurrence of haemorrhagic colitis due to the continuous stimulation and damage to the mucosa, where the head of the worm is embedded and moves in search of blood and fluid. Electrolyte imbalance can be associated with helminth infection; however, *T. vulpis* seems to be the only nematode associated with pseudo-Addison disease, as the present case seems to demonstrate.

2. Case History

An 8-year-old, spayed female Rottweiler mixed breed dog was presented to our hospital with several weeks history of intermittent vomiting and diarrhoea (watery faeces with mucus and increased stool frequency) together with dysorexia, weakness, and weight loss.

On physical examination, the patient was weak, but alert. Heart rate, respiratory rate, and temperature were within reference ranges. Mucous membranes appeared tacky and pale. The dog was slightly dehydrated, thin (body condition score: 2.5/5, 33 kg), had mild muscle wasting, and generally poor body condition. Haemocytometry and biochemistry showed normal values except for electrolyte analyses that demonstrated hyponatremia (sodium 132 mmol/L; reference interval: 140 to 155 mmol/L), hyperkalemia (potassium 5.7 mmol/L; reference interval: 3.8 to 5.2 mmol/L), with a decreased sodium/potassium (Na : K) ratio (23: reference interval 27 to 40), all suggestive of hypoadrenocorticism [1]. A direct fresh smear faecal examination yielded a negative result for intestinal nematodes. Electrolyte abnormalities were, therefore, attributed to a possible hypoadrenocorticism and evaluation of basal and post-ACTH stimulation cortisolemia was performed. Electrocardiography, carried out while awaiting ACTH stimulation results, did not show any abnormality. Thoracic radiograph showed a small cardiac silhouette with a vertebral heart scale system score of 8.2 (reference values 8.5–10.6) [2] and reduced pulmonary perfusion (Figure 1). Abdominal ultrasonography was also

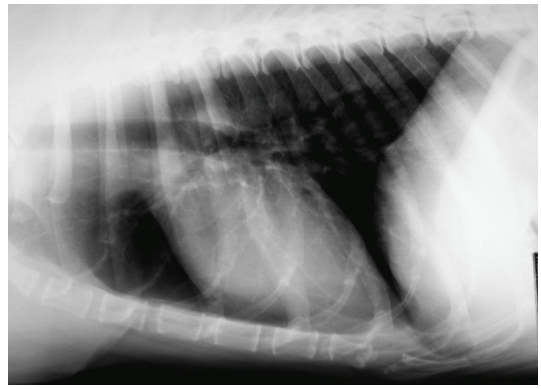
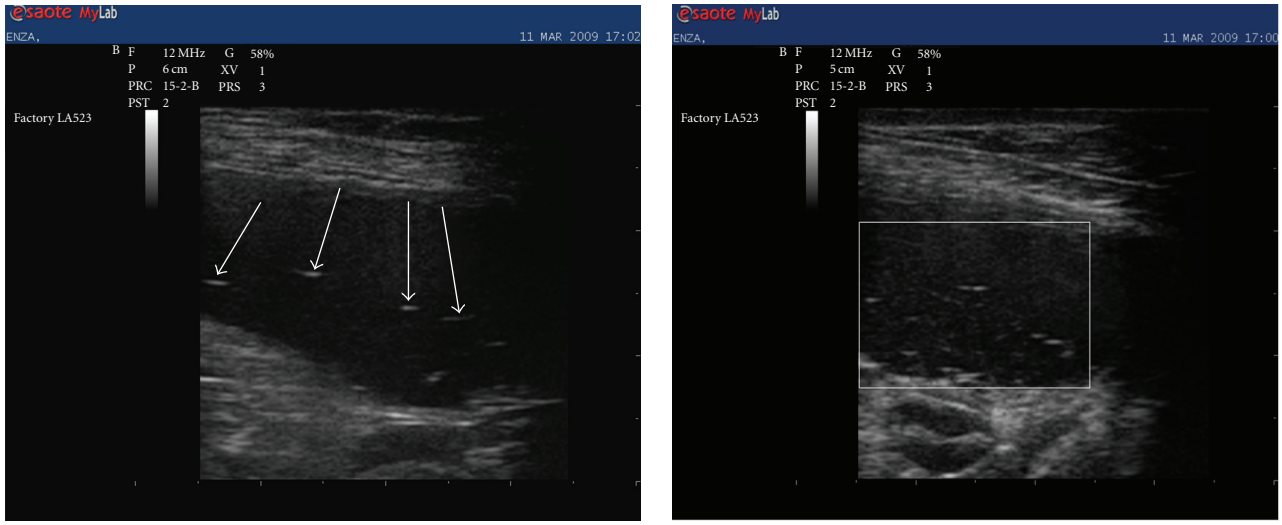


Figure 1: Thoracic radiograph laterolateral view showing the reduction of the cardiac silhouette (vertebral heart scale system score: 8.2) and pulmonary vascular hypoperfusion that both suggest reduction of plasmatic volume.



(a) (b)

Figure 2: Abdominal ultrasonography (linear probe 10 MHz). In different views, the bowel appears distended by fluid with several thin linear hyperechoic objects (arrows (a)) (white square (b)) floating within the lumen.

carried out. Adrenal glands appeared normal both for dimension and morphology, while the bowel was distended by fluid, and several thin linear hyperechoic echoes floating within the bowel lumen were observed (Figures 2(a) and 2(b)). A bowel irrigation with warm saline solution was performed, and two adult female whipworms (*Trichuris vulpis*) were collected. Centrifugation flotation faecal analysis showed a few whipworm eggs with the characteristic "double plugs", while the results of basal and post-ACTH stimulation cortisolemia were within the normal values.

The dog was treated with oral administration of milbemycine oxime (0.70 mg/kg bw; Interceptor Novartis AH) and i.v. administration of 800 mL of saline solution (Na^+Cl^- 0,9%) and discharged.

Three day later, the dog appeared clinically improved, and electrolyte analyses were completely normal. One month later, the dog was rechecked and clinical hematological exams were within the normal range; faecal analysis was negative, and body condition was clearly improved (body condition score 3/5, 37 kg). Oral milbemycine oxime at the same dose as above was prescribed once a month for an entire year in order to avoid whipworm reinfection.

3. Discussion

Although uncommon, as general rule intestinal nematode infections should be considered as a possible cause of electrolyte imbalance [3]. To the authors' knowledge, however, only *T. vulpis* infections are reported as causing hyponatremia and hyperkalemia [4–7]. Although this kind of syndrome (pseudo-Addison disease) due to *T. vulpis* infection has been reported, its pathogenesis is not well understood. The symptoms mimic those of Addison's disease with waxing and waning weakness. Severe electrolyte disturbance ultimately creates dehydration. The syndrome mimics Addison's disease in every way except that testing

for Addison's disease is negative and deworming yields a complete recovery [4–7]. In this case, the first diagnostic suspicion was hyponatremia as a consequence of the gastrointestinal losses, exacerbated by continued drinking and nonosmotic stimulation of antidiuretic hormone (ADH) release in response to volume depletion and hyperkalemia related to metabolic acidosis and decreased urinary excretion of potassium caused by reduced distal renal tubular flow rate [4, 6]. To note that this seems to happen only in the case of *T. vulpis* infection and not in other parasite infections that cause severe diarrhoea such as *Giardia intestinalis* or severe *Ancylostoma caninum* infections. Previous studies have shown that *T. vulpis* is able to induce transmural ileocolitis with severe lesions to the intestine wall and local cellular inflammation and oedema during the prepatent period of infection and fibrosis and mixed cellular inflammation of the terminal ileum, cecum, and proximal colon in response to deep penetration of adult worms, all of which could be responsible for electrolyte imbalance [8, 9].

This case report indicates the need to perform accurate faecal examination even when the dog's condition does not appear related to a parasitic infection and that dogs should be periodically monitored for helminth infection. In fact, the negative results of the fresh smear faecal examination confirmed the low sensitivity of this technique: 92.7% of whipworm false negatives when comparing direct smear to centrifugation [10].

This case report also suggests that whipworms in dogs, particularly in those cases where the bowel is distended by fluid allowing thorough examination, may be detected by ultrasonography as linear floating echoes, as previously demonstrated in humans [11].

References

[1] J. A. Adler, K. J. Drobatz, and R. S. Hess, "Abnormalities of serum electrolyte concentrations in dogs with hypoadrenocorticism," *Journal of Veterinary Internal Medicine*, vol. 21, no. 6, pp. 1168–1173, 2007.

[2] J. W. Buchanan and J. Bücheler, "Vertebral scale system to measure canine heart size in radiographs," *Journal of the American Veterinary Medical Association*, vol. 206, no. 2, pp. 194–199, 1995.

[3] S. I. Pak, "The clinical implication of sodium-potassium ratios in dogs," *Journal of Veterinary Science*, vol. 1, no. 1, pp. 61–65, 2000.

[4] S. P. DiBartola, S. E. Johnson, and D. J. Davenport, "Clinicopathologic findings resembling hypoadrenocorticism in dogs with primary gastrointestinal disease," *Journal of the American Veterinary Medical Association*, vol. 187, no. 1, pp. 60–63, 1985.

[5] T. K. Graves, W. D. Schall, K. Refsal, and R. F. Nachreiner, "Basal and ACTH-stimulated plasma aldosterone concentrations are normal or increased in dogs with trichuriasis-associated pseudohypoadrenocorticism," *Journal of Veterinary Internal Medicine*, vol. 8, no. 4, pp. 287–289, 1994.

[6] S. P. Di Bartola and H. A. De Morais, "Clinical cases," in *Fluid Therapy in Small Animal Practice*, S. P. Di Bartola, Ed., pp. 548–597, WB Saunders, Philadelphia, Pa, USA, 2nd edition, 2000.

[7] N. Ruckstuhl, A. Hoerauf, K. Tomsa, and C. Reusch, "Pseudohypoadrenocorticism in two Sibirian Huskies with intestinal parasitismPseudohypoadrenokortizismus bei zwei Siberian Huskies mit gastrointestinalen Parasitosen," *Schweizer Archiv für Tierheilkunde*, vol. 144, no. 2, pp. 75–81, 2002.

[8] R. Rubin, "Studies on the common whipworm of the dog. *Trichuris vulpis*," *The Cornell Veterinarian*, vol. 44, no. 1, pp. 36–49, 1954.

[9] W. R. Widmer and H. J. van Kruiningen, "*Trichuris* induced transmural ileocolitis in a dog: an entity mimicking regional enteritis," *Journal of the American Animal Hospital Association*, vol. 10, no. 6, pp. 581–585, 1974.

[10] M. W. Dryden, P. A. Payne, R. Ridley, and V. Smith, "Comparison of common fecal flotation techniques for the recovery of parasite eggs and oocysts," *Veterinary Therapeutics*, vol. 6, no. 1, pp. 15–28, 2005.

[11] S. B. Vijayaraghavan, "Sonographic whipworm dance in trichuriasis," *Journal of Ultrasound in Medicine*, vol. 28, no. 4, pp. 555–556, 2009.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 512154, 8 pages
doi:10.1155/2011/512154

Review Article

Excretory-Secretory Products from Hookworm L_3 and Adult Worms Suppress Proinflammatory Cytokines in Infected Individuals

Stefan Michael Geiger,[1] Ricardo Toshio Fujiwara,[1,2] Paula Albuquerque Freitas,[1] Cristiano Lara Massara,[1] Omar dos Santos Carvalho,[1] Rodrigo Corrêa-Oliveira,[1] and Jeffrey Michael Bethony[1,3]

[1] *Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Avenida Augusto de Lima 1715, 30190-002 Belo Horizonte, MG, Brazil*
[2] *Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, 31270-901 Belo Horizonte, MG, Brazil*
[3] *Department of Microbiology, Immunology, and Tropical Medicine, The George Washington University Medical Center, 2300 Eye Street NW, Washington, DC 20037, USA*

Correspondence should be addressed to Stefan Michael Geiger, stefan@cpqrr.fiocruz.br

Received 30 November 2010; Revised 18 March 2011; Accepted 29 March 2011

Academic Editor: Takeshi Agatsuma

We compared the effects of larval and adult worm excretory-secretory (ES) products from hookworm on the proliferative responses and cytokine secretion in peripheral blood mononuclear cells (PBMCs) from hookwormpatients and egg-negative, nonendemic controls. When compared with negative controls, mitogen-stimulated PBMC from hookworm-infected individuals showed a significantly reduced proliferative response when adult worm ES antigen was added to the cultures. Furthermore, in hookworm-infected individuals a significant downmodulation of inflammatory interleukin (IL)-6 and tumor necrosis factor (TNF)-α secretion resulted when PBMCs were stimulated with mitogen in combination with larval or adult worm ES. Both, interferon (IFN)-γ and IL-10 secretion were significantly lower in stimulated PBMC from infected individuals; however the IFN-γ/IL-10 ratio was much lower in hookworm-infected patients. Comparable effects, although at lower concentrations, were achieved when PBMCs from both groups were incubated with living hookworm third-stage larvae. We suggest that hookworm ES products downmodulate proliferative responses and inflammation during the chronic phase of the disease and facilitate early larval survival or adult worm persistence in the gut.

1. Introduction

Helminth excretory-secretory products (ES) contain a vast mixture of antigens, are potent modulators of the host's immune response, and therefore are important factors in worm survival and maintenance of a chronic human infection. ES products from different parasite species and different stages of parasitic development within the host were shown to downmodulate Type 1 immunity [1]. Among other mechanisms, early effects on dendritic cell function and innate immune responses have been previously described for intestinal nematode models [2], as well as for filarial infections [3, 4] with these events contributing to minimize inflammatory responses and induce a Type 2 response [1]. For hookworms, many components in ES products have been described so far [5, 6]; however, their effect on the human immune response is still not well understood and has become, more recently, a topic of intense investigation.

We have recently reported that PBMCs from *Necator americanus*-infected patients had a lower production of TNF-α and IL-10 in response to ES antigen derived from adult *Ancylostoma caninum*. Apart from the downmodulated cytokine secretion in response to ES antigen, PBMCs from endemic patients proliferated poorly in response to crude

ES antigen extracts [7]. In the present study, we compare the effects of hookworm adult and larval ES antigen preparations on polyclonal-activated lymphocytes from two different groups—(1) individuals chronically infected with hookworm, residing in an area of high transmission and (2) egg-negative ("control") and nonexposed individuals, residing in a nonendemic area. We found that proliferative responses and the secretion of proinflammatory cytokines in hookworm-infected individuals were downmodulated to a significant degree when compared to egg-negative controls.

2. Materials and Methods

2.1. Selection of Patients. In the present study, hookworm-infected adult individuals were recruited during an epidemiological field survey in 2006 in the village of Ladainha, located in the north-eastern region of Minas Gerais state, Brazil. A single stool sample was collected from individuals and eggs per gram of feces (epg) determined by the Kato-Katz fecal thick-smear technique [8] with two slides per patient. Individuals found to be monoinfected with hookworm were then enrolled into this study ($n = 10$). Blood was taken by venipuncture and the patients subsequently treated with a single dose of albendazole (400 mg) by the local health service personnel. Approximately 20 mL of blood was collected in heparinized tubes for separation of peripheral blood mononuclear cells (PBMCs). Nonendemic, egg-negative controls ($n = 7$) were recruited from the urban area of Belo Horizonte, Minas Gerais, which is considered an area of low to negligible transmission, especially outside the poor urban areas. Egg-negative controls consisted of volunteers from Centro de Pesquisas René Rachou who have not reported any intestinal helminth infection in the past. All volunteers provided written informed consent to participate in the study, and it was approved by the ethics committee from Centro de Pesquisas René Rachou-FIOCRUZ and the Brazilian "Conselho Nacional de Ética em Pesquisa" (CONEP).

2.2. Parasite ES Antigen Preparation. *Ancylostoma caninum* adult worms were recovered from the small intestines of stray dogs euthanized at the dog kennel of the Prefecture of Belo Horizonte during the regular leishmaniasis control program. Adult worm ES products (ES-AW) were obtained after incubation of the parasites for a period of 16–20 hours at 37°C in a humidified incubator and stored in aliquots at −70°C [9]. ES preparations from several days were pooled into 15 mL filtration tubes with a 5 kDa molecular weight cut-off filter (Millipore) and centrifuged for 1 hour at 4°C and 1,250 g. Antigen preparations were obtained after filtration in a 0.22 μm low-protein binding syringe filter (Millipore) and the resulting protein concentration determined using the BCA protein assay kit (Pierce).

In order to obtain L_3 for preparation of ES products, Harada-Mori fecal cultures from hookworm-infected individuals with more than 4,000 epg were performed [10]. Fifty-mili-liter plastic tubes was filled with 5 mL of tap water. Feces were distributed on filter paper strips on the upper two thirds of the filter paper, transferred to plastic tubes, and incubated in vertical position at 26–28°C for 7–10 days. The tubes were sealed with perforated Parafilm for air circulation. Fecal cultures were checked daily for water level, fungal contamination, and larvae in the water. After 7–10 days of incubation, the liquid was pooled in new tubes. To separate L_3 from fecal material and fungi the tubes were thoroughly mixed on a vortex and left on the bench during 30 minutes for sedimentation. After carefully removing the supernatant the content of the tubes were merged and the pellet with L_3 was resuspended with 40 mL of BU buffer at room temperature, as described by Hawdon et al. [11]. The solution was transferred to a small Baerman filter unit, containing several layers of gauze, and incubated for one hour at room temperature (RT). The resulting pellet of larvae was resuspended with BU buffer, mixed thoroughly, and the sedimentation step repeated. For bacterial decontamination, the pellet was incubated in BU/1% HCl buffer for 30 minutes at RT [11]. The larval suspension was transferred to a sterile 50 mL plastic tube and resuspension and sedimentation steps repeated under sterile conditions once with BU buffer, twice with minimal essential medium (Gibco), and once with RPMI-1640 medium (Gibco), containing 10% heat-inactivated normal human serum (ICN), 2% antibiotic-antimycotic solution (Sigma), and 1% L-glutamine (Winlab, Leicestershire, UK). After the last sedimentation step, the supernatant was removed, and the larvae resuspended in 5 mL complete RPMI-1640 medium (see above). For activation of larvae, 1 mL of larval suspension, containing approximately 10,000–20,000 larvae, was incubated in 3 mL of complete RPMI-1640 medium in 8-well cell multidish plates (NUNC) for 16–18 hours at 37°C and 5% CO_2. Finally, the larval suspension was removed, pooled into 1.5 mL cups, and centrifuged at 20,800 g for 3 minutes. The supernatant was carefully removed and antigen preparations (ES-L_3) from different larval cultures were pooled and stored at −70°C until used. Due to serum supplementation of cell culture medium for activation of L_3, it was not possible to determine the specific protein concentration of larval ES products.

2.3. In Vitro Lymphocyte Proliferation. Separation of PBMC was performed as described elsewhere [12]. In 96-well cell culture plates, triplicates of 250,000 cells/well were added for antigen and mitogen stimulations in a final volume of 200 μL of complete RPMI-1640 [12]. Final concentrations of stimulants determined to be optimal in cell culture were 35 μg/mL for ES-AW antigens and 2.5 μg/mL for phytohaemagglutinin (PHA)-L (Difco Laboratories, Detroit, MI, USA). For proliferation assays with ES-L_3 antigen, a dose-response curve was performed at the beginning of the experiments, in which an increasing volume of L_3 supernatant (5–50 μL) was added to mitogen-stimulated PBMCs. 25 μL of L_3 supernatant resulted in a 56% inhibition of PBMC proliferation (data not shown) and was used in all subsequent experiments of proliferation and cytokine secretion. Cells were cultured at 37°C in a humidified 5% CO_2 incubator. Tritiated thymidine (Amersham Pharmacia, São Paulo, Brazil; 0.5 μCi/culture;

specific activity 6.7 Ci/mM) was added to the cultures at 48 h, and the cells were harvested 18 h later. Incorporated tritiated thymidine was determined in a liquid scintillation counter and the data expressed as stimulation indices (SIs) (mean proliferation of stimulated culture divided by mean proliferation of unstimulated culture). The stimulation index of PHA-stimulated lymphocytes served as reference value (100%), and percentages of costimulated cell cultures, either PHA plus ES-AW or PHA plus ES-L_3, were calculated from this value.

