Cercarial penetration, in low to moderate numbers, does not cause a normal skin inflammatory response; therefore, the authors sought to determine whether cercariae can down-regulate keratinocyte activation and thus the secretion of pro-inflammatory cytokines and eicosanoids. Human living skin equivalent (LSE, Organogenesis) consisting of dermal, epidermal and stratum corneum-like layers was used as the skin substrate. The surface of the LSE membrane was exposed to 100 ng IFNγ or ~850 cercariae for 18 h. Incubation media and tissue was then assayed for IL-1α, IL-6, IL-8, TNFα, 5-HETE, 12-HETE, PGE₂, LTB₄, and LTC₄ via RIA and Western Blots. TNFα was not detected. Secreted IL-1α levels were (mean ± S.E.M. (n)): Control, 1.03 ng ± 0.15 (11); IFNγ 1.90 ng ± 0.48 (5); cercariae, 1.79 ng ± 0.22 (22). In spite of this increase, cercariae down-regulated IL-8 (cercariae 11.13 ± 1.70 ng vs. IFNγ = 16.47 ± 0.29 ng, p = 0.04) and LTB₄ (cercariae = 98.86 ± 19.65 pg/0.1 ml vs. IFNγ = 193.42 ± 44.21 pg/0.1 ml p = 0.02). No changes were seen in IL-6, 12-HETE, 5-HETE, and PGE₂ levels. It is concluded that cercarial penetration causes a release of IL-1α consistent with skin trauma; however, schistosomulae may regulate the production of chemotactic (neutrophils, macrophages, T-cells, etc.) and activation factors such as IL-8 and LTB₄.

Key words: Cercariae, Cytokines, Eicosanoids, Living Skin Equivalent, Penetration, Schistosoma mansoni

Introduction

*Schistosoma mansoni* is primarily a tropical and semi-tropical parasitic disease which infects an estimated 250–300 million people. Infection occurs when a human host comes into contact with water containing the infective cercarial stage. Cercariae penetrate the skin barrier and undergo a series of biochemical, physiological and morphological changes resulting in the schistosomulae stage. Schistosomulae remain in the skin for 24–48 h before migrating to the capillaries and eventually becoming established as adults in the hepatoporal system. Skin penetration by low to moderate (<200) numbers of *S. mansoni* cercariae does not cause a normal inflammatory response in man or most laboratory models (mice, hamsters).1–7 This lack of inflammation is remarkable for two reasons: (1) It is known that the epidermis, especially the keratinocyte, is extremely active in immunoregulation and that the first manifestation of any trauma to the skin barrier is an inflammatory response.8,9 (2) Cercariae and schistosomulae, *in vitro*, secrete a wide variety of eicosanoids which are potent inflammatory agents.10–12 Since cytokine production, most notably IL-1 and TNFα, and eicosanoid synthesis are important mediators of inflammation, the authors sought to determine whether cercariae or schistosomulae control skin inflammation by regulating their production. In order to study these interactions, the Living Skin Equivalent (LSE) manufactured by Organogenesis was used. The LSE consists of a dermal layer containing human dermal fibroblasts embedded in a collagen matrix, an epidermal layer consisting of human keratinocytes in various states of differentiation including a fully developed stratum corneum, and a basement membrane-like layer separating the dermis and epidermis.

Materials and Methods

Animals: Biomphalaria glabrata snails infected with *Schistosoma mansoni* were obtained from Dr Yung-san Liang, University of Lowell Center for Tropical Diseases. Infected snails were kept under the ideal population densities as stated by Coles.13 Snails were fed alternately TetraMin® Fish food and Romaine lettuce. In addition, low grade chalk was put into each tank as a source of calcium.

Tissue culture and host-parasite incubations: LSE was purchased from Organogenesis, Inc. (Cambridge, MA) as TESTSKIN® and was maintained in the laboratory 1 week before use according to the manufacturer’s directions (35°C, 5–8% CO₂).
Organogenesis Maintenance Media was supplemented with 3 μM arachidonic and linoleic acids. On the day of experimentation an Assay Ring (Organogenesis) was placed over the LSE tissue and sealed to the surface with sterile DuPont Silicone Sealant. The surface of the LSE (internal area of the assay ring) was then coated with 4 μg/cm² linoleic acid. The assay chambers were then placed in the Organogenesis Assay Tray (essentially a six-well tissue culture plate) containing 1.5 ml of Assay Media (1:1 mixture of low calcium Dulbecco’s Modified Eagles Medium and Hams F-12 medium containing phenol red, 1.85 g/ml of sodium bicarbonate and 50 μg/ml gentamicin sulphate) and stabilized in this medium for 1 h. At the end of this time the assay Media was changed and either approximately 850 cercariae in 200 μl aged tap water, or 100 ng IFNγ were placed in the centre of the assay ring. Control assays contained only 4 μg/cm² linoleate. Incubation was for 18 to 20 h at 35°C and 6-8% CO₂. At the end of the incubation period, the media in each assay well was divided into three 0.5 ml samples and frozen at −80°C. The tissue was homogenized in 5 ml of assay media, divided into three aliquots, and frozen at −80°C.