2.4. Cytokine Detection in Cell Culture Supernatants. For production of cytokines and chemokines, 5×10^5 PBMCs were cultivated in 48-well tissue culture plates (Costar, Corning, NY, USA) at a total volume of 400 μL in complete RPMI-1640 for 2 days, using the same final mitogen and antigen concentrations as described above. Cell-free supernatants were stored at −70°C until cytokine quantification. Concentrations for IL-1β, IL-5, IL-6, IL-10, IFN-γ, and TNF-α were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, USA). When necessary, samples were diluted with PBS in order to obtain a value within the range of the standard curve. ELISAs were performed in duplicate according to the manufacturer's protocols, using a total volume of 25 μL per well in high-binding half-area plates (COSTAR, Corning, NY, USA). On each plate, serial dilutions of standards were run to construct standard curves with the following ranges of concentration: IL-1β (3.9–500 pg/mL); IL-5 (11.7–1,500 pg/mL); IL-6 (4.7–600 pg/mL); IL-10 (23.4–3,000 pg/mL); IFN-γ (7.8–1,000 pg/mL); TNF-α (7.8–1,000 pg/mL). The sensitivity for all ELISAs was lower than the last standard dilution. The colorimetric reaction was determined in an automated ELISA reader at 450 nm. Back calculations of cytokine concentrations from mean optical density values were interpolated from the standard curves by using a 4-parameter curve fitting program (SOFTmax PRO 3.1.2).

2.5. Cultivation of Lymphocytes and Living L_3. For co-cultivation of PBMC with infective hookworm larvae, L_3 from *Ancylostoma caninum*-infected dogs were obtained after fecal cultures (see above) and were a kind donation of Professor Walter dos Santos Lima and Professor Joziana Barçante (Federal University of Minas Gerais—UFMG). The *in vitro* cell cultures, 5×10^5 PBMCs, were cultivated in 24-Transwell plates (pore size 3.0 μm, COSTAR, Corning, NY, USA), at a total volume of 400 μL in complete RPMI-1640. Cells were incubated, either in direct contact or separated by the plate insert, with approximately 20 L_3 per well during 48 hours. Cell supernatants were obtained as described above and were stored at −70°C for future cytokine determination. Cytokine ELISAs were performed as described above.

2.6. Statistical Methods. For statistical evaluation, an SPSS 12.0 software package was used. Values from proliferation assays and cytokine concentrations were checked for normal distribution and were subsequently analysed by the non-parametric Mann-Whitney *U*-test for the comparison of two groups. Differences with a *P* value of less than .05 were considered significant and are indicated in the text, table, or figure.

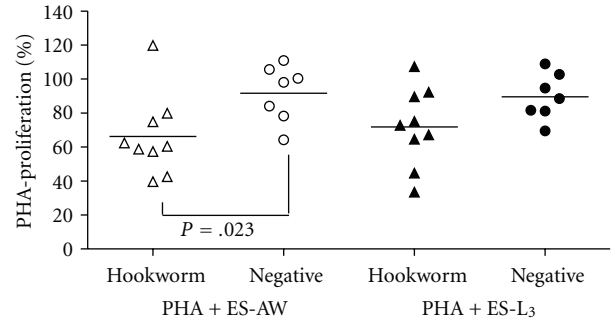


Figure 1: Inhibition of mitogen-stimulated PBMC proliferation after costimulation with adult worm ES (ES-AW, open symbols) and third-stage larval ES (ES-L_3, full symbols) antigen in hookworm-infected (▲; $n = 9$) and egg-negative (•; $n = 7$) individuals. Symbols show individual percentages of proliferation in comparison with PHA-stimulated cultures only, and horizontal bars indicate mean values for each group. Significant differences between groups are indicated.

3. Results

3.1. Patients and Parasitological Exams. The mean age of hookworm-infected patients from Ladainha was higher than that in negative, nonendemic controls from Belo Horizonte (44.4 ± 13.8 years versus 29.4 ± 5.8 years); however this difference was not significant ($P = .055$). Also, nonendemic controls have been living in the urban area of Belo Horizonte. For the egg-negative group, helminth infections were not diagnosed at the time of the study and no past records of helminth infections were stated by the volunteers from the hypoendemic area. The geometric mean of hookworm egg counts was 789 epg (range: 11,892–684), with 7 patients harbouring light, 2 moderate, and 1 patient with heavy hookworm infections, according to the classification by the World Health Organization [13].

3.2. Lymphocyte Proliferation. Figure 1 shows the individual percentages of PBMC proliferation after costimulation with PHA and hookworm ES-AW or ES-L_3 in comparison to PHA-stimulated lymphocytes only. PBMCs from hookworm patients showed a lower percentage of proliferation when hookworm ES antigens were added to the lymphocyte cultures. After addition of ES-AW to PHA-stimulated lymphocytes the percentage of proliferation was significantly lower ($P = .023$) in hookworm-infected individuals. Notably, when PBMC cultures were stimulated with PHA only, no significant differences in the stimulation index resulted between the two patient groups (data not shown).

3.3. Cytokine Secretion. Cytokine concentrations detected in cell culture supernatants are shown in Table 1.

In unstimulated PBMC cultures from negative, nonendemic individuals lower concentrations of inflammatory IL-1β, IL-6, and TNF-α were detected when compared

Table 1: Cytokine concentrations (pg/mL) in supernatants from PHA-stimulated PBMC cultures with or without costimulation by adult worm ES (ES-AW) or third-stage larval ES (ES-L_3) antigen. Values from hookworm-infected individuals ($n = 7$) and negative controls ($n = 6$) are compared and indicated as mean values ± standard errors. Significant differences between groups are indicated with an asterisk ($^*P < .01$; $^{**}P < .05$).

	Group	Control	PHA	PHA+ES-AW	PHA+ES-L_3
IL-1β	Hookworm	$1,034 \pm 239^*$	$1,446 \pm 176^*$	$1,421 \pm 265$	$1,456 \pm 320$
	Negative controls	$27 \pm 27^*$	$215 \pm 114^*$	$1,562 \pm 424$	785 ± 340
IL-6	Hookworm	$52,382 \pm 14,323$	$26,553 \pm 12,000^{**}$	$27,865 \pm 7,464^*$	$22,056 \pm 4,763^*$
	Negative controls	$18,452 \pm 4,400$	$63,409 \pm 14,530^{**}$	$102,499 \pm 11,146^*$	$87,143 \pm 14,244^*$
TNF-α	Hookworm	$3,630 \pm 2,621^{**}$	$297 \pm 185^{**}$	$741 \pm 300^*$	$885 \pm 333^{**}$
	Negative controls	$306 \pm 114^{**}$	$2,104 \pm 538^{**}$	$5,638 \pm 1,385^*$	$2,820 \pm 832^{**}$
IL-5	Hookworm	$35 \pm 19^{**}$	40 ± 20	12 ± 7	53 ± 40
	Negative controls	$5 \pm 5^{**}$	45 ± 22	7 ± 6	37 ± 17
IL-10	Hookworm	63 ± 26	113 ± 48	$225 \pm 69^{**}$	$310 \pm 120^{**}$
	Negative controls	0	157 ± 33	$526 \pm 58^{**}$	$776 \pm 89^{**}$
IFN-γ	Hookworm	43 ± 22	$119 \pm 69^*$	$230 \pm 165^*$	$158 \pm 99^*$
	Negative controls	0	$3,288 \pm 1,046^*$	$3770 \pm 899^*$	$2,168 \pm 796^*$

with participants with hookworm infection. For IL-1β and TNF-α, these differences were statistically significant ($P < .01$ and $P < .05$, resp.). When stimulated with PHA, PBMC from hookworm-infected individuals secreted significantly more IL-1β ($P < .01$) than egg-negative control subjects. Opposite to unstimulated control cultures, IL-6 and TNF-α secretions in PHA-stimulated or costimulated cultures were significantly lower in PBMCs from hookworm patients than in egg-negative individuals. For IL-5, low concentrations were detected in both, control and stimulated lymphocyte cultures from the two groups. However, unstimulated PBMCs from hookworm patients secreted significantly more IL-5 ($P < .05$) than PBMCs from egg-negative individuals. After addition of either ES-AW or ES-L_3 antigen to PHA-stimulated cell cultures, individuals in both groups showed increased IL-10 secretion. However, this increase was significantly ($P < .05$) higher in the egg-negative controls. The IFN-γ secretion in participants with hookworm infection was significantly ($P < .01$) lower in all stimulated lymphocyte cultures when compared with egg-negative individuals. Figure 2 shows the paired IFN-γ and IL-10 secretion for the two groups of patients separated in egg-negative controls (2A) and hookworm-infected individuals (2B). Upon stimulation, PBMC from egg-negative individuals secreted high levels of IFN-γ and considerably lower levels of IL-10. In contrast, PBMC from hookworm patients secreted equally low concentrations of IFN-γ and IL-10 (Figures 2(a) and 2(b)).

3.4. Cultivation of Lymphocytes and Living L_3. Table 2 shows the results obtained for cytokine secretion levels from PBMC cocultured with living L_3 of *A. caninum*. Although the concentrations are lower if compared with the previous data, the differences in the secretion of inflammatory cytokines (IL-6, TNF-α) after co-cultivation with living L_3 showed the same trend as described for soluble ES-L_3 preparations from *N. americanus* (Table 1). These results show that this regulatory effect may be mediated by soluble factors secreted

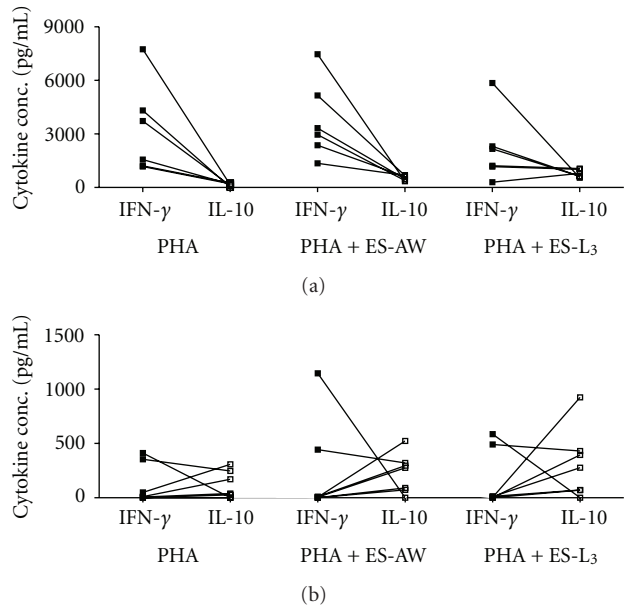


Figure 2: IFN-γ and IL-10 secretion in pg/mL in PBMC stimulated with PHA, PHA+ adult worm ES (ES-AW), or PHA+ ES-L_3. (a) Paired IFN-γ and IL-10 cytokine concentrations in PBMCs from egg negative, nonendemic controls and (b) in hookworm-infected patients from an endemic area.

by the L_3. On the other hand and in contrast with the soluble antigen preparations, co-cultivation of PBMC and L_3 did not induce an increase in IL-10 secretion and cytokine concentrations remained low with no significant differences between the two groups (Table 2). IL-5 and IFN-γ secretion in PBMC cocultured with living L_3 remained at marginal levels in both patient groups (data not shown).

4. Discussion

Suppressed cellular responsiveness, either antigen-specific or polyclonal, and a skewed Th2 immune response are two of

Table 2: Cytokine concentrations (pg/mL) in supernatants of PBMC cultures cocultured with living L_3 from *Ancylostoma caninum*. Cells were either separated from the L_3 by a membrane insert (cells/L_3) or were cultivated in direct contact with the parasites (cells+L_3). Values from hookworm-infected individuals ($n = 7$) and negative controls ($n = 6$) are compared and indicated as mean values ± standard errors. Significant differences between patient groups are indicated with an asterisk ($^*P < .01$; $^{**}P < .05$).

	Group	Control	Cells/L_3	Cells+L_3
IL-1β	Hookworm	$1,034 \pm 239^*$	833 ± 149	$1,161 \pm 218$
	Negative controls	$27 \pm 27^*$	424 ± 134	$1,470 \pm 494$
IL-6	Hookworm	$52,382 \pm 14,323$	$8,703 \pm 2,258^*$	$12,124 \pm 2,548^*$
	Negative controls	$18,452 \pm 4,400$	$62,750 \pm 18,488^*$	$59,185 \pm 15,443^*$
TNF-α	Hookworm	$3,630 \pm 2,621^{**}$	$351 \pm 110^*$	$404 \pm 94^*$
	Negative controls	$306 \pm 114^{**}$	$1,784 \pm 362^*$	$2,163 \pm 221^*$
IL-10	Hookworm	63 ± 26	86 ± 48	37 ± 26
	Negative controls	0	18 ± 16	92 ± 31

the hallmarks of helminth infections and have been extensively described for filarial infections [14, 15] and schistosomes [16–18]. In two independent studies, we have found reduced antigen-specific cellular responsiveness in hookworm patients in response to larval and adult worm soluble extracts [7, 19]. Interestingly, proliferative responses and cytokine secretion patterns were quite distinct for the different antigen preparations, with adult ES antigen inducing low type 1 and inflammatory responses especially in hookworm—infected individuals [7].

In the present paper, we have focused on the cellular response to ES antigenic preparations from two developmental stages of hookworms—infective third-stage larvae (L_3) and adult worms (AWs). We were able to show that both ES preparations, L_3 and AW, induce a considerable reduction in cell proliferative responses of polyclonally stimulated lymphocytes from hookworm-infected patients. The fact that we observe a reduction on polyclonal activation of PBMCs in infected participants with L_3 ES antigen, suggests that down-modulatory mechanisms occur at the early L_3 parasitic stage of infection in individuals previously sensitized with parasite antigens or that have a current infection. This observation is novel for hookworm infections and is certainly of major importance for vaccine development. In this context, induction of regulatory/suppressor responses induced by ES L_3 antigens may have significant effect on the maintenance of parasite survival as well as on reinfection in endemic human populations. Importantly, Loukas et al. [20] showed that L_3 and adult worms from *A. caninum* share ES and somatic antigens; these results together with the results presented in this paper suggest that common mechanisms of L_3 and adult worms on the regulation of the immune response may facilitate parasite escape and survival. In contrast, it has also been shown by others that mechanisms of immunomodulation and the induction of pathological changes cannot be generalised between different hookworm species and that the induced inflammatory response in humans infected with *N. americanus* is much more subtle than, for example, after enteric *A. caninum* infections [21]. Also, in primary experimental infections in human volunteers and in hamsters it was shown that a state of reduced cellular responsiveness builds up slowly during the onset of patency, most probably with the more important contribution of the adult worm population that builds up with time [22, 23].

Due to the obvious difficulties in obtaining sufficient ES material from living human hookworm species, we used in our experiments adult worm ES products, as well as living larvae from *A. caninum* and compared the human cellular response to that of L_3 ES products from *N. americanus*. We are well aware of the differences between human and dog hookworm infections [24] and that ES products from adult *A. caninum*, and *N. americanus* have been shown to have individual protein patterns and bind to distinct leukocyte populations [25] and different components might therefore have distinct effects on the human immune system [26]. However, the effects of total ES extracts, as described here, seem to be comparable for both dog and human hookworm ES product preparations. We are also aware of statistical limitations due to the low number of individuals in each group, which might impair to discover small differences between groups. However, we feel that in the present experimental setting with the unspecific polyclonal stimulation of lymphocytes by the mitogen PHA we are able to draw the presented conclusions, even if limited quantities of ES antigens forced us to use blood from a restricted number of individuals.

Experimental human infections monitored by capsule endoscopy have shown that adult worms still cause a significant degree of intestinal inflammation and newly arriving premature worms are expelled from the small intestine in the course of acute eosinophilic enteritis [27]. Nevertheless, adult hookworms have also been shown to resist intestinal inflammation and continued to parasitize the small intestine [28]. Furthermore, in repeatedly administered experimental human infections, even with as much as 250 L_3, initial intestinal symptoms seem to vanish with every newly applied infection [29]. In our view, this indicates time- and dose-dependent mechanisms of hookworm immunosuppression with decreased intestinal inflammation, resulting in parasite persistence. There are already described mechanisms by which adult worm ES components induce suppression of the human immune response, for example, by the induction of apoptosis in reactive T cells, which avoids the infiltration

of reactive host leukocytes to the place of adult worm attachment and facilitates worm survival [30], or by affecting dendritic cell maturation and differentiation of regulatory T cells [31].

We observed elevated levels of IL-1β, IL-6, and TNF-α concentrations in unstimulated PBMC cultures from infected individuals indicating that these volunteers have ongoing inflammatory processes. However, after *in vitro* PBMC stimulation infected patients showed a reduced capacity in secreting IL-6 and TNF-α either after PHA stimulation or in combination with ES antigen from both parasitic stages. Together with a low IFN-γ secretion in individuals with hookworm infection, our results point to a major role of ES products for reduced inflammatory/type 1 immune responses in the course of human hookworm infection. On the other hand, low to absent IL-5 secretion, either in response to ES preparations or in response to living parasites, respectively, suggests that ES antigens are poor inducers of type 2 immunity. This was also confirmed by previous antigen-specific stimulations of PBMC from hookworm-infected subjects [7], and similar results were described in PBMCs from nonexposed individuals which were incubated with live L_3 from the filarial parasite *Brugia malayi* [32]. As other mechanism influencing the immunoregulation and the cytokine and chemokine milieu, it was reported that host eotaxin is specifically cleaved by metalloproteases from adult *N. americanus* ES secretions, which might be important in preventing recruitment and activation of eosinophils and even may influence Th2 responses [33]. Therefore, it would be interesting in future studies to collect additional information on IL-4 or IL-13 lymphocyte secretion patterns, or even on IL-21, a Th2 cytokine identified to be highly relevant in helminth infections [34]. Furthermore, during schistosome infection and migration through the skin, IL-10 seems to be a key regulator of the immune response [35]. However, in this study we observed a significantly lower IL-10 secretion in PBMC cultures from hookworm-infected patients when compared to negative individuals. On the other hand, a resulting immune response is the interplay between different and also counteracting cytokines. As such, the paired IFN-γ and IL-10 secretion for each patient upon stimulation of PBMC with ES antigens showed a completely different pattern between the two patient groups; for example, upon stimulation there is a relatively higher IFN-γ than IL-10 level in egg-negative controls and equally low levels of IFN-γ and IL-10 in hookworm-infected individuals. In our view, this ES antigen-induced low IFN-γ/IL-10 ratio in hookworm-infected patients may contribute to the enhanced suppression of the immune response, with reduced cellular reactivity, and a diminished inflammatory response. Interestingly, an elegant study in *Heligmosomoides polygyrus*-infected mice has recently shown that adult worm ES products from this rodent nematode inhibit the maturation and function of dendritic cells and may drive the differentiation of IL-10 producing T_{reg} cells. As a result, T cell, cytokine, and antibody responses are suppressed in a generalized manner [36]. The putative role of an altered phenotype and function of dendritic cells in hookworm-infected patients on the observed suppression/regulation of the immune response in these patients has recently been published by our group [37]. Also, the important contribution of alternatively activated macrophages in the course of intestinal helminth infections has recently been reviewed and emphasized by Kreider et al. [38] and might be important in directing the immune response during human hookworm infections. As such, the effect of hookworm ES products on antigen presenting cells, as well as the induction of regulatory T cell cytokines, such as TGF-β or IL-17, deserves further investigation.

In summary, we were able to show that hookworm ES products from *A. caninum* adult worms or L_3 induce immune mechanisms that significantly reduce proliferative responses in mitogen-activated PBMC from hookworm-infected individuals. Furthermore, a significant downmodulation of inflammatory cytokine secretion, as well as a lower IFN-γ/IL-10 ratio, resulted in stimulated PBMC from hookworm patients, when compared to nonendemic, egg-negative controls, factors that might be decisive for early larval survival or adult worm persistence in the gut.

Acknowledgments

The authors are grateful to the patients from Ladainha and the nonendemic volunteers at Centro de Pesquisas René Rachou for their collaboration. Financial support for the study was provided by the J. Fogarty Foundation and by the PAPES V program from Fiocruz/CNPq. Also, They would like to thank Professor Walter dos Santos Lima and Professor Joziana Barçante (Federal University of Minas Gerais—UFMG) for the donation of infective hookworm larvae. S. M. Geiger and R. T. Fujiwara contributed equally to this work.

References

[1] R. M. Maizels, A. Balic, N. Gomez-Escobar, M. Nair, M. D. Taylor, and J. E. Allen, "Helminth parasites—masters of regulation," *Immunological Reviews*, vol. 201, pp. 89–116, 2004.

[2] A. Balic, Y. Harcus, M. J. Holland, and R. M. Maizels, "Selective maturation of dendritic cells by *Nippostrongylus brasiliensis*-secreted proteins drives Th2 immune responses," *European Journal of Immunology*, vol. 34, no. 11, pp. 3047–3059, 2004.

[3] R. T. Semnani, M. Law, J. Kubofcik, and T. B. Nutman, "Filaria-induced immune evasion: suppression by the infective stage of *Brugia malayi* at the earliest host-parasite interface," *Journal of Immunology*, vol. 172, no. 10, pp. 6229–6238, 2004.

[4] H. S. Goodridge, F. A. Marshall, K. J. Else et al., "Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62," *Journal of Immunology*, vol. 174, no. 1, pp. 284–293, 2005.

[5] A. Loukas and P. Prociv, "Immune responses in hookworm infections," *Clinical Microbiology Reviews*, vol. 14, no. 4, pp. 689–703, 2001.

[6] A. L. Williamson, P. J. Brindley, D. P. Knox, P. J. Hotez, and A. Loukas, "Digestive proteases of blood-feeding nematodes," *Trends in Parasitology*, vol. 19, no. 9, pp. 417–423, 2003.

[7] S. M. Geiger, I. R. Caldas, B. E. Mc Glone et al., "Stage-specific immune responses in human *Necator americanus* infection," *Parasite Immunology*, vol. 29, no. 7, pp. 347–358, 2007.

[8] N. Katz, A. Chaves, and J. Pellegrino, "A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni," *Revista do Instituto de Medicina Tropical de São Paulo*, vol. 14, no. 6, pp. 397–400, 1972.

[9] A. Carr and D. I. Pritchard, "Antigen expression during development of the human hookworm, *Necator americanus* (Nematoda)," *Parasite Immunology*, vol. 9, no. 20, pp. 219–234, 1987.

[10] L. R. Ash and T. C. Orihel, *In Atlas of Human Parasitology*, ASCP Press, Chicago, Ill, USA, 4th edition, 1997.

[11] J. M. Hawdon, S. Narasimhan, and P. J. Hotez, "Ancylostoma secreted protein 2: cloning and characterization of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*," *Molecular and Biochemical Parasitology*, vol. 99, no. 2, pp. 149–165, 1999.

[12] S. M. Geiger, C. L. Massara, J. Bethony, P. T. Soboslay, O. S. Carvalho, and R. Corrêa-Oliveira, "Cellular responses and cytokine profiles in *Ascaris lumbricoides* and *Trichuris trichiura* infected patients," *Parasite Immunology*, vol. 24, no. 11-12, pp. 499–509, 2002.

[13] World Health Organization, *Prevention and Control of Schistosomiasis and Soil-Transmitted Helminthiasis*, WHO Technical Report Series, vol. 912, World Health Organization, Geneva, Switzerland, 2002.

[14] E. A. Ottesen, P. F. Weller, and L. Heck, "Specific cellular immune unresponsiveness in human filariasis," *Immunology*, vol. 33, no. 3, pp. 413–421, 1977.

[15] W. F. Piessens, P. B. McGreevy, P. W. Piessens et al., "Immune responses in human infections with *Brugia malayi*. Specific cellular unresponsiveness to filarial antigens," *Journal of Clinical Investigation*, vol. 65, no. 1, pp. 172–179, 1980.

[16] I. R. C. Viana, A. Sher, O. S. Carvalho et al., "Interferon-gamma production by peripheral blood mononuclear cells from residents of an area endemic for *Schistosoma mansoni*," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 88, no. 4, pp. 466–470, 1994.

[17] C. L. King, A. Medhat, I. Malhotra et al., "Cytokine control of parasite-specific anergy in human urinary schistosomiasis. IL-10 modulates lymphocyte reactivity," *Journal of Immunology*, vol. 156, no. 12, pp. 4715–4721, 1996.

[18] J. L. Grogan, P. G. Kremsner, A. M. Deelder, and M. Yazdanbakhsh, "Elevated proliferation and interleukin-4 release from CD4+ cells after chemotherapy in human *Schistosoma heamatobium* infection," *European Journal of Immunology*, vol. 26, no. 6, pp. 1365–1370, 1996.

[19] S. M. Geiger, C. L. Massara, J. Bethony, P. T. Soboslay, and R. Corrêa-Oliveira, "Cellular responses and cytokine production in post-treatment hookworm patients from an endemic area in Brazil," *Clinical and Experimental Immunology*, vol. 136, no. 2, pp. 334–340, 2004.

[20] A. Loukas, J. Croese, J. Opdebeeck, and P. Prociv, "Immunologic incrimination of *Ancylostoma caninum* as a human enteric pathogen," *American Journal of Tropical Medicine and Hygiene*, vol. 50, no. 1, pp. 69–77, 1994.

[21] P. Prociv and J. Croese, "Human enteric infection with *Ancylostoma caninum*: hookworms reappraised in the light of a 'new' zoonosis," *Acta Tropica*, vol. 62, no. 1, pp. 23–44, 1996.

[22] V. Wright and Q. Bickle, "Immune responses following experimental human hookworm infection," *Clinical and Experimental Immunology*, vol. 142, no. 2, pp. 398–403, 2005.

[23] S. Mendez, J. G. Valenzuela, W. Wu, and P. J. Hotez, "Host cytokine production, lymphoproliferation, and antibody responses during the course of *Ancylostoma ceylanicum* infection in the Golden Syrian Hamster," *Infection and Immunity*, vol. 73, no. 6, pp. 3402–3407, 2005.

[24] R. T. Fujiwara, S. M. Geiger, J. Bethony, and S. Mendez, "Comparative immunology of human and animal models of hookworm infection," *Parasite Immunology*, vol. 28, no. 7, pp. 285–293, 2006.

[25] G. C.-F. Hsieh, A. Loukas, A. M. Wahl et al., "A secreted protein from the human hookworm *Necator americanus*binds selectively to NK cells and induces IFN-γ production," *Journal of Immunology*, vol. 173, no. 4, pp. 2699–2704, 2004.

[26] H. J. McSorley and A. Loukas, "The immunology of human hookworm infections," *Parasite Immunology*, vol. 32, no. 8, pp. 549–559, 2010.

[27] J. Croese, M. J. Wood, W. Melrose, and R. Speare, "Allergy controls the population density of *Necator americanus* in the small Intestine," *Gastroenterology*, vol. 131, no. 2, pp. 402–409, 2006.

[28] J. M. Behnke, R. Rose, and J. Little, "Resistance of the hookworms *Ancylostoma ceylanicum* and *Necator americanus* to intestinal inflammatory responses induced by heterologous infection," *International Journal for Parasitology*, vol. 24, no. 1, pp. 91–101, 1994.

[29] B. M. Ogilvie, A. Bartlett, R. C. Godfrey, J. A. Turton, M. J. Worms, and R. A. Yeates, "Antibody responses in self-infections with *Necator americanus*," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 72, no. 1, pp. 66–71, 1978.

[30] S. C. Chow, A. Brown, and D. I. Pritchard, "The human hookworm pathogen *Necator americanus* induces apoptosis in T lymphocytes," *Parasite Immunology*, vol. 22, no. 1, pp. 21–29, 2000.

[31] C. Cuéllar, W. Wu, and S. Mendez, "The hookworm tissue inhibitor of metalloproteases (Ac-TMP-1) modifies dendritic cell function and induces generation of CD4 and CD8 suppressor T cells," *PLoS neglected tropical diseases*, vol. 3, no. 5, p. e439, 2009.

[32] S. Babu and T. B. Nutman, "Proinflammatory cytokines dominate the early immune response to filarial parasites," *Journal of Immunology*, vol. 171, no. 12, pp. 6723–6732, 2003.

[33] F. J. Culley, A. Brown, D. M. Conroy, I. Sabroe, D. I. Pritchard, and T. J. Williams, "Eotaxin is specifically cleaved by hookworm metalloproteases preventing its action in vitro and in vivo," *Journal of Immunology*, vol. 165, no. 11, pp. 6447–6453, 2000.

[34] J. Pesce, M. Kaviratne, T. R. Ramalingam et al., "The IL-21 receptor augments Th2 effector function and alternative macrophage activation," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 2044–2055, 2006.

[35] S. J. Jenkins, J. P. Hewitson, G. R. Jenkins, and A. P. Mountford, "Modulation of the host's immune response by schistosome larvae," *Parasite Immunology*, vol. 27, no. 10-11, pp. 385–393, 2005.

[36] M. Segura, Z. Su, C. Piccirillo, and M. M. Stevenson, "Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression," *European Journal of Immunology*, vol. 37, no. 7, pp. 1887–1904, 2007.

[37] R. T. Fujiwara, G. G. L. Cançado, P. A. Freitas et al., "*Necator americanus* infection: a possible cause of altered dendritic cell differentiation and eosinophil profile in chronically infected individuals," *PLoS Neglected Tropical Diseases*, vol. 3, no. 3, article e399, 2009.

[38] T. Kreider, R. M. Anthony, J. F. Urban, and W. C. Gause, "Alternatively activated macrophages in helminth infections," *Current Opinion in Immunology*, vol. 19, no. 4, pp. 448–453, 2007.

Hindawi Publishing Corporation
Journal of Parasitology Research
Volume 2011, Article ID 214174, 19 pages
doi:10.1155/2011/214174

Review Article

Parasites or Cohabitants: Cruel Omnipresent Usurpers or Creative "Éminences Grises"?

Marcos A. Vannier-Santos[1, 2] and Henrique L. Lenzi[3]

[1] *Laboratório de Biomorfologia Parasitária, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz (FIOCRUZ), Rua Waldemar Falcão 121, Brotas, 40295-001 Salvador, BA, Brazil*
[2] *Instituto Nacional para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica, Conselho Nacional de Desenvolvimento Científico e Tecnológico/MCT, Rio de Janeiro, RJ, Brazil*
[3] *Laboratório de Patologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Marcos A. Vannier-Santos, marcos.vannier@pesquisador.cnpq.br

Received 20 January 2011; Accepted 6 April 2011

Academic Editor: Bernard Marchand

This paper presents many types of interplays between parasites and the host, showing the history of parasites, the effects of parasites on the outcome of wars, invasions, migrations, and on the development of numerous regions of the globe, and the impact of parasitic diseases on the society and on the course of human evolution. It also emphasizes the pressing need to change the look at the parasitism phenomenon, proposing that the term "cohabitant" is more accurate than parasite, because every living being, from bacteria to mammals, is a consortium of living beings in the pangenome. Even the term parasitology should be replaced by cohabitology because there is no parasite alone and host alone: both together compose a new adaptive system: the parasitized-host or the cohabitant-cohabited being. It also suggests switching the old paradigm based on attrition and destruction, to a new one founded on adaptation and living together.

1. Introduction

> *"It is derogatory that the Creator of countless systems of worlds should have created each of the myriads of creeping parasites and slimy worms which have swarmed each day of life... on this one globe."* Charles Robert Darwin.

The words quoted above suggest that Darwin was rather concerned about parasites. If he had seen the hematophagous finches *Geospiza nebulosa*, also known as "vampire birds", of Wolf Island in the Galapagos Archipelago [1], he would have presumably been overwhelmed. Many features of the parasitic life style can indubitably surprise most people regardless naturalist or not.

2. Parasitism and Symbiology

The term parasite (Latin *parasites* – Greek παράσιτος-*parasitos*, παρα- (*para*-, beside) + σίτος (*sitos*, wheat, food) "person who eats at the table of the bystander" "feeding beside") is employed here in the traditional sense, but it must be stated that such concepts are only communication tools to be used in a flexible and relative way, as are the biological phenomena. In the words of van Beneden, the differences among parasites, mutualists, and free-living organisms are "almost insensible", and according to Noël Bernard there is "no absolute distinction to be made" between *symbiosis and disease* [2].

From the study of lichens derived the concept of "consortium" to express the associations between phylogenetically distinct organisms that ranged from the loosest to the most intimate and essential, and the most antagonistic and one-sided to the most beneficial for the well-being of the both associates [3]. Albert Bernhard Frank (1877) at Leipzig coined the word *Symbiotismus*: "We must bring all the cases where two different species live on or in one another under a comprehensive concept which does not consider the role which the two individuals play but is based on the mere

coexistence and for which the term *Symbiotismus* is to be recommended." Though Frank's studies were well known, and he became one of the chief advocates of the view that many associations involving microorganisms could not be labeled parasitism, the term "*symbiosis*" was credited to Heinrich Anton de Bary to describe organisms coexisting or living together [3]. Thus we attempted to avoid the misleading aspects of *strictu sensu* definitions such as mutualist, pathogen, or parasite, used to characterize species which flow in a role-exchanging dynamic continuum [2–5], compared to a marriage, where it is difficult to measure the gains of respective partners [2].

The term "*cohabitant*" is often more accurate than *parasite* [6], because it is increasingly clear that every living being, from bacteria to mammals, is a consortium of living beings [6, 7]. Even we propose that the term parasitology should be replaced by *Cohabitology because there is no parasite and host alone: both together compose a new adaptive system: the parasitized-host or the cohabitant-cohabited being* [6]. Symbiosis is a cyclical and permanent phenomenon in evolution [8]. The ubiquitous symbiotic consortia played a pivotal role in the prokaryote-to-eukaryote transition, and the term "*symbiogenesis*" was coined by Konstantine Merezhkovskii to designate "...the origin of organisms through combination and unification of two or more beings" [3]. Therefore, symbiosis, which is often under laid by hostility [9], is a powerful source of biodiversity inside the *pangenome* [10]. According to this concept, the Pangenome is the common (collective) genetic system of all living organisms, the organic molecules, and their complexes (DNA-and RNA-containing viruses, plasmids, transposons, insertion sequences) involved in the storage and transmission processes of genetic information. In fact, genes are remarkably outnumbered by retrotransposons and other types of mobile elements [10–12]. The navigation of mobile elements in the pangenome of the living beings is made by *infectrons*. Tosta coined this term to encompass the broad array of exogenous DNAs that invade a genome and interfere with its structure or organization, and, therefore with its function [13]. In fact, infectious agents are everywhere and they dwell the uppermost of our individuality: our genome. Indeed, it has been recognized that about 40% of mammalian genome is composed of retrotransposons derived from retroviruses [13–15]. *Symbiology* plays a central role in ecology and on the overall understanding of Biology. Nowadays it is widely accepted that most if not all metazoan organisms and many microorganisms harbor different microbes, mainly prokaryotes. These may be harmless commensals, mutually advantageous mutualists, or virulent pathogens, depending on the milieu [16, 17]. Microbes may account for up to half the weight of insects such as termites [2]. Some of these cohabitants present their own endosymbionts which produce enzymes that breakdown lignin and cellulose. Over 90% of the plants are associated with mycorrhizal fungi which help absorbing nutrients, but may act parasitically, depending on the environment conditions [18–20].

Most if not all known species are involved in parasitism, acting as parasites and/or hosts. It is estimated that *circa* 80% of the known species, which are found in many different taxa, are parasitic [20]. Parasitology (Cohabitology) plays a central role in biological sciences, not only because parasites constitute the great majority of the living beings [21], but also because they regulate countless populations in numerous ecosystems.

According to Dr. J. R. Lichtenfels, "If every part of the Earth were to disappear magically except for parasites, one would still see the outline of the planet". He also states that we need to understand the parasites because without the knowledge about the "enemy" and its strategies, we will hardly be able to win the battle [22]. Nevertheless, this bellicose view which dates from the times of Pasteur may have hindered the understanding of the innovative potential of these creative organisms upon life as we know [2, 3, 5, 6]. Microbes were and are still largely seen as agents of disease and death rather than dynamic factors in transformation and evolution [2]. As will be seen below, this belligerent point of view, common in the literature, may be driven by parasites themselves.

About 374 parasite species infect the *Homo sapiens sapiens* being more than 300 agents of zoonosis [23]. This is not surprising if we keep in mind that among our 25.000 genes only about 1% is exclusively human and more than 99% homology was observed between the human and chimpanzee genomes [24].

In the *latu sensu,* Parasitology would focus viruses, bacteria, fungi, animals, and plants with parasitic way of life. For methodological rather than conceptual reasons, viruses, bacteria, fungi, and sometimes even protozoa are focused by Microbiology and its branches Virology, Bacteriology and Mycology. Thus these sciences present considerable overlapping. The modern Parasitology (Cohabitology) constitutes much more than a branch of biological sciences, congregating, in elegant and complex fashion, diverse areas of the knowledge for example, Zoology, Ecology, Pathology, Molecular Biology, Biochemistry, Epidemiology, Immunology, Systems Biology, and others, thus constituting a rich multidisciplinary collection. The parasitism phenomenon comprises an intricate web of interactions in which the parasite not only is fed, sheltered, and transported through its host, but is also able to significantly modify its physiology, behavior, and even direct the routes of its evolution.

Symbiotic consortia involving bacteria and blue-green algae had probably originated, respectively, mitochondria and chloroplasts of the eukaryotic organisms. This perception arose from the similarities between microbes and organelles pointed out by Joshua Lederberg and further examined by Wakeford [2] and Margulis [25]. The prokaryote-organelle continuum is the main stream of eukaryotic cell evolution and even the eukaryotic cell nucleus may have had a prokaryotic origin [26, 27] or even a viral origin [28, 29].

Besides different types of endosymbiont, protozoa may be associated with ectosymbiont spirochaetes that function as undolipodia or locomotion structures, which may have given rise to cilia and flagella of the higher organisms [30]. It is worth mentioning that mitochondria as well as pathogens like *Listeria*, *Shigella*, *Rickettsiae,* and vaccinia virus induce the polymerization of actin tails in the cytoplasm of the "host" cell [31]. This "evolutionary scar" may have been

useful only for pathogen spread at first, but now it is also used for organelle translocation [31, 32] as in the spermiogenesis regulation in *Drosophila* [33]. It was hypothesized that the infection vestiges of the bacteria of the spotted fever group would play a pivotal role in the origin of sexual reproduction [34]. Sterrer [35] also proposed that infection may have originated sex. It should be kept in mind that sexual reproduction is involved in parasite resistance (*vide infra*), and the parasite-host interface is frequently depicted as a red queen race [36, 37] but we would rather use the Sisyphus punishment metaphor. Sisyphus, founder and king of Corinth (or Ephyra as it was called in those days), was condemned in Tartarus to an eternity of rolling a boulder uphill then watching it roll back down again (the Sisyphus metaphor was previously used to depict the parasite-host interplay [38].) In these consortia both species strive to overcome the opponent strategies, keeping a dynamic adaptive balance. In this kind of dance, the cohabitants beings suffer adaptive epigenetic changes, that is, they never retain the same initial state as is expressed by the red queen metaphor.

Symbionts can cross continuum between commensals, mutualists, and pathogens in both directions. Also, parasitic and predatory life styles may be exchangeable and sometimes overlapping. Therefore, it is necessary to analyze the ecology of these symbiotic associations in a broader and dynamic form [2, 3].

Protozoa of the Phylum Apicomplexa such as *Toxoplasma gondii* and *Plasmodium* sp. present an organelle of vegetal origin, bounded by four membranes, called apicoplast, which presumably appeared from successive phagocytic processes involving an algal cell, before these protozoa adopted the parasitic way of life. Today these compartments constitute a chemotherapy target [39]. Similarly many invertebrate species including filarid nematode parasites such as *Wuchereria bancrofti* were infected by the bacterium *Wolbachia pipientis* and depend on the prokaryote for optimal reproduction [40], and this bacterium enhances the *Tribolium confusum* male beetle fertility [41].

Lichens are generally regarded as a classical example of mutualistic symbiosis, but the very description of the dualistic nature of lichens in 1869 ("*Die Flechten als Parasiten der Algen*", *Verhandlungen der Schweizerischen Naturforschenden Gesellschaft*—"The Lichens as *Parasites* of Algae", Proceedings of the Association of Swiss Natural Scientists) by the Swiss botanist Simon Schwendener (1829–1919) clearly declares the parasitic nature of the fungi and compares the fungus to a "spider" that slaughters its "victim" since the algae are penetrated by suctorial hyphae, termed haustoria. Actually the algal cells react to refrain the hyphal invasion, but are eventually killed during the fungi saprobic feeding. The axenic cultivation of each partner is not always simple, but the algae are more easily isolated and maintained (less dependent). These facts point to the parasitic nature of fungi. Phylogenetic studies using small subunit ribosomal DNA (SSU rDNA) indicate that lichen symbionts arose from parasitic fungi, and that there is no general evolutionary progression from parasitism to mutualism [42]. These authors propose that neither mutualism nor parasitism should be seen as endpoints in the evolution, and symbiosis and mutualism may give rise to parasitism, causing human diseases [5, 43]. Interestingly lichens are classified taxonomically, although made-up of two distinct species belonging to different kingdoms. Therefore, two species form a third one. That may be considered symbiogenesis. Similarly, the parasite-host biocartel is suggested to be the target of natural selection [20]. These consortia may arise from infection and/or predatism and the symbionts may become strictly interdependent. Animals that feed on algae, such as *Elysia viridis*, may preserve functioning chloroplasts and perform photosynthesis, and these organelles can even multiply in the marine ciliate *Mesodinium*. This kind of consortium is so common among marine zooplankton that it was asked why the cows are not green? [44]. Presumably because they are not translucent, but this problem was solved, at least in part, by the giant clam, the bivalve *Tridacna maxima*, by the development of hyaline organs, which scatter sunlight to the neighboring aggregated photosynthetic dinoflagellates or zooxantellae [45]. Most corals present zooxantellae, and these microorganisms play a pivotal role in the energy flow in the reefs, ecosystems of rich biodiversity.