**Western Blots:** Initial screening for cytokines was done by Western Blot and then quantitated by RIA. Samples were separated using the SDS-PAGE system described by Laemmli with minimal changes. Optimal conditions were a 13% acrylamide gel (0.15 M Tris/HCl pH 8.8, 0.1% SDS) overlaid with a 4% acrylamide stacking gel (0.05 M Tris/HCl pH 6, 0.1% SDS) cast using 140 mm × 160 mm × 1.5 mm plates. Typical running conditions for two 1.5 mm thick gels were 6 h at 60 mA constant current after an initial current of 40 mA for 1 h. Western Blots were done on nitrocellulose membranes (BA-S NC, 0.2 μm pore size, Schleicher & Schuell, NH) using the Towbin system described by Laemmli with minimal changes. Optimal conditions were a 13% acrylamide gel (0.15 M Tris/HCl pH 8.8, 0.1% SDS) overlaid with a 4% acrylamide stacking gel (0.05 M Tris/HCl pH 6, 0.1% SDS) cast using 140 mm × 160 mm × 1.5 mm plates. Typical running conditions for two 1.5 mm thick gels were 6 h at 60 mA constant current after an initial current of 40 mA for 1 h. Western Blots were done on nitrocellulose membranes (BA-S NC, 0.2 μm pore size, Schleicher & Schuell, NH) using the Towbin buffer system (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) in a Trans-Blot Electrophoresis Transfer Cell (BioRad, CA) at 30 V constant voltage overnight. The membrane was blocked with 3% gelatin in 20 mM Tris and 500 mM NaCl. The first antibody solution was 20 μl Tris, 500 mM NaCl, 0.05% Tween 20, 1% gelatin containing the following antibodies at a concentration of 1 mg/100 ml: rabbit anti-human IL-1α (Endogen P-420A), rabbit anti-human IL-8 (Endogen P-801), rabbit anti-human TNFα (Endogen P-300A), rabbit anti-human IL-6 (Endogen P-620). Incubation in the first antibody solution was for 2 h at room temperature. The second antibody solution contained the same buffer as the first with a 1:3,000 dilution of goat anti-rabbit IgG (H + L) alkaline phosphatase (BioRad). Incubation was for 1 h at room temperature. Colour was developed using an NBT/DCIP system as supplied in the BioRad Immuno-Blot Assay Kit. This system was easily able to separate and detect purified standards of IL-1α (pro ~ 31 KDa, expressed 17.5 KDa), IL-6 (pro 26 KDa, expressed 20.5 KDa), IL-8 (8.5 KDa), and TNFα (17 KDa).

**Radioimmunoassays:** RIAs were done using commercially available kits according to the manufacturer’s instructions. Interleukin-1α [125I], Interleukin-6 [125I], Interleukin-8 [125I], and TNFα [125I] assay systems were purchased from Amersham (Arlington Heights, IL). All eicosanoid kits were titrated and purchased from Advanced Magnetics (Cambridge, MA).

**Statistics:** Statistics were calculated using Microsoft Excel version 4.0 using the t test: two-sample assuming equal variances function. Significance was calculated at the α = 0.05 level.

**Results**

**Cercarial penetration of LSE:** Cercarial penetration into LSE membranes averaged 81 ± 2.6% as determined by recovery of cercariae from the membrane surface.