Photosynthetic symbionts also nourish animal species such as *Hydra viridis* and *Convoluta roscoffensis.* Although the latter can be 15 mm-long, this turbellarian flatworm is devoid of functional pharynx and mouth, so, unable to perform heterotrophic nutrition, relies solely on their endosymbionts for survival [45]. In addition symbiotic microbes take part in blood meal digestion in the lice *Pediculus* sp., the triatomine bugs that transmit *Trypanosoma cruzi* among vertebrate hosts [45].

3. Parasitism Ecology

Since parasitology approaches the interactions among species and their environment (which may be our very bodies), it is considered an area of ecology [46]. In fact parasitism, as well as other types of ecological relations, is considered symbiotic consortia. Currently, the evolutionary biologists are beginning to recognize parasitism as an important creative force of biodiversity [47, 48].

When our societies moved from the hunter-gatherer way of life to farming, our populations reached much higher numbers, supporting many epidemics and keeping the sedentary humans in close contact with flock animals as well as with its feces, urine, tissues, and so forth [49]. Zoonosis such as plague, measles, tuberculosis, smallpox, leptospirosis, flu, and pertussis was acquired from domesticated/domiciliary animals such as rats, cows, pigs, chickens, ducks, and dogs. Infections from wild animals such as AIDS, schistosomiasis, leishmaniasis, tularemia, and many hemorrhagic fevers comprise important sources of emergent and reemergent diseases.

Parasites are continually exploring new available ecological niches in our organism and therefore originating emergent diseases. Protozoa such as *Giardia* are increasing its prevalence due to the vacant niches left by helminthes, as a result of the more efficient antihelminthic compounds used in both humans [50, 51] and domesticated animals

[52]. New vacant niches are eventually tried by parasites as in the reports of enteric parasitism of human beings by *Ancylostoma caninum* adult worms [53], as well as the human infection by monogenetic trypanosomatids [54, 55], normally found infecting insects.

Emergent diseases can both regulate the biodiversity of the wild life and threaten human beings [56] as well as other animals. Species invading new areas that leave parasites behind and encounter few new parasites can experience demographic overgrowth and become a pest [57]. In addition, invasive plant species that are more completely free from pathogens are more widely reported as harmful invaders of both agricultural and natural ecosystems. Therefore, invasive plants' impacts may be function of release from of natural enemies, including pathogens, causing their accumulation [58]. This indicates that parasite loss in animal and plant species invading new areas may confer significant competitive advantage, rendering them pests menacing the new ecosystem biodiversity. Zoonosis such as brucelosis, leptospirosis, salmonelosis, tuberculosis, and echinococcosis can cause considerable mortality and morbidity to human beings. Tuberculosis control in British flocks may have resulted in reduced risk of human infection [59] but the cowpox infection protected humans from smallpox, helping Sir Edward Jenner develop its vaccine ("*vacca*" = cow). It is worth noting that about two thirds of the emergent diseases had a zoonotic origin [58]. Parasitic diseases such as the different types of malaria had probably evolved from primate or avian infections [60, 61]. The recent outbreak of avian Influenza in Hong-Kong, with some serious and even fatal human cases, brings to mind the pandemic of Influenza that caused the death of more than 20 million people and had an avian origin, whereas the emergent H1N1 appears to be derived from swine infections [62].

The cuckoo birds (cuculids as *Cuculus canorus* L.) are neither endo-nor ecto-parasites and may be much larger than its hosts, but it has a typically parasitic behavior, in which the females lay eggs in the nests of other birds that will feed its offspring, often causing the death of the original younglings. Thus these species act as parasites and parasitoids or necrotrophs [63] at the same time. The parasitic infection, frequently deleterious at the individual level, can not only be advantageous for the populations, but even for the organisms individually [64]. These ecological relations can evolve into advantageous balances for both partners. Trophozoites of *Entamoeba gingivalis* and *Trichomonas tenax*, found in the human oral cavity, can help controlling the bacterial populations. These, in turn, produce proteins that function as "vaccines" against pathogenic bacteria. Some bacteria of our intestinal flora produce compounds useful for our metabolism, including glycosylhydrolases required for the optimum digestive system functioning. Enteric bacteria also induce and regulate the expression of many genes in the gut, such as fucosyl transferase enzyme characteristic of mouse intestinal villi [65], colipase, which is important in nutrient absorption, angiogenin-4, which helps to form blood vessels, and Small proline-rich protein 2A (Sprr2a), that fortify matrices that line the intestine [66–68]. The Gram-negative anaerobe *Bacteroides thetaiotaomicron* even promotes the development of the intestine's submucosal capillaries network [69–71]. Stappenbeck et al. [67] determined that the Paneth cells were required for the induction of the capillary network. These cells respond to *B. thetaiotaomicron* by transcribing the gene encoding angiogenin-4, a protein known to induce blood vessel formation [68–70]. Microbial community plays also a role in the development of Gut-associated Lymphoid Tissue (GALT) and, particularly, in the B immune system [71, 72]. There is growing recognition that microbial residents of the gastrointestinal tract might be important for both our understanding and treatment of obesity. However, many questions remain to be answered about the possible mechanisms [69, 70]. The intestinal flora can be affected by nematode infection as *Angiostrongylus costaricensis*, in mice [73].

The human gut may present 500–1000 bacterial species and the number of microorganisms associated to our mucosae can be tenfold higher than the total number of human cells (around ten trillion, 10^{13}, summing up nearly 1.5 kg microorganisms). The pattern of the gut microflora is peculiar for each individual [74]. Whenever imbalanced, the intestinal flora can be highly harmful and thousands of children die every year due to bacteria such as enteropathogenic or enterotoxigenic *Escherichia coli* [75–77] (it should be noted that multiple pathogens are often associates simultaneously [78].) However, the normal intestinal flora presenting bifidobacteria and lactobacilli has great metabolic importance, including the vitamin production. In general the excrements of an animal have more of certain vitamins than its food, a fact that explains why so many species carry out coprofagy. Vitamin K (menaquinones) is produced by intestinal bacteria such as *Bacteroides*, *Eubacterium*, *Propionibacterium*, *Fusobacterium*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Enterococcus*, *Streptococcus*, and others. The lack of these bacteria in neonates kept in incubators or subjected to antibiotic therapy may lead to the deficiency of menaquinone-dependent coagulation factors and hemorrhagic disorders.

The normal microflora can also confer resistance to infections by microbes such as *Salmonella*, *Yersinia*, *Listeria*, *Vibrio* spp., *Clostridium difficile* [72, 73], and even cancer [76]. Because of that, it is important to manage the flora composition to furnish therapeutic strategies using probiotic, prebiotic, and symbiotic approaches. In fact, the bacteria of the digestive tract are fundamental to the proper development of the mammalian immune system [72]. By helping in the development of the host immune system, the symbiotic bacteria are in fact aiding in the construction of their own niche, protecting themselves from both foreign competitors and possible detrimental attacks from their host [68]. Angiogenin-4, like other mouse and human angiogenin, is a member of the RNAse superfamily. In humans, two members of the RNAse family, eosinophil-derived neurotoxin and eosinophil cationic protein, exhibit antibacterial and antiviral activities [79, 80]. Angiogenin-4 was found to have microbicidal activity against the pathogenic Gram-positive bacteria *Enterococcus faecalis* and *Listeria monocytogenes*, reducing the populations of each of these bacteria

by more than 99% after just two hours of Angiogenin-4 exposure [71].

The lactic fermentation by *Lactobacillus acidophilus* keeps the acid pH of the vaginal mucosa, helping to protect the organism from pathogens such as *Trichomonas* and *Candida*. Thus the excessive hygienization, particularly employing bactericidal products, may not only predispose to other infections but also select drug-resistant phenotypes.

Pathogenic bacteria, such as *Pasteurella multocida*, inoculated by the bite of the Komodo dragons (*Varanus komodoensis*) help its hosts, killing evading preys by sepsis and subsequent bacteremia [81]. Analogously, the polyDNA viruses, inoculated together with eggs of the brachonid parasitoid wasps (*Cotesia* sp.) on the caterpillar (Lepidoptera), aid immunosuppressing and blocking the endocrine homeostasis of the larval host, granting the success of the hymenopteran. These necrotrophic parasitoids consume the less essential parts of its hosts, sparing particularly the nervous and circulatory systems, in order to allow a prolonged survival, consequently optimizing the development of the pathogens. Larvae of parasitoid insects such as *Nemeritis canescens* present an even more hateful behavior, using long jaws to attack individuals of the same species in a severe intraspecific competition [82].

Plants such as the *Ipomopsis aggregata* and *Gentianella campestris* attacked by herbivores or parasites present increased development [82]. The infection of *Spermophilus richardsonii* squirrels by the *Trypanosoma otospermophili* is normally harmful to hosts, but under a vitamin B6 (piridoxine)-deficient diet, the parasitized animals have increased survival and growth. Parasitic/cohabitant nematodes and cestodes can accumulate highly toxic heavy metals such as lead and cadmium, favoring the survival of the hosts in polluted environments [83–85]. Similarly, the plasmids are normally deleterious to the bacteria, but in the presence of antibiotics they can confer resistance [86].

Moreover, pathogens such as *Bacillus thuringiensis* and the parasitoid or necrotrophic hymenoptera are used in biological control of plagues [87]. In addition, parasites may be used in monitoring environmental pollution [88].

It is interesting to notice that mutualism can generate a great dependence between symbionts and often most of the endosymbiont DNA is transferred to the host nucleus. Lateral or trans-species gene transfers between prokaryotes and eukaryotes take place from organelle to nucleus or between diverse microbes [89]. Because of multiple genome fusions, the evolutionary trees or dendrograms obtained via conventional phylogenetic algorithms may be converted to "rings of life" explaining the origins of chimaeric eukaryotes [90]. More than 8% of the human genome had a retroviral origin [91] and maybe about 40 genes were transferred from bacteria [92]. Some of these retrotransposons may be associated with mammalian malignancies and autoimmune disorders [93], but can be otherwise beneficial (*vide infra* placental formation). Up to 17% of the *Escherichia coli* genome may had been transferred [94]. The parasitic protozoa *Trichomonas vaginalis*, *Entamoeba histolytica,* and *Giardia lamblia* also display bacterial genes [95–97].

Interestingly, horizontal DNA transfer may take place between a eukaryotic protozoan and mammalian and avian hosts. In infected human macrophage lines, rabbits, birds, and patients, the *Trypanosoma cruzi* minicircle sequences can integrate into the genome of the infected hosts [98–101]. This is the first evidence in the literature of lateral and vertical DNA transfer from a protozoan to host. Naturally occurring human infections by *T. cruzi* were documented, where mitochondrial minicircles integrated mainly into retrotransposable LINE-1 of various chromosomes [101]. The fact that integration occurred almost always into LINE-1 reveals another original finding, reproducing a secondary parasitic transfer into previous mobile genetic element (primary transfer) similar to what happens with SINEs. These are short sequences (typically 100–200 bp) that appear to be parasites of LINE elements. Alu elements, a type of SINE, comprise fully 10.6% of the draft sequence of the human genome [11]. Then Tc kDNA and SINE are hitchhikers of LINE-1. Probably, the occurrence of oxidative stress during the infection can act as a genotoxic stress that threatens the integrity of the genome, creating DNA double-strand breaks (DSB) that activate mobile elements, such LINE-1 [102]. Moreover, LINE-1 presents some sequence homologies with *T.cruzi* kDNA, favoring the secondary parasite insertion or transference. There is evidence that L1 retrotransposition may be involved in the origin of illegitimate rearrangements and may contribute to DSB repair and genomic instability in mice [99]. Other contribution of these original works on *T. cruzi* kDNA transfer to human genome, based on the endosymbiotic theory of mitochondria, is the rarity of the phenomenon of gene transfer from bacteria to eukaryotes via endosymbionts: fully 223 human genes were identified in the sequence that had the strongest similarity to bacterial genes, suggesting that the genes were imported from bacteria into vertebrate lineage. Reanalysis of these data left only about 40 genes with bacterial closest relatives, and it seems likely that this number will decline still further [11]. It is noteworthy that the kinetoplast is a portion of the single trypanosomatid mitochondrion, an organelle with presumed endosymbiotic origin. Different mechanisms have been hypothesized for the transfer of foreign DNA to eukaryotes, including phagocytosis, infection, and symbiosis [13].

We may presume that after acquiring Chagas disease in the Beagle voyage to South America (e.g., [102–105], but see also [106]) (there is a great debate about the chronic disease Darwin presented and there are several authors supporting the Chagas disease hypothesis (considering indicative symptoms and his own report of being attacked by triatomine bugs), whereas other possible maladies include anxiety, panic disorder with agoraphobia, hypochondria, arsenic poisoning, chronic allergy, Crohn's disease, lactose intolerance. The dispute would presumably only be solved with the PCR examination of his remains from the Westminster Abbey, which was not allowed.), Charles Darwin did not expect to become a trans-kingdom chimera, resultant of an evolutionary mechanism that only now begins to be understood.

Similarly, *Agrobacterium tumefaciens* DNA is transferred to vegetal host cells leading to the formation of tumor-like

galls [107], where the pathogen proliferates and this process may pose valuable biotechnological applications. These harmonious relations could have resulted from either predatism or parasitism events. Cultures of *Amoeba proteus* had been accidentally contaminated in 1966 with a bacterium that infected the protozoan with high virulence. In some years, however, this deleterious effect had been reduced and currently this protozoan depends on hosting the procaryote. Therefore, a parasitic symbiosis can originate a mutualistic one [2]. The opposite is also true as mutualists may become adversaries. The red billed oxpecker *Buphagus erythrorhynchus* removes arthropod ectoparasites such as ticks from large African mammals and even use their 360° sight to warn the host of advancing predators. Because of ingesting blood-engorged ticks, they gained a "vampirish taste" and learned to take blood from opened wounds, delaying healing. Similarly, the New Zealand parrot *Nestor notabilis* feeds on sheep ectoparasites, but whenever there is a food supply shortage, they feed on the host subcutaneous tissues [108]. Life is often not as harmonious as it seems. Organelles [109, 110] and gene *loci* [111] may compete within a single "organism" cells.

It is interesting to notice that in many cases of symbiosis between bacteria and protozoa, the prokaryotes are found within vacuoles that do not fuse with lysosomes. Similar nonfusogenic parasitophorous vacuoles are observed in infections by Mycobacteria and *Toxoplasma gondii*. The free-living amoebae *Acanthamoeba* spp. can harbor and even increase the virulence of prokaryotes such as *Legionella* spp. and Mycobacteria [112–114]. The protozoan host may protect *Legionella* from *Bdelovibrio* (a microbial parasitoid/predator of microbes) attack, but under stress conditions it may digest the hosted bacteria. Mycorrhizae may also act as either mutualistic or parasitic symbionts, depending on environmental conditions [2].

Protozoan parasites as *T. vaginalis* can be parasitized by the pathogen *Mycoplasma hominis* [115]. This phenomenon is called "hyperparasitism" [116]. Similarly *Ancylostoma* sp. may be infected by *Giardia lamblia* [117]. In the words of Swift [118]: "*So naturalists observe, A flea hath smaller fleas that on him prey; and these have smaller still to bite 'em; And so proceed ad infinitum.*"

Thus, the ecosystems today, like many societies, maybe somewhat like "dog-eat-dog" and the early ones were presumably a "microbe-eat-microbe" world.

The types of symbiosis are in constant transformation; virulence of the parasites is always varying according to the infection strategies and environment, including the host organism. Parasitism is not always harmful to the host and, depending on the environment conditions, it can be beneficial for both symbionts [119], giving rise to mutualism [120]. To be parasitized can confer the host a competitive advantage upon other more susceptible organisms. Just like the European settlers involuntary made use of its pathogens to decimate their opponents [49], *Paramecium tetraurelia* uses the taeniospiralis bacteria endosymbionts *Caedibacter* as an armament against susceptible strains. Similarly, *Parelaphostrongylus tenuis* worms were differentially advantageous in the population competition between cervids of the North America [119, 121]. The dynamics of competing species such as rabbits (*Oryctolagus cuniculus*) and hares (*Lepus europaeus*) is determined by pathogens such as myxoma virus and the helminth *Graphidium strigosum*. Likewise the competitive success between the coleopterans *Tribolium confusum* and *T. castaneum* is determined by presence of the sporozoan *Adelina tribolli* [122].

The parasites frequently cause greater morbidity and/or lethality in the new or accidental hosts than in the usual one, in which they have evolved (i.e., coevolved). Thus, domestic cats (*Felis catus*) infected with *T. gondii* can seriously threaten species that have remained geographically isolated as, for instance, wild beasts of Australia.

Protozoa [123], viruses [124], and bacteria [125–127] can be of therapeutical utility. In the past malaria was used as a sort of treatment, called "malariotherapy", for the neurosyphilis, that presented high mortality [128, 129]. Although the infection by *T. gondii* is associated with the formation of tumors, mainly in immunosupressed patients [130], the chronic infection can be antitumoral [131]. This parasite can even reverse the multidrug resistance of human and murine tumoral cells [132]. The medicinal leech (*Hirudo medicinalis*) that had been very useful in the past, saving many lives, has returned to use nowadays and helps preventing postsurgical venous congestion. However, these annelids require the aid of the bacterium *Aeromonas* for digesting blood meals [44]. This prokaryote in turn can provoke infections and even septicemia in the individuals submitted to the bleeding by the hirudine, being, therefore mutualistic for the invertebrate host and accidental pathogen for the vertebrate [133]. Other hematophagous parasites such as *Ancylostoma caninum* are studied aiming medical applications on the lucrative market of anticoagulants and can even have inhibitory effect upon human melanoma metastasis *in vivo* [134, 135].

The disequilibrium in the pathogen-host interface, which results from a long coevolution process, can generate pathological alterations such as allergies, asthma [136, 137], and autoimmune manifestations including type I diabetes [138–140] and systemic lupus erythematosus [141]. Some studies [142] indicate that the elimination of the intestinal helminthes promotes autoimmune diseases, such as ulcerative colitis, Crohn's disease, and perhaps multiple sclerosis [143], that remain rare in underdeveloped areas where intestinal parasites are highly prevalent. The chronic helminth infections can revert autoimmune disorders preventing Th1-driven self-aggressions [144, 145] by induction of antiinflammatory cytokines as IL-10 and TGF-β [146, 147] and protect humans from cerebral malaria [148, 149]. Thus these parasites may become commensals and/or mutualistic. In this regard about 80% of human B lymphocytes are associated to the intestinal mucosa and each meter of intestine produces about 0.8 g of IgA daily, approximately as much as a mammary gland during lactation. *Necator americanus* parasitism may be also converting into a mutualistic consortium with humans [150].

It is provoking to keep in mind that ecological terminology could be applied to man as well. Most authors consider the cuckoos as parasites since they are fed by other species,

but for feeding on milk, honey, eggs, and tissues of other "solicitous" species (e.g., bees, livestock and vegetables that are consumed without killing the individual plant) or slaving dogs, hawks and eagles for hunting, as well as pigs or dogs for finding truffles do not we parasitize them for their particular feeding abilities? Although it may seem heretical, are we not somewhat parasitic? Similarly, we do not think of phoresis when watching a Western movie cowboy riding a horse or of commensalism for having puppies or kittens at home.

4. History of Parasites

Symbiosis and Parasitism certainly preceded the rise of the first terrestrial organisms. The first evidence of bacterial parasitism is one billion years old [151].

The most primitive mycorrhizae have been found in fossil fungi dating from 460 million years ago, and 400 million years old lichen fossils were documented and it is worth mentioning that fungi important to humanity, such as *Penicillium* and *Aspergillus*, derived from lichen-forming ancestors [152].

The perception of parasitic disease and the attempts to control it may have outdated humankind. The medical use of medicinal plants by chimpanzees in the wild may have resulted in the very first origins of herbal medicine [153, *vide infra*].

The knowledge on Parasitology, particularly on larger parasites, is also antique. Egyptian papyri from the period 2,000–1,500 b. C., including the papyrus discovered in 1862 by Professor Georg Ebers in Tebas, dated of 1,500 b. C., describe parasitism by intestinal helminthes and *Schistosoma haematobium* [154]. Manuscripts found in India and China, dating about 2,500 and 3,000 b. C., respectively, describe observations of parasitic diseases and presumably comprise the earliest medical texts known. Hippocrates (460–375 b. C.) described aspects of the malaria and hydatidosis. Hippocrates and Aristotle (348–372 b. C.) named the intestinal *Teaniae* cestodes (taenia (Gr.) *ταινία* = ribbon), but the *Taenia* species that infect humans were only described in detail in 1758 by Linnaeus.

Biblical texts presumably describe the Guinea or Medina worms *Dracunculus medinensis* as "fiery serpents". It states that the Lord said to Moses "Make a fiery serpent and set it on a pole, and it shall be that everyone who is bitten, when he looks at it, shall live" (Numbers XXI, 6–8). According to a number of authors [45, 155], the historical removal of this parasitic nematode, with the aid of a wooden stick, may have originated the pictorial representation of the medicine symbol, the Aesculapius staff, the caduceus.

The history of the discoveries in Parasitology, particularly in the field of Protozoology, was usually related to the development of light and electron microscopy techniques. The first cells seen by microscopy were not the cellulosic cell walls ((Latin) "*cellula*" = small cell or chamber) observed in the cork by Robert Hook, but bacteria and trophozoites of *Giardia lamblia* that Antoni van Leeuwenhoek (1632–1723), a skillful and inquiring draping shop owner in Delft, Netherlands, collected from his own feces and those of his horse. Leeuwenhoek also observed *Opalina*, *Nyctotherus* and oocysts of rabbit coccid(s). At this time, microscopy was considered a hobby rather than a *bona fide* scientific activity and the embryos of Parasitology, Microscopy, and Cell Biology were twin born. It is important to point out that parasites comprise valuable experimental models in different fields of modern Biology. The use of microorganisms to approach fundamental aspects of Cell Biology has been termed "Cellular Microbiology" [156] and many discoveries were made via studies on parasitic protozoa [157–159]. Discoveries such as meiosis, continuity of chromosomes, cytochromes and electron transport system, among many others, were made on parasite-focusing studies [155]. Viral infections prompted the development of cloning and transfection techniques leading to the birth of Biotechnology and Molecular Biology.

5. Parasites of History

> "*Ingenuity, knowledge, and organization alter but cannot cancel humanity's vulnerability to invasion by parasitic forms of life. Infectious disease which antedated the emergence of humankind will last as long as humanity itself, and will surely remain, as it has been hitherto, one of the fundamental parameters and determinants of human history.*" McNeill in *Plagues and Peoples*, 1976 [160].

Parasitic and infectious diseases have played a profound role in the outcome of wars, invasions, and migrations and in the development of numerous regions of the globe, thus determining the course of history [161, 162]. The most notorious conqueror of history, Alexander the Great died at the age of 32 following a two-week febrile illness. Speculated causes of his death have included poisoning, assassination, and a number of infectious diseases including typhoid fever, malaria [163], or West Nile Virus [164], among other (not so great) hypothesized etiologies.

It is noteworthy that sometimes morbidity may be more decisive in war outcome than lethality. A sick or slowly dying soldier will have to be cared for by the others often expending scarce resources and sick men maybe more vulnerable to die from wounding. That is why biological weapons are frequently intended to debilitate the health (keeping the victim alive) rather than killing. Numerous pathogens have been tested and used as biological weapons and the consequences of their use (including bioterrorism) are notorious. Interestingly, as for parasitic diseases resistance (*vide infra*), the natural selection of immune variants in our progeny may comprise a pivotal defense against bioweapons [165].

Parasitic and/or infectious diseases are responsible for more deaths than disasters, catastrophes, and wars altogether. The Sumatra 2004 Tsunami claimed the life of about 225000, people and the Japan 2011 Earthquake and Tsunami death toll exceeds 10000, whereas malaria causes nearly one million deaths annually (WHO, World malaria report 2010) (about 781,000 deaths worldwide were estimated in 20009, but a remarkable subnotification should be considered in many

regions World Malaria Report, 2010—World Health Organization, available at http://www.who.int/malaria/world_malaria_report_2010/en/index.html.), amazingly not reaching the headlines, not to mention posttsunami malaria and dengue or other slaughtering infections such as tuberculosis.

The bubonic plague, also known as black death, transmitted by rodents through *Xenopsylla cheopis* fleas, claimed about 25 million lives in Europe, corresponding to approximately half the deaths in II World War. The death of 1/4 of the European population including 1/3 of the English, resulted in deep transformations to the society. The decline of the feudal system was promoted by the death of millions causing the shortage of available labor and land under cultivation began to fall. Therefore, local lords and aristocracy began to lose wealth and power. After that Europe was ready to enter the renaissance [166, 167].

Even the Church paid its tribute. At least 6 popes, cardinals, and other clergymen have died due to malaria, then also called Roman fever. The death of priests forced the Vatican to speed up the ordainment and even women, whose participation in the Church activities had often been limited, had been authorized to give the last rites to the uncountable dying. Because of the high plague mortality in Southern France, the Pope consecrated the Rhône River so that bodies thrown in its stream could have a "Christian burial" [166]. Mortality among clergy members comprised a great embarrassment (Christianity sense of guilt is more psychologically damaging than the Muslim "insh Allah" [Arabic] God willing [168].) since diseases were then frequently seen as God's wrath sent as punishment for sin and immoral behavior. Religious fanaticism grew and gave rise to sects of Flagellants. Flagellants wandered throughout Europe whipping themselves, recruiting followers, urging people to be penitent and spreading plague during their wanderings. According to several authors, the rise in religious extremism hastened the splintering of the Catholic Church, strengthening of the Reformist movement and the growth of Protestantism as an alternate belief system [167, 168] (according to McGrew (1960) [168], the appearance of the reform bill during the 1832 cholera pandemic was not accidental. The strike of cholera in Europe was associated to a stern abomination between the classes. In France, as well as other countries cholera was particularly common among the working classes, which believed that the bourgeoisie conspiring with the authorities was poisoning them for Malthusian reasons. This feeling spread through Europe as rapidly as the disease *per se*.).

Malaria killed emperors and Pontiffs. The knowledge of its treatment had a strategic role for the Church. For a long time, the quinine obtained by *Cinchona officinalis* cork, was a secret kept by the Jesuits and in the XVIII century Protestants refused to recognize its antimalarial properties, resulting in needless suffering and deaths [169].

The 1918 Influenza pandemics killed 21 million people, being responsible for three times more deaths than the World War I and almost the same as World War II. Interestingly it is believed that about 30 million people succumbed to typhus during World War I. It is noteworthy that Influenza killed so many people in only one year [170].

Comparatively tuberculosis killed 2- or 10-fold more people than the World Wars II and I, respectively. As we can see, humankind has suffered much more from parasitic and infectious diseases than from political and social conflicts worldwide. In the words of Sir William Osler (1849–1919) "Humanity has but three great enemies: *fever*, *famine* and *war*; of these by far the greatest, by far the most terrible, is *fever*". These enemies correspond to the knights of the apocalypse who lead to the fourth knight: "death". Rather than independent, these flagella of mankind are intimately linked. War produces hunger and pestilence. These last ones, in turn dictate the routes of development and the outcome of wars [161].

Colonizers and priests, besides swords and crucifixes, brought pathogens, such as smallpox virus, which devastated indigenous nations in the Americas. A deliberate attempt to cause epidemics occurred when the British troops supplied Amerindians with blankets used by smallpox victims [169]. In past, war outcomes were not determined solely by the best tactics or weaponry, but often by the nastier pathogens [49]. During Mexico invasion in 1519, 2/3 of the Cortés Spaniards were killed by the belligerent Aztecs, but afterwards about 12 million Aztecs including the Emperor Cuitláhuac perished from smallpox. Similarly, before the conquest of Peru by Pizarro in 1531, much of the Inca population including the Emperor Huayna Capac and his successor were killed by smallpox. It is estimated that the New World Indian population declined as much as 95% in the years following Columbus's arrival [49].

The decay of the powerful Roman Empire may have been related to malaria ("*mala*" + "*aria*" = bad air; flowers at the lapel or by the windows and doors were introduced because of the belief that diseases were transmitted by the air). During the first century b. C. the agrarian districts at the periphery of Rome witnessed the appearance of a malaria epidemic, then called "Roman fever", that lasted 500 years. Mortality was so high among children that many men were brought from German tribes to compose the fearsome Roman centuriae [166].

The occupation of great part of Africa and India by European colonizers was impaired by the severe flagellum of diseases such as malaria, cholera, and yellow fever. Some African leaders had considered malaria a protection against the European invader [45]. The Portuguese expression "de cabo a rabo" very common in Brazil, meant South to North Africa (from *Cape* Town, South Africa, to *Rabat*, Morrocco), which was not entirely dominated because of the "microbial soldiers". Similarly, *Toxoplasma gondii* was suggested to be useful to protect our planet from an eventual extraterrestrial invasion and therefore it should be preserved as a possible interplanetary "weapon" [171].

Besides the conspicuous effect of the high mortality due to several infections, parasitic diseases such as ancylostomosis may cause insidious and cumulative morbidity producing a great impact on the host, at both individual and population levels. The Caucasoid southern USA population was considered indolent, irresponsible, and even assigned as "poor white trash" [45]. In fact, most of these people suffered from hookworm infection by *Necator americanus* (*necator* (Latin) = killer). In this regard the Brazilian countryside

man, skillfully depicted in the Monteiro Lobato, famous Brazilian writer, character Jéca Tatú, was considered lazy and was in fact ancylostomotic [172]. Southern US populations were also afflicted by typhus and malaria.

The outcome of the American civil war should not come as a surprise. The number of soldiers killed in combat or from wounds was about 110,000, whereas about 224,000 people died from diseases. It is estimated that diarrhea and typhoid killed 35,127 and 29,336 Union Soldiers, respectively. Another 14,379 died of malaria and 9,431 of dysentery. 7,058 troops succumbed to smallpox and 5,177 were defeated by measles (war Casualties—Spartacus Educational http://www.spartacus.schoolnet.co.uk/USACWcasualties.htm).

Similarly, 20,356 French died from wounds in the Crimean war, whereas 49,815 died from diseases and 196,430 were sick. Typhus and dysentery also affected French soldiers during the invasion of Russia by Napoleon and after the battle of Ostrowo, 80,000 out of 450,000 men were sick. Perhaps more important than the participation of the so-called "Jack Frost" or "general winter" in Waterloo, had been the attack of the "general *Rickettsia prowazekii*", which claimed the life of numerous men. From the 460,000 troops that marched from France in 1812, only 6,000 returned from the four-month stay in Moscow [162].

Indirectly parasites also determined the trends of war. Sex evolved because of parasitic infections (*vide infra*) and the struggle for women often caused conflicts among people. From the mythological war of Troy, a dispute for Helen, to the nowadays Yanomami in Venezuela, men fight to get women, as many other animals do [37].

In the past, infections traveled by train or ship together with people or as *Yersinia* in the fleas on furs or riding clandestine rodents. Currently, clandestine mosquitoes carrying clandestine parasites take few hours' airplane flights to reach distant continents. For this reason, in the past, the epidemics followed the maritime routes or railways, but nowadays cases of different infections in the neighborhoods of the airports in nonendemic areas are common. The infection can take place in the very airplanes, as in the cases of malaria among passengers traveling between Switzerland and Germany, in an aircraft coming of the South America. The air-conditioning system of the commercial aircrafts also propitiates efficient virus propagation. The recent, simultaneous incidence of severe acute respiratory syndrome (SARS) in China and Canada, clearly demonstrate that, in a globalizing world, we need a globalized epidemiology [173].

The parasitism of vegetal organisms also had a great impact in the history of the humanity. The Peruvian fungus *Phytophtora infestans* that infects potatoes provoked a disaster in European economy in the period of 1845-1846. The hunger was so devastating, that more than a million out of about eight million Irish starved to death. This fact caused the Irish Diaspora and thousands of people migrated for other countries such as the United States. Among the moving families, were the Fitzgerald and the Kennedy [161]. The political and historical implications of the mentioned parasitism are obvious. Another plant pathogen is responsible for the tea drinking tradition in England, where tea and coffee used to be consumed in approximately equal amounts up to the middle IX century. The parasitic fungus *Hemileia vastatrix* drastically reduced the coffee production in countries such as Ceylon and then Brazil became the main coffee-producing country in the world. This was interesting for the Brazilian economy, but many British had to change their beverage habits [45].

Infections in vegetables by the ergot fungus *Claviceps purpurea* could provoke the ergotism known as "Saint Antonio's fire" in the Middle Ages. It could have caused the strange behavior of young girls leading to a brutal witch-hunt in 1692 in the city of Salem, USA ([174, 175], but see also [176]). After the trial, 20 innocent, ergot-intoxicated people were executed for the crime of practicing witchcraft. Effects of mycotoxins are usually more pronounced in children and the Biblical story of death of first-born (that received double rations) in Egypt during captivity of Hebrews (the tenth plague abated on the Egyptians), also may be related to the presence of fungi on food, since stored grain would go moldy and presented deadly mycotoxins. Interestingly, other plagues were possibly related to an outbreak of a vector-born disease [177]. The 3rd (lice) and the 4th (flies or gnats) plagues may be involved in the transmission of microorganisms, and the 5th (livestock struck by pestilence or murrain) and 6th (boils and blains that break in sores on man and beast) ones may comprise the veterinary and human infections, respectively. The epidemiological hypothesis for the plagues is based on an ecological disequilibrium leading to algae proliferation (1st plague) with the consequent alteration in the populations and behavior of frogs (2nd plague) and insects (often preyed by frogs), some of which vectors of infectious diseases. Unfortunately these facts are generally either obscured or overviewed in history books. Otherwise the governments might be more concerned about infections.

6. Parasitism and Society

> "*The role of the infinitely small is infinitely large*"
> Louis Pasteur.

The impact of parasitic/infectious diseases to our contemporaneous society can be demonstrated by the social and economic losses due to about two-three million deaths worldwide every year and nearly 3/4 of the human population is infected by some sort of pathogen. It is estimated that 500 million people are infected with *Plasmodium*, resulting in over 2,000 deaths every day (98 deaths/h) mostly among African children (WHO, 2010). We usually underestimate the effects of parasites on the course of human evolution. The increased resistance to malaria, largely due to the higher frequencies of hemoglobin disorders such as sickle cell anemia or glucose-6-phosphate dehydrogenase deficiency in African populations, where the malaria is endemic, clearly demonstrates that primarily deleterious mutations may be favored in response to the parasite stress. Thus in the parasite-host interface, both sides maybe submitted to intense selective pressures.

Humans have developed "weapons" such as the behavioral strategies (i.e., not relying on mutations favored by

natural selection) including the use of natural or synthetic antiparasitic substances. However, as parasites generally present shorter generation times, they can evolve and adapt more quickly, thus making use of a much diversified, renewable and efficient "arsenal" of virulence factors. Therefore, it seems that we will never get rid of pathogens and always be faced with this "arms race". Interestingly, the use of natural products to fight parasitic diseases, that is, *therapy*, may have preceded human beings. Chimps use plants such as such as *Aspilia ossabicensis*, *Aneilema aequinoctiale,* and *Vernonia amygdalina* to fight intestinal parasites [178–180]. Even foraging mammals may use plants to fight parasites [181]. These animals may avoid diseases by keeping distant from feces, but parasitic nematodes take a ride in the spores of fungi which are propelled to several meters away by bursting sporangia.

Readily treatable diseases such as roundworm and hookworm infections affect one billion and 900 million people, respectively. These parasitic nematodes may be responsible for, respectively, 10,000 and 60,000 annual deaths, mainly in the poor countries (*vide infra*). Parasitic infections can drastically reduce the physical and mental development of children, as well as the productivity of adults [182]. Low physical and cognitive capacities render parasitized people less proficient and thus restricted to less remunerated occupations. It reduces their access to good health and sanitary conditions, increasing risk of new infections, maintaining a cruel vicious cycle of social exclusion. Infectious diseases are responsible for about 80% of the deaths in underdeveloped countries, but present a minor importance for the public health of the present days First World nations [183]. The pharmaceutical industries, therefore, have had little concern for the development of new drugs for the treatment of these diseases. Although about 90% of human diseases are caused by infective agents, less than 5% of the research and development are dedicated to the resolution of these infections [184]. Less than 1% of the drugs incorporated in our therapeutic arsenal in the last decade are directed to tropical diseases [185]. Without the involvement of the public sector, generating social justice and the ultimate escape from misery it will not be possible to save our "human capital" [186, 187]. The little interest on the so-called diseases of the poverty or "neglected diseases" is astonishing, considering that one out of five people in the world lives in conditions of absolute poverty. There is evidence that poverty diseases and its research is neglected even by reputed medical journals [188].

Parasitology is of great relevance particularly, but not only, to the countries of the Third World, where more than a million people die annually victims of infections, being half of that due to infantile malaria. It is estimated that far more than 50% of the world mortality and morbidity is due to infectious and/or parasitic diseases accounting for 16 million annual deaths. For comparative purpose, cancer kills approximately six million people annually. In this regard, at least 15% of the human tumors have a viral etiology [189] and parasites including helminthes may also promote tumors [190]. Besides the spoliation frequently associated to parasitism, *Trichomonas vaginalis*, *Schistosoma haematobium,* and *S. mansoni* can induce cancer in infected tissues. *T. vaginalis* infection, the main nonviral sexually transmitted disease in humans, favors the transmission of HIV and human papilloma virus [191]. However, parasitic infections remain considered diseases of poverty and thus receive less attention from governments and industries than less prevalent, but profitable, disorders, with much smaller social impact.

Although widely employed, the expression "tropical medicine" is not conceptually accurate, since it focuses illnesses not restricted to the tropics. In the past, the so-called tropical diseases as plague, typhoid fever, and malaria were found throughout the world. Malaria has been a serious endemic disease in countries such as United States, Canada, and England. Several Shakespeare plays mention malaria, then called "ague", which comprised an infection with high lethality in England until the end of the XVIII century. The first quinine trials were carried out in Essex, about 50 km from the center of London [192]. The current prevalence of many infections reflects the efficiency of the hygiene conditions and control measures rather than climatic or geographic properties [193]. However, the existing climatic and sanitary conditions in the majority of the tropical countries favor the advance of disorders of infectious and parasitic etiologies. The global warming by the emission of gases, such as carbon dioxide, is promoting the greenhouse effect or global warming. This phenomenon can increase the distribution of mosquitoes such as *Anopheles* sp., *Culex* sp., and *Aedes* sp., enhancing the incidence of malaria, dengue, and yellow fever, among other infections [194].

The unplanned urban superpopulation associated to the frequently poor sanitary and housing conditions in underdeveloped countries (a large extent in tropical climate), favors the proliferation of pathogens and vectors, constituting an important factor in the high prevalences of parasitic diseases in these regions. Due to the environmental impact of society, typically rural diseases such as schistosomiasis, malaria, and visceral leishmaniasis are getting urbanized in different countries.

About 45% of the world population is concentrated in the urban environment, whereas in Brazil this percentage may reach 80% [195]. Dense agglomerations strongly favor the transmission of infectious agents, especially considering that sanitary conditions of the majority of the cities in the Third World remain as in Biblical or medieval times and, therefore, besides the emerging diseases, we are still faced with old-fashioned infections as leprosy, tuberculosis, dengue, and others.

The indiscriminate use of microbicidal and insecticidal compounds (in medicines and hygiene products) has prompted the appearance of resistant organisms, hindering therapeutical and prophylactic measures. Organ and tissue transplants or blood transfusions facilitate the transmission of parasites such as *Toxoplasma gondii*, *Plasmodium* sp. *Trypanosoma cruzi*, *Leishmania* sp. as well as numerous viral infections [196–198].

Besides the direct impact of the parasitic infections on host health, parasitism can subvert immune mechanisms

critical to resistance to other infections. African regions with high incidences of intestinal nematodes, such as roundworm, whipworm, and hookworm, present higher prevalence and severity of tuberculosis cases and HIV infections [199]. This effect cannot be attributed to the nutritional status. It is noteworthy that AIDS pandemic promotes the spread of opportunistic parasites such as *Toxoplasma gondii*, *Pneumocystis carinii*, *Cryptosporidium parvum*, *Leishmania* sp., and many others, which are often called opportunistic agents. However, the classification of such microorganisms as "opportunistic" was often inadequate and misleading, as some so-called "opportunistic" microorganisms can also cause disease in normal hosts [17, 200].

7. Parasites: Singular and Prevailing Driving Forces in the Evolution of Man and Society

> *"We cannot fathom the marvellous complexity of an organic being; but on the hypothesis here advanced this complexity is much increased. Each living creature must be looked at as a microcosm—a little universe, formed of a host of self-propagating organisms, inconceivably minute and as numerous as the stars in heaven."* Charles Darwin.

Microorganisms played a major role in the evolution of higher organisms, including the wise, "modestly" and redundantly self-denominated *Homo sapiens sapiens*. Curiously the human body presents at least an order of magnitude more bacterial than human cells [201]. The "human wildlife" may be comprised by 10 to 100 million species living in and on us [11]. We resemble "microbial quarters" rather than perfectly designed masterpieces.

It is presently clear that *Helicobacter pylori* can cause the gastritis, peptic ulcers, and even neoplasy. An increasing number of chronic nosologic entities believed to have genetic and/or environmental nature as acute rheumatic arthritis, arteriosclerosis, multiple sclerosis, schizophrenia and Alzheimer's [202] had been implicated. Even obesity may have an infective etiology (infectobesity). At least eight types of obesity-inducing virus (*in animal*: canine distemper virus, Rous-associated virus type 7, Borna disease virus, scrapie agent, SMAM-1 aviary adenovirus; *in Human*: adenoviruses 5, 36, 37) have been identified in animals, especially poultry and mice. Studies on humans are far less convincing; however, two adenoviruses, Ad-36 and SMAM-1, have shown adipogenic properties [203, 204]. In addition to the viruses, *Chlamydophila pneumonia* (*Chlamydia pneumonia*) and the gut microbiota can contribute to regulation of fat storages [70, 205]. Viral infections also shaped human evolution and history [206, 207]. Interestingly a protein of a viral origin may mediate sperm-egg fusion in mammals [208, 209], and endogenous retroviruses play a pivotal role in both placental morphogenesis and suppressing the maternal immune response against the embryo [210–212]. These retrotransposon-like sequences which may account for over 40% of the human genome and had been termed "infectrons" [7, 13] as mentioned above. The retroelements profoundly manipulated our genetic inheritance, thus were termed "human genome sculptors" [213].

Different symptoms of infection may be a sophisticated physiological manipulation to promote parasite dispersal. Coughing, sneezing, and diarrhea effectively mediate the shedding and environmental dispersal of respiratory or enteric pathogens, respectively. Biting may spread the rabies virus from an infected animal. Even the immune response may be exploited in the parasite life cycles. Hypersensitivity reactions lead to scratching and scarifications permitting pathogen invasion of subcutaneous tissues and spread. The discomfort produced in the immune reaction to *Dracunculus medinensis* leads the definitive host to immerse the affected limb in ponds, where the juvenile form of the parasite can encounter offspring-copepod host [155]. Fever will promote vector-borne diseases via enhanced hematophagy by mosquitoes by both rising of temperature (thermotropism) and sweating which is associated with the typical smell (chemotropism) produced by the activity of skin-dwelling microorganisms such as staphylococci and *Corynebacterium*.

The paresis and allopecia produced on mammals with visceral leishmaniasis may be useful for "serving" the sand fly a parasite-supplemented blood meal. A hairless, nonmoving animal will be far more suitable for insect "safe landing" and feeding. The protozoan even damages the insect gut enhancing its appetite [214]. Similarly, the bird parasite *Trichomonas gallinae*, usually transmitted via drinking water, produces polydipsia (chronic excessive thirst) in the infected animal.

Schistosoma sp. eggs are laid within the vascular compartment and require the host immune response to reach the environment via feces or urine. Strikingly, *S. mansoni* uses development signals from hepatic $CD4^+$ lymphocytes [215].

Ectoparasitism and parasite cleaning produced numerous mutualistic consortia among many vertebrate and invertebrate species. The act of grooming ecto-parasites assumed a very important social function among primates. Inducing endorphin release, this behavior can reduce tensions and therefore lead to intraspecific cooperation, ultimately promoting social behavior [216].

Parasites may influence the host behavior in order to facilitate their reproduction and dispersal. Acanthocephala can reverse the negative phototropism of Isopoda and Amphipoda crustaceans rendering them more easily predated by the birds, which will then harbor the adult worms [217]. Similarly, the lancet fluke *Dicrocoelium dendriticum* programs ants to offer themselves as food for grazing herbivores such as lambs and cows [216].

The behavior of vertebrates may also be affected. Trematodes may drastically alter the swimming pattern of killifishes, which may expose their shinny bellies on the water surface and therefore are easily engulfed by sea birds [216]. One of the most amazing "alien-driven" responses takes place in rodents. These little quick animals are always alert and pragmatically scared by the presence (and smell) of feline predators. Nevertheless, when they are infected by

Toxoplasma gondii they not only cease to flee but also may challenge death by approaching a cat, the protozoan definitive host. *Toxoplasma* may similarly modify human behavior [218]. Infected people may become more agitated, less neophobic, aggressive and more prone to take part in traffic accidents [219]. *Toxoplasma*-infected women show different personality profiles tend to be less moralistic, disregarding rules, and taking more risks [219]. It was even anecdotally suggested that the typical attitude of the British and the French might be due, at least in part, to parasitism by *T. gondii* since in the former population the seroprevalence is under 20%, whereas in the latter it is over 80% of the population [220]. Xenophobia and racial discrimination may have risen as primitive strategies to avoid alien parasites within alien populations.

Pathogen resistance may have promoted kin altruism [221] and kin selection may have played a role in the origin of monogamy [222]. In social insects, especially ants, it is likely that variation in polygyny is primarily driven by factors other than those responsible for variation in polyandry, but both may be under selection by parasitism in more complex ways than generally appreciated until now, requiring further thorough investigation [223]. Among humans, the more polygamous a society, the greater its parasite burden, though meaning of this observation is not clear [37, 224, 225]. This may be due at least in part to the higher male susceptibility to infections [226, 227]. In polygamous animals, the variation in number of sex partners is greater among members of one sex than the other [228]. Therefore, if a sexually transmitted parasite can recognize the sex of its host, the reproductive rate of that parasite is expected to be greater in the sex that potentially has the larger number of partners; given the preponderance of polygyny among animals [228], sexually transmitted parasites should be more virulent in males than in females [229]. Sex differences in parasite infection rates, intensities, or population patterns are common in a wide range of taxa [229]. Ecological view usually postulated that sex differences in parasite infestation were due to differences in the life histories of males and females, with one sex perhaps eating more or different prey, and thus ingesting more infective stages, or perhaps inhabiting an area with greater tendency to harbor parasites, such as a stream margin [229]. Another tradition explained the sex differences to parasite infections based on the sexual dimorphism that are important to the host-parasite relationship, such as endocrine-immune interactions and mortality or senescence rates [229–234]. Some of the most clinically important parasitic diseases of humans, including ascariasis, leishmaniasis, malaria, schistosomiasis, trypanosomiasis, and a variety of helminthes show significant differences in male and female infection rates [229]. Hormones in general and sex steroids specifically may affect the genes, immune responses, and behaviors that influence susceptibility and resistance to infection [230]. In addition to sex steroids, several other steroid hormones, including glucocorticoids, pituitary-derived peptide hormones, such as follicle-stimulating hormone, luteinizing hormone, adrenocorticotropin hormone, and prolactin may influence sex differences in infection [230].

8. Conclusion and Perspectives

There is a pressing need of "change the look" at the cohabitation phenomenon between humans and parasites. A better cohabitation will depend on a better comprehension of the relational mechanisms between cohabitant(s) and the host(s). The following papers and books will contribute to change the look perspective at parasitic phenomenon: Ferreira [46], Trager [234], Brooks & McLennan [235], Sapp [3], Lenzi et al. [236], Paracer & Ahmadjian [237], Tosta [7, 13], Bush et al. [238], Margulis & Sagan [239], Bushman [11], Moore [217], Combe [5] and Combes [240], Lenzi & Vannier-Santos [6], Ulvestad [38], Rollinson & Hay [241], Ward [242], Jablonka & Lamb [243].

The new look guides, according to Carlos Eduardo Tosta (personal communication), to switch the current paradigm to a new one.

The current paradigm presents the following concepts: (a) infectious agents are enemies that should be destroyed; (b) the function of the immune system is to destroy the infectious agents and to maintain the organism free of them; (c) genomes of the host and the infectious agents are closed structures without relationship among them; (d) the vaccines should maintain the organism free from infectious agents; (e) theoretical base: *attrition* and *destruction*.

Otherwise, the new paradigm proposes: (a) "infectious agents" [*cohabitant/symbiont*] are coevolutionary partners; (b) the function of the immune system is to maintain the molecular individuality of the organism and to promote its adaptation to the infectious agents; (c) the host and infectious agents genomes are connected by mutual activation and infectrons exchange [they live in the pangenome], and they are organized in *coevolutionary networks*; (d) vaccines should contribute to the best adaptation of the "infectious agents" [cohabitant/symbiont] to the organism*: adaptive vaccines*; (e) theoretical base: *adaptation* and *living together* [=*cohabitation*].

In conclusion, the cohabitation of cohabitant (symbiont) and cohabited being raises the emergency of a new and *complex adaptive system: the parasitized-host* [244]. There is no parasite without host and *vice versa*. The future understanding of the parasitism phenomenon (cohabitation) through the Systems Biology perspective, will need the cooperation among parasitologists, infectologists, immunologists, pathologists, experimental and theoretical biologists, ecologists, mathematicians, physicists, experts in artificial intelligence and in computation science, and others [245, 246]. There is still a long way ahead before we can understand the language of the parasites: *The parascript*. The parascript was formulated by Harold W. Manter, and referred by Brooks and McLennan [235]: "*Parasites-furnish information about present-day habits and ecology of their individual hosts. These same parasites also hold promise of telling us something about host and geographical connections of long ago. They are simultaneously the product of an immediate environment and of a long ancestry reflecting associations of millions years. The messages they carry are thus always bilingual and usually garbled. Eventually there may be enough pieces to form a meaningful language which could be called*

parascript—the language of parasites, which tells of themselves and their hosts both of today and yesteryear."

> "*We can be born 100% human, but we will die 90% bacterial—a true complex organism.*" *[247]*.
>
> "*The cohabitation symbiont-cohabited host is an unfinished symphony, so fascinating as Schubert's unfinished symphony. It never will have an end. This symphony is continuously played in two different tones: one is harmonious (Gaia tone) [248] and the other, noxious and dramatic (Medea tone) [242].*"

Acknowledgments

This paper was kindly revised by Drs. Vivian Rumjanek, Jane Arnt Lenzi, Eline Deccache-Maia, Aline Fagundes, and Ana Lúcia Matos. The authors are CNPq fellows recipient of grants from CNPq, FIOCRUZ, PP-SUS, CAPES, FAPESB, and INCT-INPeTAm/CNPq/MCT.

References

[1] P. Hayward, "Darwin's charm: charm (n), a group of finches," *The Lancet*, vol. 372, supplement 1, pp. S50–S56, 2008.

[2] T. Wakeford, *Liaisons of Life: From Hornworts to Hippos, How the Unassuming Microbe has Driven Evolution*, John Wiley & Sons, New York, NY, USA, 2001.

[3] J. Sapp, *Evolution by Association: A History of Symbiosis*, Oxford University Press, New York, NY, USA, 1994.

[4] P. W. Price, "The web of life: development over 3.8 billion years of trophic relationships," in *Symbiosis as a Source of Evolutionary Innovation: Speciation and Morphogenesis*, L. Margulis and R. Fester, Eds., pp. 262–272, The MIT Press, Cambridge, MA, USA, 1991.

[5] C. Combe, *The Art of Being a Parasite*, The University of Chicago Press, Chicago, Ill, USA, 2005.

[6] H. L. Lenzi and M. A. Vannier-Santos, "Parasite-host interface: cohabitology—a different look at the parasitism phenomenon," in *Dinâmica das Doenças Infecciosas e Parasitárias*, J. R. Coura, Ed., pp. 19–44, Guanabara-Koogan, Rio de Janeiro, Brazil, 2005.

[7] C. E. Tosta, "Coevolutionary networks: a novel approach to understanding the ralationships of humans with the infectious agents," *Memórias do Instituto Oswaldo Cruz*, vol. 96, no. 3, pp. 415–425, 2001.

[8] L. Margulis and R. Fester, "Symbiosis as a source of evolutionary innovation: speciation and morphogenesis," *The MIT Press*, 1991.

[9] D. A. Relman, "The human body as microbial observatory," *Nature Genetics*, vol. 30, no. 2, pp. 131–133, 2002.

[10] V. V. Tetz, "The pangenome concept: a unifying view of genetic information," *Medical Science Monitor*, vol. 11, no. 7, pp. HY24–HY29, 2005.

[11] F. Bushman, *Lateral DNA Transfer: Mechanisms and Consequences*, Harbor Laboratory Press, New York, NY, USA, 2002.

[12] J. P. Gogarten, "Gene transfer: gene swapping craze reaches eukaryotes," *Current Biology*, vol. 13, no. 2, pp. R53–R54, 2003.

[13] C. E. Tosta, "Infectrons and coevolution," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 34, no. 1, pp. 1–3, 2001.

[14] H. H. Kazazian Jr., "L1 retrotransposons shape the mammalian genome," *Science*, vol. 289, no. 5482, pp. 1152–1153, 2000.

[15] H. H. Kazazian Jr., "Mobile elements: drivers of genome evolution," *Science*, vol. 303, no. 5664, pp. 1626–1632, 2004.

[16] A. F. Smit, "Interspersed repeats and other mementos of transposable elements in mammalian genomes," *Current Opinion in Genetics and Development*, vol. 9, no. 6, pp. 657–663, 1999.

[17] A. Casadevall and L. A. Pirofski, "The damage-response framework of microbial pathogenesis," *Nature Reviews Microbiology*, vol. 1, no. 1, pp. 17–24, 2003.

[18] P. Bonfante and I. A. Anca, "Plants, mycorrhizal fungi, and bacteria: a network of interactions," *Annual Review of Microbiology*, vol. 63, pp. 363–383, 2009.

[19] V. Merckx, M. I. Bidartondo, and N. A. Hynson, "Myco-heterotrophy: when fungi host plants," *Annals of Botany*, vol. 104, no. 7, pp. 1255–1261, 2009.

[20] D. A. Windsor, "Biocartels: units of ecology and evolution based on host-symbiont interrelationships," *Archives of the SciAesthetics Institute*, vol. 1, pp. 4–12, 2000.

[21] D. A. Windsor, "Most of the species on Earth are parasites," *International Journal for Parasitology*, vol. 28, no. 12, pp. 1939–1941, 1998.

[22] A. Araújo and L. F. Ferreira, "Dos caçadores de micróbios à paleoparasitologia molecular," *Ciência Hoje*, vol. 26, no. 152, pp. 32–38, 1999.

[23] J. Ackerman, "Parasites looking for a free lunch," *National Geographic*, vol. 192, no. 4, pp. 74–91, 1997.

[24] H. Kehrer-Sawatzki and D. N. Cooper, "Understanding the recent evolution of the human genome: insights from human-chimpanzee genome comparisons," *Human Mutation*, vol. 28, no. 2, pp. 99–130, 2007.

[25] L. Margulis, *Symbiotic Planet: A New Look at Evolution*, Basic Books, New York, NY, USA, 1998.

[26] T. Cavalier-Smith, "The origin of nuclei and of eukaryotic cells," *Nature*, vol. 256, no. 5517, pp. 463–468, 1975.

[27] T. Horiike, K. Hamada, S. Kanaya, and T. Shinozawa, "Origin of eukaryotic cell nuclei by symbiosis of Archaea in Bacteria is revealed by homology-hit analysis," *Nature Cell Biology*, vol. 3, no. 2, pp. 210–214, 2001.

[28] P. J. L. Bell, "Viral eukaryogenesis: was the ancestor of the nucleus a complex DNA virus?" *Journal of Molecular Evolution*, vol. 53, no. 3, pp. 251–256, 2001.

[29] J. M. Claverie, "Viruses take center stage in cellular evolution," *Genome Biology*, vol. 7, no. 6, Article ID 110, p. 110, 2006.

[30] L. Margulis and D. Sagan, *Origins of Sex*, Yale University Press, London, UK, 1986.

[31] K. L. Fehrenbacher, I. R. Boldogh, and L. A. Pon, "Taking the A-train: actin-based force generators and organelle targeting," *Trends in Cell Biology*, vol. 13, no. 9, pp. 472–477, 2003.

[32] K. Fehrenbacher, T. Huckaba, H. C. Yang, I. Boldogh, and L. Pon, "Actin comet tails, endosomes and endosymbionts," *Journal of Experimental Biology*, vol. 206, pp. 1977–1984, 2003.

[33] C. Bazinet and J. E. Rollins, "*Rickettsia*-like mitochondrial motility in *Drosophila* spermiogenesis," *Evolution and Development*, vol. 5, no. 4, pp. 379–385, 2003.

[34] C. Bazinet, "Endosymbiotic origins of sex," *BioEssays*, vol. 26, no. 5, pp. 558–566, 2004.

[35] W. Sterrer, "On the origin of sex as vaccination," *Journal of Theoretical Biology*, vol. 216, no. 4, pp. 387–396, 2002.

[36] W. D. Hamilton, R. Axelrod, and R. Tanese, "Sexual reproduction as an adaptation to resist parasites (a review)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 9, pp. 3566–3573, 1990.

[37] M. Ridley, *The Red Queen: Sex and the Evolution of Human Nature*, Penguin Books, New York, NY, USA, 1993.

[38] E. Ulvestad, *Defending Life. The Nature of Host-Parasite Relations*, Springer, Dordrecht, The Netherland, 2007.

[39] J. Wiesner and F. Seeber, "The plastid-derived organelle of protozoan human parasites as a target of established and emerging drugs," *Expert Opinion on Therapeutic Targets*, vol. 9, no. 1, pp. 23–44, 2005.

[40] M. J. Taylor, C. Bandi, and A. Hoerauf, "*Wolbachia* bacterial endosymbionts of filarial nematodes," *Advances in Parasitology*, vol. 60, pp. 245–284, 2005.

[41] M. J. Wade and N. W. Chang, "Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite *Wolbachia*," *Nature*, vol. 373, no. 6509, pp. 72–74, 1995.

[42] A. Gargas, P. T. DePriest, M. Grube, and A. Tehler, "Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny," *Science*, vol. 268, no. 5216, pp. 1492–1495, 1995.

[43] P. W. Ewald, "Transmission modes and evolution of the parasitism-mutualism continuum," *Annals of the New York Academy of Sciences*, vol. 503, pp. 295–306, 1987.

[44] R. A. Lewin, "Why are cows not green?" *Nature*, vol. 326, no. 6115, pp. 743–744, 1987.

[45] C. P. Read, *Parasitism and Symbiology*, The Ronald Press, New York, NY, USA, 1970.

[46] L. F. Ferreira, "O Fenômeno Parasitismo," *Revista da Sociedade Brasileira de Medicina Tropical*, vol. 7, no. 4, pp. 261–277, 1973.

[47] K. Summers, S. McKeon, J. Sellars et al., "Parasitic exploitation as an engine of diversity," *Biological Reviews of the Cambridge Philosophical Society*, vol. 78, no. 4, pp. 639–675, 2003.

[48] P. J. Hudson, A. P. Dobson, and K. D. Lafferty, "Is a healthy ecosystem one that is rich in parasites?" *Trends in Ecology and Evolution*, vol. 21, no. 7, pp. 381–385, 2006.

[49] J. Diamond, *Guns Germs, and Steel: The Fates of Human Societies*, W. W. Norton & Co, New York, NY, USA, 1999.

[50] E. K. Rousham, "An increase in *Giardia duodenalis* infection among children receiving periodic Anthelmintic treatment in Bangladesh," *Journal of Tropical Pediatrics*, vol. 40, no. 6, pp. 329–333, 1994.

[51] C. A. Northrop-Clewes, E. K. Rousham, C. G. N. Mascie-Taylor, and P. G. Lunn, "Anthelmintic treatment of rural Bangladeshi children: effect on host physiology, growth, and biochemical status," *American Journal of Clinical Nutrition*, vol. 73, no. 1, pp. 53–60, 2001.

[52] R. J. Bugg, I. D. Robertson, A. D. Elliot, and R. C. A. Thompson, "Gastrointestinal parasites of urban dogs in Perth, Western Australia," *Veterinary Journal*, vol. 157, no. 3, pp. 295–301, 1999.

[53] P. Prociv and J. Croese, "Human enteric infection with *Ancylostoma caninum*: hookworms reappraised in the light of a "new" zoonosis," *Acta Tropica*, vol. 62, no. 1, pp. 23–44, 1996.

[54] J. P. Dedet and F. Pratlong, "*Leishmania, Trypanosoma* and monoxenous trypanosomatids as emerging opportunistic agents," *Journal of Eukaryotic Microbiology*, vol. 47, no. 1, pp. 37–39, 2000.

[55] R. S. Pacheco, M. C. A. Marzochi, M. Q. Pires, C. M. M. Brito, M. D. F. Madeira, and E. G. O. Barbosa-Santos, "Parasite genotypically related to a monoxenous trypanosomatid of dog's flea causing opportunistic infection in an HIV positive patient," *Memórias do Instituto Oswaldo Cruz*, vol. 93, no. 4, pp. 531–537, 1998.

[56] P. Daszak, A. A. Cunningham, and A. D. Hyatt, "Emerging infectious diseases of wildlife—threats to biodiversity and human health," *Science*, vol. 287, no. 5452, pp. 443–449, 2000.

[57] M. E. Torchin, K. D. Lafferty, A. P. Dobson, V. J. McKenzie, and A. M. Kuris, "Introduced species and their missing parasites," *Nature*, vol. 421, no. 6923, pp. 628–630, 2003.

[58] C. E. Mitchell and A. O. Power, "Release of invasive plants from fungal and viral pathogens," *Nature*, vol. 421, no. 6923, pp. 625–627, 2003.

[59] P. J. Cripps, "Veterinary education, zoonoses and public health: a personal perspective," *Acta Tropica*, vol. 76, no. 1, pp. 77–80, 2000.

[60] R. E. Ricklefs and D. C. Outlaw, "A molecular clock for malaria parasites," *Science*, vol. 329, no. 5988, pp. 226–229, 2010.

[61] S. Blanquart and O. Gascuel, "Mitochondrial genes support a common origin of rodent malaria parasites and *Plasmodium falciparum's* relatives infecting great apes," *BMC Evolutionary Biology*, vol. 11, no. 1, p. 70, 2011.

[62] Y. Guan, D. Vijaykrishna, J. Bahl, H. Zhu, J. Wang, and G. J. Smith, "The emergence of pandemic influenza viruses," *Protein & Cell*, vol. 1, no. 1, pp. 9–13, 2010.

[63] L. Margulis and M. J. Chapman, "Endosymbioses: cyclical and permanent in evolution," *Trends in Microbiology*, vol. 6, no. 9, pp. 342–345, 1998.

[64] A. P. Møller, "Interactions between interactions: predator-prey, parasite-host, and mutualistic interactions," *Annals of the New York Academy of Sciences*, vol. 1133, pp. 180–186, 2008.

[65] L. V. Hooper, M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon, "Molecular analysis of commensal host-microbial relationships in the intestine," *Science*, vol. 291, no. 5505, pp. 881–884, 2001.

[66] Y. Umesaki, "Immunohistochemical and biochemical demonstration of the change in glycolipid composition of the intestinal epithelial cell surface in mice in relation to epithelial cell differentiation and bacterial association," *Journal of Histochemistry and Cytochemistry*, vol. 32, no. 3, pp. 299–304, 1984.

[67] T. S. Stappenbeck, L. V. Hooper, and J. I. Gordon, "Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15451–15455, 2002.

[68] L. V. Hooper, T. S. Stappenbeck, C. V. Hong, and J. I. Gordon, "Angiogenins: a new class of microbicidal proteins involved in innate immunity," *Nature Immunology*, vol. 4, no. 3, pp. 269–273, 2003.

[69] J. Xu and J. I. Gordon, "Honor thy symbionts," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10452–10459, 2003.

[70] B. Dixon, "How do gut bacteria affect obesity?" *The Lancet Infectious Diseases*, vol. 10, no. 6, p. 372, 2010.

[71] B. Crabtree, D. E. Holloway, M. D. Baker, K. R. Acharya, and V. Subramanian, "Biological and structural features of murine angiogenin-4, an angiogenic protein," *Biochemistry*, vol. 46, no. 9, pp. 2431–2443, 2007.

[72] K. J. Rhee, P. Sethupathi, A. Driks, D. K. Lanning, and K. L. Knight, "Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody

repertoire," *Journal of Immunology*, vol. 172, no. 2, pp. 1118–1124, 2004.

[73] V. Nobre, J. C. Serufo, O. D. S. Carvalho et al., "Alteration in the endogenous intestinal flora of Swiss Webster mice by experimental *Angiostrongylus costaricensis* infection," *Memórias do Instituto Oswaldo Cruz*, vol. 99, no. 7, pp. 717–720, 2004.

[74] G. W. Tannock, "What immunologists should know about bacterial communities of the human bowel," *Seminars in Immunology*, vol. 19, no. 2, pp. 94–105, 2007.

[75] D. K. Lanning, K. J. Rhee, and K. L. Knight, "Intestinal bacteria and development of the B-lymphocyte repertoire," *Trends in Immunology*, vol. 26, no. 8, pp. 419–425, 2005.

[76] C. D. Davis and J. A. Milner, "Gastrointestinal microflora, food components and colon cancer prevention," *Journal of Nutritional Biochemistry*, vol. 20, no. 10, pp. 743–752, 2009.

[77] F. Baldi, M. A. Bianco, G. Nardone, A. Pilotto, and E. Zamparo, "Focus on acute diarrhoeal disease," *World Journal of Gastroenterology*, vol. 15, no. 27, pp. 3341–3348, 2009.

[78] C. L. F. Walker, D. Sack, and R. E. Black, "Etiology of diarrhea in older children, adolescents and adults: a systematic review," *PLoS Neglected Tropical Diseases*, vol. 4, no. 8, p. e768, 2010.

[79] H. F. Rosenberg and J. B. Domachowske, "Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens," *Journal of Leukocyte Biology*, vol. 70, no. 5, pp. 691–698, 2001.

[80] K. D. Dyer and H. F. Rosenberg, "The RNAse a superfamily: generation of diversity and innate host defense," *Molecular Diversity*, vol. 10, no. 4, pp. 585–597, 2006.

[81] J. M. Montgomery, D. Gillespie, P. Sastrawan, T. M. Fredeking, and G. L. Stewart, "Aerobic salivary bacteria in wild and captive Komodo dragons," *Journal of Wildlife Diseases*, vol. 38, no. 3, pp. 545–551, 2002.

[82] P. H. Harvey and L. Partridge, "Murderous mandibles and black holes in hymenopteran wasps," *Nature*, vol. 326, no. 6109, pp. 128–129, 1987.

[83] J. Torres, J. Peig, C. Eira, and M. Borrás, "Cadmium and lead concentrations in *Skrjabinotaenia lobata* (Cestoda: Catenotaeniidae) and in its host, *Apodemus sylvaticus* (Rodentia: Muridae) in the urban dumping site of Garraf (Spain)," *Environmental Pollution*, vol. 143, no. 1, pp. 4–8, 2006.

[84] M. Malek, M. Haseli, I. Mobedi, M. R. Ganjali, and K. MacKenzie, "Parasites as heavy metal bioindicators in the shark *Carcharhinus dussumieri* from the Persian Gulf," *Parasitology*, vol. 134, no. 7, pp. 1053–1056, 2007.

[85] I. Jankovsá, D. Miholová, V. Bejcek et al., "Influence of parasitism on trace element contents in tissues of red fox (*Vulpes vulpes*) and its parasites *Mesocestoides* spp. (Cestoda) and *Toxascaris leonina* (Nematoda)," *Archives of Environmental Contamination and Toxicology*, vol. 58, no. 2, pp. 469–477, 2010.

[86] S. Fellous and L. Salvaudon, "How can your parasites become your allies?" *Trends in Parasitology*, vol. 25, no. 2, pp. 62–66, 2009.

[87] N. M. Rosas-García, "Biopesticide production from Bacillus *thuringiensis*: an environmentally friendly alternative," *Recent Patents on Biotechnology*, vol. 3, no. 1, pp. 28–36, 2009.

[88] B. Sures, "Environmental parasitology: relevancy of parasites in monitoring environmental pollution," *Trends in Parasitology*, vol. 20, no. 4, pp. 170–177, 2004.

[89] L. A. Katz, "Lateral gene transfers and the evolution of eukaryotes: theories and data," *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, no. 5, pp. 1893–1900, 2002.

[90] M. C. Rivera and J. A. Lake, "The ring of life provides evidence for a genome fusion origin of eukaryotes," *Nature*, vol. 431, no. 7005, pp. 152–155, 2004.

[91] E. S. Lander, L. M. Linton, B. Birren et al., "Initial sequencing and analysis of the human genome," *Nature*, vol. 409, no. 6822, pp. 860–921, 2001.

[92] S. L. Salzberg, O. White, J. Peterson, and J. A. Eisen, "Microbial genes in the human genome: lateral transfer or gene loss?" *Science*, vol. 292, no. 5523, pp. 1903–1906, 2001.

[93] R. Druker and E. Whitelaw, "Retrotransposon-derived elements in the mammalian genome: a potential source of disease," *Journal of Inherited Metabolic Disease*, vol. 27, no. 3, pp. 319–330, 2004.

[94] O. G. Berg and C. G. Kurland, "Evolution of microbial genomes: sequence acquisition and loss," *Molecular Biology and Evolution*, vol. 19, no. 12, pp. 2265–2276, 2002.

[95] A. P. de Koning, F. S. L. Brinkman, S. J. M. Jones, and P. J. Keeling, "Lateral gene transfer and metabolic adaptation in the human parasite *Trichomonas vaginalis*," *Molecular Biology and Evolution*, vol. 17, no. 11, pp. 1769–1773, 2000.

[96] B. Loftus, I. Anderson, R. Davies et al., "The genome of the protist parasite *Entamoeba histolytica*," *Nature*, vol. 433, no. 7028, pp. 865–868, 2005.

[97] H. G. Morrison, A. G. McArthur et al., "Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*," *Science*, vol. 317, no. 5846, pp. 1921–1926, 2007.

[98] A. R. L. Teixeira, R. J. Nascimento, and N. R. Sturm, "Evolution and pathology in Chagas disease—a review," *Memórias do Instituto Oswaldo Cruz*, vol. 101, no. 5, pp. 463–491, 2006.

[99] A. R. L. Teixeira, N. Nitz, M. C. Guimaro, C. Gomes, and C. A. Santos-Buch, "Chagas disease," *Postgraduate Medical Journal*, vol. 82, no. 974, pp. 788–798, 2006.

[100] A. Simoes-Barbosa, E. R. Arganaraz, A. M. Barros et al., "Hitchhiking *Trypanosoma cruzi* minicircle DNA affects gene expression in human host cells via LINE-1 retrotransposon," *Memórias do Instituto Oswaldo Cruz*, vol. 101, no. 8, pp. 833–843, 2006.

[101] M. M. Hecht, N. Nitz, P. F. Araujo et al., "Inheritance of DNA transferred from American trypanosomes to human hosts," *PLoS One*, vol. 5, no. 2, article e9181, 2010.

[102] E. A. Farkash and E. T. L. Prak, "DNA damage and L1 retrotransposition," *Journal of Biomedicine and Biotechnology*, vol. 2006, no. 1, p. 37285, 2006.

[103] S. Adler, "Darwin's, illness," *British Medical Journal*, vol. 1, no. 5444, pp. 1249–1250, 1965.

[104] R. E. Bernstein, "Darwin's illness: Chagas' disease resurgens," *Journal of the Royal Society of Medicine*, vol. 77, no. 7, pp. 608–609, 1984.

[105] H. A. G. Teive and W. O. Arruda, "Did Darwin have syncope caused by Chagas' disease?" *Arquivos de Neuro-Psiquiatria*, vol. 67, no. 2A, p. 371, 2009.

[106] A. W. Woodruff, "Darwin's health in relation to his voyage to South America," *British Medical Journal*, vol. 1, no. 5437, pp. 745–750, 1965.

[107] A. Das, "DNA transfer from *Agrobacterium* to plant cells in crown gall tumor disease," *Subcellular Biochemistry*, vol. 29, pp. 343–363, 1998.

[108] B. E. Matthews, *An Introduction to Parasitology, Studies in Biology*, Cambridge University Press, Cambridge, UK, 1998.

[109] W. G. Eberhard, "Evolutionary consequences of intracellular organelle competition," *The Quarterly Review of Biology*, vol. 55, no. 3, pp. 231–249, 1980.

[110] W. G. Eberhard, "Intraorganism competition involving eukaryotic organelles," *Annals of the New York Academy of Sciences*, vol. 361, pp. 44–52, 1981.

[111] W. R. Rice and B. Holland, "The enemies within: intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen," *Behavioral Ecology and Sociobiology*, vol. 41, no. 1, pp. 1–10, 1997.

[112] M. R. W. Brown and J. Barker, "Unexplored reservoirs of pathogenic bacteria: protozoa and biofilms," *Trends in Microbiology*, vol. 7, no. 1, pp. 46–50, 1999.

[113] J. D. Cirillo, S. Falkow, L. S. Tompkins, and L. E. Bermudez, "Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence," *Infection and Immunity*, vol. 65, no. 9, pp. 3759–3767, 1997.

[114] J. D. Cirillo, S. Falkow, L. S. Tompkins, S. L. G. Cirillo, and L. E. Bermudez, "Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*," *Infection and Immunity*, vol. 67, no. 9, pp. 4427–4434, 1999.

[115] P. Rappelli, M. F. Addis, F. Carta, and P. L. Fiori, "*Mycoplasma hominis* parasitism of *Trichomonas vaginalis*," *The Lancet*, vol. 352, no. 9136, p. 1286, 1998.

[116] M. G. Boosalis, "Hyperparasitism," *Annual Review of Phytopathology*, vol. 2, pp. 363–376, 1964.

[117] L. R. Ranford, M. K. Jones, and P. Prociv, "*Giardia trophozoites* in dog hookworm, *Ancylostoma caninum*: accident or hyperparasitism?" *Acta Tropica*, vol. 80, no. 1, pp. 77–79, 2001.

[118] J. Swift, in *A New Dictionary of Quotations on Historical Principles from Ancient and Modern Sources*, H. L. Mencken, Ed., p. 1712, Knopf, New York, NY, USA, 1942.

[119] F. Thomas, R. Poulin, J.-F. Guégan, Y. Michalakis, and F. Renaud, "Are there pros as well as cons to being parasitized?" *Parasitology Today*, vol. 16, no. 12, pp. 533–536, 2000.

[120] N. Yamamura, "Vertical transmission and evolution of mutualism from parasitism," *Theoretical Population Biology*, vol. 44, no. 1, pp. 95–109, 1993.

[121] R. D. Holt and J. H. Lawton, "The ecological consequences of shared natural enemies," *Annual Review of Ecology and Systematics*, vol. 25, pp. 495–520, 1994.

[122] P. Hudson and J. Greenman, "Competition mediated by parasites: biological and theoretical progress," *Trends in Ecology and Evolution*, vol. 13, no. 10, pp. 387–390, 1998.

[123] D. E. Vaccaro, "Symbiosis therapy: the potential of using human protozoa for molecular therapy," *Molecular Therapy*, vol. 2, no. 6, pp. 535–538, 2000.

[124] M. S. Rolph and I. A. Ramshaw, "Recombinant viruses as vaccines and immunological tools," *Current Opinion in Immunology*, vol. 9, no. 4, pp. 517–524, 1997.

[125] C. Locht, "Live bacterial vectors for intranasal delivery of protective antigens," *Pharmaceutical Science and Technology Today*, vol. 3, no. 4, pp. 121–128, 2000.

[126] J. Flo, S. Tisminetzky, and F. Baralle, "Oral transgene vaccination mediated by attenuated *Salmonellae* is an effective method to prevent *Herpes simplex* virus-2 induced disease in mice," *Vaccine*, vol. 19, no. 13-14, pp. 1772–1782, 2001.

[127] T. H. Flo, K. D. Smith, S. Sato et al., "Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron," *Nature*, vol. 432, no. 7019, pp. 917–921, 2004.

[128] E. Chernin, "The malariatherapy of neurosyphilis," *Journal of Parasitology*, vol. 70, no. 5, pp. 611–617, 1984.

[129] S. C. Austin, P. D. Stolley, and T. Lasky, "The history of malariotherapy for neurosyphilis: modern parallels," *Journal of the American Medical Association*, vol. 268, no. 4, pp. 516–519, 1992.

[130] P. Ryan, S. F. Hurley, A. M. Johnson et al., "Tumours of the brain and presence of antibodies to *Toxoplasma gondii*," *International Journal of Epidemiology*, vol. 22, no. 3, pp. 412–419, 1993.

[131] F. K. Conley and J. S. Remington, "Nonspecific inhibition of tumor growth in the central nervous system: observations of intracerebral ependymoblastoma in mice with chronic *Toxoplasma* infection," *Journal of the National Cancer Institute*, vol. 59, no. 3, pp. 963–973, 1977.

[132] A. Varga, W. Sokolowska-Kohler, W. Presber et al., "*Toxoplasma* infection and cell free extract of the parasites are able to reverse multidrug resistance of mouse lymphoma and human gastric cancer cells *in vitro*," *Anticancer Research*, vol. 19, no. 2A, pp. 1317–1324, 1999.

[133] S. M. Levine, S. G. Frangos, B. Hanna, K. Colen, and J. P. Levine, "*Aeromonas* septicemia after medicinal leech use following replantation of severed digits," *American Journal of Critical Care*, vol. 19, no. 5, pp. 469–471, 2010.

[134] K. M. Donnelly, M. E. Bromberg, A. Milstone et al., "*Ancylostoma caninum* anticoagulant peptide blocks metastasis in vivo and inhibits factor Xa binding to melanoma cells in vitro," *Journal of Thrombosis and Haemostasis*, vol. 79, no. 5, pp. 1041–1047, 1998.

[135] P. Stanssens, P. W. Bergum, Y. Gansemans et al., "Anticoagulant repertoire of the hookworm *Ancylostoma caninum*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 5, pp. 2149–2154, 1996.

[136] N. R. Lynch, J. Goldblatt, and P. N. Le Souëf, "Parasite infections and the risk of asthma and atopy," *Thorax*, vol. 54, no. 8, pp. 659–660, 1999.

[137] J. V. Weinstock, R. Summers, and D. E. Elliott, "Helminths and harmony," *Gut*, vol. 53, no. 1, pp. 7–9, 2004.

[138] M. Pietropaolo and M. Trucco, "Viral elements in autoimmunity of type I diabetes," *Trends in Endocrinology and Metabolism*, vol. 7, no. 4, pp. 139–144, 1996.

[139] T. A. Chowdhury, C. H. Mijovic, and A. H. Barnett, "The aetiology of type I diabetes," *Best Practice & Research Clinical Endocrinology & Metabolism*, vol. 13, no. 2, pp. 181–195, 1999.

[140] M. S. Horwitz, C. Fine, A. Ilic, and N. Sarvetnick, "Requirements for viral-mediated autoimmune diabetes: beta-cell damage and immune infiltration," *Journal of Autoimmunity*, vol. 16, no. 3, pp. 211–217, 2001.

[141] M. Van Ghelue, U. Moens, S. Bendiksen, and O. P. Rekvig, "Autoimmunity to nucleosomes related to viral infection: a focus on hapten-carrier complex formation," *Journal of Autoimmunity*, vol. 20, no. 2, pp. 171–182, 2003.

[142] J. V. Weinstock, R. W. Summers, D. E. Elliott, K. Qadir, J. F. Urban Jr., and R. Thompson, "The possible link between de-worming and the emergence of immunological disease," *Journal of Laboratory and Clinical Medicine*, vol. 139, no. 6, pp. 334–338, 2002.

[143] D. E. Elliott, J. F. Urban Jr., C. K. Argo, and J. V. Weinstock, "Does the failure to acquire helminthic parasites predispose to Crohn's disease?" *The FASEB Journal*, vol. 14, no. 12, pp. 1848–1855, 2000.

[144] A. Newman, *In Pursuit of an Autoimmune Worm Cure*, The New York Times, New York, NY, USA, 1999.

[145] R. W. Summers, D. E. Elliott, K. Qadir, J. F. Urban Jr., R. Thompson, and J. V. Weinstock, "*Trichuris suis* seems to be safe and possibly effective in the treatment of inflammatory

bowel disease," *The American Journal of Gastroenterology*, vol. 98, no. 9, pp. 2034–2041, 2003.

[146] M. Yazdanbakhsh, A. van den Biggelaar, and R. M. Maizels, "Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease," *Trends in Immunology*, vol. 22, no. 7, pp. 372–377, 2001.

[147] M. Yazdanbakhsh, P. G. Kremsner, and R. van Ree, "Immunology: allergy, parasites, and the hygiene hypothesis," *Science*, vol. 296, no. 5567, pp. 490–494, 2002.

[148] M. Nacher, F. Gay, P. Singhasivanon et al., "*Ascaris lumbricoides* infection is associated with protection from cerebral malaria," *Parasite Immunology*, vol. 22, no. 3, pp. 107–113, 2000.

[149] M. Nacher, P. Singhasivanon, B. Traore et al., "Helminth infections are associated with protection from cerebral malaria and increased nitrogen derivatives concentrations in Thailand," *American Journal of Tropical Medicine and Hygiene*, vol. 66, no. 3, pp. 304–309, 2002.

[150] D. I. Pritchard and A. Brown, "Is *Necator americanus* approaching a mutualistic symbiotic relationship with humans?" *Trends in Parasitology*, vol. 17, no. 4, pp. 169–172, 2001.

[151] G. Greub and D. Raoult, "History of the ADP/ATP-translocase-encoding gene, a parasitism gene transferred from a Chlamydiales ancestor to plants 1 billion years ago," *Applied and Environmental Microbiology*, vol. 69, no. 9, pp. 5530–5535, 2003.

[152] F. Lutzoni, M. Pagel, and V. Reeb, "Major fungal lineages are derived from lichen symbiotic ancestors," *Nature*, vol. 411, no. 6840, pp. 937–940, 2001.

[153] B. L. Hart, "The evolution of herbal medicine: behavioural perspectives," *Animal Behaviour*, vol. 70, no. 5, pp. 975–989, 2005.

[154] A. R. David, "5000 years of schistosomiasis in Egypt," *Chungara, Revista de Antropología Chilena*, vol. 32, no. 1, pp. 133–135, 2000.

[155] L. S. Roberts, J. Janovy Jr., and G. D. Schmidt, *Gerald D. Schmidt & Larry S. Roberts' Foundations of Parasitology*, William C. Brown, Chicago, Ill, USA, 5th edition, 1996.

[156] P. Cossart, P. Boquet, S. Normak, and R. Rappuoli, "Cellular microbiology emerging," *Science*, vol. 271, no. 5247, pp. 315–316, 1996.

[157] D. Camus, M. G. Zalis, M. A. Vannier-Santos, and D. M. Banic, "The art of parasite survival," *Brazilian Journal of Medical and Biological Research*, vol. 28, no. 4, pp. 399–413, 1995.

[158] W. de Souza, "Special organelles of some pathogenic protozoa," *Parasitology Research*, vol. 88, no. 12, pp. 1013–1025, 2002.

[159] M. A. Vannier-Santos, A. Martiny, and W. de Souza, "Cell biology of *Leishmania* spp.: invading and evading," *Current Pharmaceutical Design*, vol. 8, no. 4, pp. 297–318, 2002.

[160] W. H. McNeill, *Plagues and Peoples*, Garden City, New York, NY, USA, 1976.

[161] B. Dixon, *Power Unseen: How Microbes Rule the World*, WH Freeman, New York, NY, USA, 1994.

[162] K. F. Kiple and K. C. Ornelas, "Typhus, ships and soldiers," in *Plague, Pox and Pestilence: Disease in History*, K. F. Kiple, Ed., pp. 104–109, Barnes and Noble, New York, NY, USA, 1997.

[163] B. A. Cunha, "The death of Alexander the Great: malaria or typhoid fever?" *Infectious Disease Clinics of North America*, vol. 18, no. 1, pp. 53–63, 2004.

[164] J. S. Marr and C. H. Calisher, "Alexander the Great and West Nile virus encephalitis," *Emerging Infectious Diseases*, vol. 9, no. 12, pp. 1599–1603, 2003.

[165] S. N. Rumyantsev, "The best defence against bioweapons has already been invented by evolution," *Infection, Genetics and Evolution*, vol. 4, no. 2, pp. 159–166, 2004.

[166] F. F. Cartwright and M. D. Biddiss, *Diseases and History*, Barnes and Noble, New York, NY, USA, 1991.

[167] P. Ziegler, *The Black Death*, Allan Sutton, Gloucestershire, UK, 1991.

[168] R. S. Bray, *Armies of Pestilence: The Impact of Disease on History*, Barnes and Noble, New York, NY, USA, 1996.

[169] R. J. Doyle and N. C. Lee, "Microbes, warfare, religion, and human institutions," *Canadian Journal of Microbiology*, vol. 32, no. 3, pp. 193–200, 1986.

[170] A. Crosby, "Influenza: in the grip of the grippe," in *Plague, Pox and Pestilence: Disease in History*, K. F. Kiple, Ed., pp. 148–153, Barnes and Noble, New York, NY, USA, 1997.

[171] R. D. Meléndez, "*Toxoplasma gondii*: the best terrestrial biological weapon against extraterrestrial invaders?" *Parasitology Today*, vol. 12, no. 4, p. 166, 1996.

[172] Lobato M, *Idéias de Jeca Tatú*, Editora Brasiliense, São Paulo, Brazil, 1956.

[173] M. Cupa, "Air transport, aeronautic medecine, health," *Bulletin de la Académie Nationale de Médecine*, vol. 193, no. 7, pp. 1619–1631, 2009.

[174] N. P. Spanos and J. Gottlieb, "Ergotism and the Salem Village witch trials," *Science*, vol. 194, no. 4272, pp. 1390–1394, 1976.

[175] L. R. Caporael, "Ergotism: the satan loosed in Salem?" *Science*, vol. 192, no. 4234, pp. 21–26, 1976.

[176] A. Woolf, "Witchcraft or mycotoxin? The Salem witch trials," *Journal of Toxicology—Clinical Toxicology*, vol. 38, no. 4, pp. 457–460, 2000.

[177] J. S. Marr and J. Baldwin, *The Eleventh Plague*, HarperCollins, New York, NY, USA, 1998.

[178] M. Jisaka, M. Kawanaka, H. Sugiyama et al., "Antischistosomal activities of sesquiterpene lactones and steroid glucosides from *Vernonia amygdalina*, possibly used by wild chimpanzees against parasite-related diseases," *Bioscience, Biotechnology, and Biochemistry*, vol. 56, no. 5, pp. 845–846, 1992.

[179] M. Jisaka, H. Ohigashi, K. Takegawa, M. A. Huffman, and K. Koshimizu, "Antitumoral and antimicrobial activities of bitter sesquiterpene lactones of *Vernonia amygdalina*, a possible medicinal plant used by wild chimpanzees," *Bioscience, Biotechnology, and Biochemistry*, vol. 57, no. 5, pp. 833–834, 1993.

[180] K. Koshimizu, H. Ohigashi, and M. A. Huffman, "Use of *Vernonia amygdalina* by wild chimpanzee: possible roles of its bitter and related constituents," *Physiology and Behavior*, vol. 56, no. 6, pp. 1209–1216, 1994.

[181] M. R. Hutchings, S. Athanasiadou, I. Kyriazakis, and I. J. Gordon, "Can animals use foraging behaviour to combat parasites?" *Proceedings of the Nutrition Society*, vol. 62, no. 2, pp. 361–370, 2003.

[182] H. Guyatt, "Do intestinal nematodes affect productivity in adulthood?" *Parasitology Today*, vol. 16, no. 4, pp. 153–158, 2000.

[183] G. A. Schmuñis and J. F. L. Antuñano, "World-wide importance of parasites," in *Topley & Wilson's Microbiology & Microbial Infections Vol 5: Parasitology*, L. Collier, A. Balows, M. Sussman, F. E. G. Cox, J. P. Kreier, and D. Wakelin, Eds., vol. 5, pp. 19–38, Arnold, London, UK, 9th edition, 1998.

[184] T. Godal, "Fighting the parasites of poverty: public research, private industry, and tropical diseases," *Science*, vol. 264, no. 5167, pp. 1864–1866, 1994.

[185] T. Hubbard and J. Love, "A new trade framework for global healthcare R&D," *PLoS Biology*, vol. 2, no. 2, p. e52, 2004.

[186] E. Jimenez, *Human an Physical Structure: Public Investment and Pricing Policies in Developing Countries*, The World Bank Policy Research Departnent, Poverty and Human Resources Division, Washington, DC, USA, 1994.

[187] F. Noorbakhsh, A. Paloni, and A. Youssef, "Human capital and FDI inflows to developing countries: new empirical evidence," *World Development*, vol. 29, no. 9, pp. 1593–1610, 2001.

[188] R. Horton, "Medical journals: evidence of bias against the diseases of poverty," *The Lancet*, vol. 361, no. 9359, pp. 712–713, 2003.

[189] J. S. Butel, "Viral carcinogenesis: revelation of molecular mechanisms and etiology of human disease," *Carcinogenesis*, vol. 21, no. 3, pp. 405–426, 2000.

[190] L. A. Herrera and P. Ostrosky-Wegman, "Do helminths play a role in carcinogenesis?" *Trends in Parasitology*, vol. 17, no. 4, pp. 172–175, 2001.

[191] M. W. Lehker and J. F. Alderete, "Biology of trichomonosis," *Current Opinion in Infectious Diseases*, vol. 13, no. 1, pp. 37–45, 2000.

[192] P. Reiter, "From Shakespeare to Defoe: malaria in England in the Little Ice Age," *Emerging Infectious Diseases*, vol. 6, no. 1, pp. 1–11, 2000.

[193] F. E. G. Cox, "History of human parasitology," in *Topley & Wilson's Microbiology & Microbial Infections Vol 5: Parasitology*, L. Collier, A. Balows, M. Sussman, F. E. G. Cox, J. P. Kreier, and D. Wakelin, Eds., vol. 5, pp. 3–18, Arnold, London, UK, 9th edition, 1998.

[194] P. R. Epstein, "Is global warming harmful to health," *Scientific American*, vol. 283, no. 2, pp. 50–57, 2000.

[195] Instituto Brasileiro de Geografia e Estatística (IBGE), http://www.ibge.gov.br/.

[196] R. S. Barsoum, "Parasitic infections in organ transplantation," *Experimental and Clinical Transplantation*, vol. 2, no. 2, pp. 258–267, 2004.

[197] R. S. Barsoum, "Parasitic infections in transplant recipients," *Nature Clinical Practice Nephrology*, vol. 2, no. 9, pp. 490–503, 2006.

[198] F. Derouin and H. Pelloux, "Prevention of toxoplasmosis in transplant patients," *Clinical Microbiology and Infection*, vol. 14, no. 12, pp. 1089–1101, 2008.

[199] D. Bundy, A. Sher, and E. Michael, "Good worms or bad worms: do worm infections affect the epidemiological patterns of other diseases?" *Parasitology Today*, vol. 16, no. 7, pp. 273–274, 2000.

[200] A. von Graevenitz, "The role of opportunistic bacteria in human disease," *Annual Review of Microbiology*, vol. 31, pp. 447–471, 1977.

[201] D. C. Savage, "Microbial ecology of the gastrointestinal tract," *Annual Review of Microbiology*, vol. 31, pp. 107–133, 1977.

[202] C. Zimmer, "Do chronic diseases have an infectious root?" *Science*, vol. 293, no. 5537, pp. 1974–1977, 2001.

[203] M. Pasarica and N. V. Dhurandhar, "Infectobesity: obesity of infectious origin," *Advances in Food and Nutrition Research*, vol. 52, pp. 61–102, 2007.

[204] H. L. Suplicy and A. Bornschein, "Infeccions as the etiology for obesity," *Arquivos Brasileiros de Endocrinologia e Metabologia*, vol. 53, no. 2, pp. 159–164, 2009.

[205] F. Bäckhed, H. Ding, T. Wang et al., "The gut microbiota as an environmental factor that regulates fat storage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 44, pp. 15718–15723, 2004.

[206] E. S. Leal and P. M. A. Zanotto, "Viral disease and human evolution," *Memórias do Instituto Oswaldo Cruz*, vol. 95, supplement 1, pp. 193–200, 2000.

[207] E. C. Ujvari, "The history of dissemination of microorganisms," *Estudos Avançados*, vol. 22, no. 64, pp. 171–182, 2008.

[208] C. P. Blobel, T. G. Wolfsberg, C. W. Turck, D. G. Myles, P. Primakoff, and J. M. White, "A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion," *Nature*, vol. 356, no. 6366, pp. 248–252, 1992.

[209] T. G. Wolfsberg, J. F. Bazan, C. P. Blobel, D. G. Myles, P. Primakoff, and J. M. White, "The precursor region of a protein active in sperm-egg fusion contains a metalloprotease and a disintegrin domain: structural, functional, and evolutionary implications," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 22, pp. 10783–10787, 1993.

[210] L. P. Villareal, "On viruses, sex, and motherhood," *Journal of Virology*, vol. 71, no. 2, pp. 859–865, 1997.

[211] S. Mi, X. Lee, X. P. Li et al., "Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis," *Nature*, vol. 403, no. 6771, pp. 785–789, 2000.

[212] M. Renard, P. F. Varela, C. Letzelter, S. Duquerroy, F. A. Rey, and T. Heidmann, "Crystal structure of a pivotal domain of human syncytin-2, a 40 million years old endogenous retrovirus fusogenic envelope gene captured by primates," *Journal of Molecular Biology*, vol. 352, no. 5, pp. 1029–1034, 2005.

[213] S. D. J. Pena, "Parasitos e Hospedeiros: evolução genômica sob o jugo da rainha vermelha," in *Dinâmica das Doenças Infecciosas e Parasitárias*, J. R. Coura, Ed., pp. 143–147, Guanabara-Koogan, Rio de Janeiro, Brazil, 2005.

[214] Y. Schlein, R. L. Jacobson, and G. Messer, "*Leishmania* infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 20, pp. 9944–9948, 1992.

[215] S. J. Davies, J. L. Grogan, R. B. Blank, K. C. Lim, R. M. Locksley, and J. H. McKerrow, "Modulation of blood fluke development in the liver by hepatic CD4+ lymphocytes," *Science*, vol. 294, no. 5545, pp. 1358–1361, 2001.

[216] C. Zimmer, *Parasite Rex: Inside the Bizarre World of Nature's Most Dangerous Creatures*, The Free Press, New York, NY, USA, 2000.

[217] J. Moore, *Parasites and the Behavior of Animals*, Oxford University Press, Oxford, UK, 2002.

[218] R. E. Holliman, "Toxoplasmosis, behaviour and personality," *Journal of Infection*, vol. 35, no. 2, pp. 105–110, 1997.

[219] J. Flegr, P. Kodym, and V. Tolarova, "Correlation of duration of latent *Toxoplasma gondii* infection with personality changes in women," *Biological Psychology*, vol. 53, no. 1, pp. 57–68, 2000.

[220] T. Schetters, "Why are the French different from the British?" *Trends in Parasitology*, vol. 17, no. 10, p. 467, 2001.

[221] K. Lewis, "Pathogen resistance as the origin of kin altruism," *Journal of Theoretical Biology*, vol. 193, no. 2, pp. 359–363, 1998.

[222] J. R. Peck and M. W. Feldman, "Kin selection and the evolution of monogamy," *Science*, vol. 240, no. 4859, pp. 1672–1674, 1988.

[223] P. Schmid-Hempel and R. H. Crozier, "Polyandry versus polygyny versus parasites," *Philosophical Transactions of the Royal Society B*, vol. 354, no. 1382, pp. 507–515, 1999.

[224] B. S. Low, "Marriage systems and pathogen stress in human societies," *American Zoologist*, vol. 30, no. 2, pp. 325–339, 1990.

[225] B. S. Low, *Why Sex Matters: A Darwinian Look at Human Dehavior*, Princeton University Press, Princeton, NJ, USA, 2000.

[226] S. L. Moore and K. Wilson, "Parasites as a viability cost of sexual selection in natural populations of mammals," *Science*, vol. 297, no. 5589, pp. 2015–2018, 2002.

[227] I. P. F. Owens, "Ecology and evolution. Sex differences mortality rate," *Science*, vol. 297, no. 5589, pp. 2008–2009, 2002.

[228] M. Anderson, *Sexual Selection*, Princeton University Press, Princeton, NJ, USA, 1994.

[229] M. Zuk and K. A. McKean, "Sex differences in parasite infections: patterns and processes," *International Journal of Parasitology*, vol. 26, no. 10, pp. 1009–1023, 1996.

[230] S. L. Klein, "The effects of hormones on sex differences in infection: from genes to behavior," *Neuroscience and Biobehavioral Reviews*, vol. 24, no. 6, pp. 627–638, 2000.

[231] C. J. Grossman, "Interactions between the gonadal steroids and the immune system," *Science*, vol. 227, no. 4684, pp. 257–261, 1985.

[232] H. Snider, C. H. Lezama-Davila, J. Alexander, and A. R. Satoskar, "Sex hormones and modulation of immunity against leishmaniasis," *Neuroimmunomodulation*, vol. 16, no. 2, pp. 106–113, 2009.

[233] G. Escobedo, C. W. Roberts, J. C. Carrero, and J. Morales-Montor, "Parasite regulation by host hormones: an old mechanism of host exploitation?" *Trends in Parasitology*, vol. 21, no. 12, pp. 588–593, 2005.

[234] W. Trager, *Living Together—The Biology of Animal Parasitism*, Plenum Press, New York, NY, USA, 1986.

[235] D. R. Brooks and D. A. McLennan, *Parascript—Parasites and the Language of Evolution*, Smithsonian Institution Press, Washington, DC, USA, 1993.

[236] H. L. Lenzi, R. G. Pacheco, M. Pelajo-Machado, M. S. Panasco, W. S. Romanha, and J. A. Lenzi, "Immunological system and *Schistosoma mansoni*: co-evolutionary immunobiology. What is the eosinophil role in parasite-host relationship?" *Memórias do Instituto Oswaldo Cruz*, vol. 92, supplement 2, pp. 19–32, 1997.

[237] S. Paracer and V. Ahmadjian, *Symbiosis—An Introduction to Biological Associations*, Oxford University Press, Oxford, UK, 2nd edition, 2000.

[238] A. O. Bush, J. C. Fernández, G. W. Esch, and J. R. Seed, *Parasitism: The Diversity and Ecology of Animal Parasites*, Cambridge University Press, Cambridge, UK, 2001.

[239] L. Margulis and D. Sagan, *Acquiring Genomes—A Theory of the Origins of Species*, Basic Books, New York, NY, USA, 2002.

[240] C. Combes, *Interactions Durables: Écologie et Évolution du Parasitism*, Masson, Paris, France, 1995.

[241] D. Rollinson and S. Hay, *Advances in Parasitology: Natural History of Host-Parasite Interactions*, Academic Press, London, UK, 2009.

[242] P. Ward, *The Medea Hypothesis—Is Life on Earth Ultimately Self-Destructive?* Princeton University Press, Princeton, NJ, USA, 2009.

[243] E. Jablonka and M. J. Lamb, *Evolution in Four Dimensions: Genetic, Epigenetic, Behavioral, and Symbolic Variation in the History of Life*, The MIT Press, Cambridge, Mass, USA, 2005.

[244] P. Horwitz and B. A. Wilcox, "Parasites, ecosystems and sustainability: an ecological and complex systems perspective," *International Journal for Parasitology*, vol. 35, no. 7, pp. 725–732, 2005.

[245] M. J. Fraunholz, "Systems biology in malaria research," *Trends in Parasitology*, vol. 21, no. 9, pp. 393–395, 2005.

[246] K. Aggarwal and K. H. Lee, "Functional genomics and proteomics as a foundation for systems biology," *Briefings in Functional Genomics and Proteomics*, vol. 2, no. 3, pp. 175–184, 2003.

[247] R. Goodacre, "Metabolomics of a superorganism," *Journal of Nutrition*, vol. 137, no. 1, pp. 259S–266S, 2007.

[248] J. Lovelock, *Gaia: A New Look at Life on Earth*, Oxford University Press, Oxford, UK, 2000.