**Regulation of cytokine production:** Initial screening by Western Blots showed the presence of IL-1 (31 KDa form), IL-6 and IL-8 in all samples; however, the Western Blot did not prove sensitive enough to determine quantitative differences between these three cytokines. TNFα was not detected by Western Blot. Cytokine quantitation by RIA is shown in Fig. 1 (secreted) and Fig. 2 (tissue). These results show that cercarial penetration of LSE and topical treatment with IFNγ caused an increase in IL-1α secretion over controls (controls = 1.03 ± 0.15 ng vs. IFNγ = 1.90 ± 0.48 ng, p = 0.02; and cercariae = 1.79 ± 0.22 ng, p = 0.02). There was no significant difference in IL-1α secretion between IFNγ treatment and cercarial penetration. IL-6 production was not significantly different between all three groups (average of 1.97 ± 0.35 ng). Cercariea down-regulated the production of IL-8 when compared to IFNγ (cercariae = 11.13 ± 1.70 ng vs. IFNγ = 16.47 ± 0.29 ng, p = 0.04). IL-8 levels in LSE exposed to cercariae were not significantly different from untreated controls (cercariae = 11.13 ± 1.70 ng vs. controls = 8.48 ± 1.47 ng, p = 0.12). Tissue cytokine levels remained relatively constant regardless of treatment. TNFα was not detected by RIA, confirming the Western Blot analysis.

**Regulation of eicosanoid production:** Figure 3 shows the results of cercarial regulation of eicosanoid
Regulation of LSE cytokine and eicosanoids

FIG. 1. Living Skin Equivalent (LSE, Organogenesis) secretion of cytokines into the assay media. The air interface side of the LSE (stratum corneum side) was coated with 4 μg/cm² linoleic acid and then exposed to either 100 ng IFNγ (IFN), 850 S. mansoni cercariae (CER), or nothing (CTRL) for 18–20 h at 35°C. Cytokine levels in the assay media were measured directly by RIA. Numbers represent the number of replications for each group. Error bars are mean ± S.E.M. *p < 0.05 as compared with controls. Students test. Nonspecific binding (assay media only) has been subtracted.

FIG. 2. Tissue cytokine levels in Living Skin Equivalent (LSE, Organogenesis) exposed to either 100 ng IFNγ, 850 S. mansoni cercariae (CER), or nothing (CTRL). Details as in Fig. 1 except that LSE tissue was homogenized in 5 ml of assay media and cytokines levels of the homogenate were measured by RIA. Numbers represent the number of replications for each group. Error bars are mean ± S.E.M. *p < 0.05 as compared with controls. Students t-test. Nonspecific binding (assay media only) has been subtracted.

production. IFNγ was used as a control inflammatory agent. IFNγ treatment caused an increase in LTB₄ of almost 260% although no significant increase was seen in the other eicosanoids examined. Cercarial penetration caused a decrease in LTB₄ production when compared with the increase caused by IFNγ (control = 73.9 ± 30.42 pg/0.1 ml vs. IFNγ = 193.42 ± 44.21 pg/0.1 ml vs. cercariae = 98.86 ± 19.65 pg/0.1 ml). There were no statistically significant changes in 12-HETE, 5-HETE, PGE₂, and LTC₄ production in LSE exposed to either IFNγ or cercariae.

Discussion

The skin is a formidable barrier against infection and it is now known that the keratinocyte plays an active and key role in skin immunology. Keratinocytes are normally found in a resting state. In this state keratinocytes contain internal stores of IL-1, relatively few IL-1 receptors, and lack the enzymes necessary to convert pro IL-1β to its active form. In addition, resting keratinocytes also secrete an IL-1r antagonist (IL-1ra) to further prevent response to IL-1 under normal (resting) conditions. As keratinocytes differentiate into the stratum corneum, the IL-1 still remains associated with the cell. Both the stratum corneum and sweat have been shown to contain high levels of IL-1α and IL-1β. When the stratum corneum layer is disrupted and/or keratinocytes are damaged this causes a release of proinflammatory IL-1. IL-1α (pro or processed) then binds to receptors contained on nearby keratinocytes. This initiates the up-
regulation of IL-1 receptors (IL-1r), the presumed down-regulation of IL-1ra, and the synthesis and secretion of additional IL-1 by neighbouring keratinocytes. The keratinocyte is now considered 'activated'. Keratinocytes can also be activated by PGE (increase IL-1r), LTB_4 (induces secretion of IL-1) or IFNγ (induces both IL-1 secretion and increase in IL-1r).

Once keratinocytes are 'activated' they synthesize and secrete a wide variety of cytokine and eicosanoid factors involved in inflammation. These factors include those chemotactic for various inflammation cells (IL-8: neutrophils, macrophages, T-cells; 12-(R)HETE: neutrophils; LTB_4: neutrophils, eosinophils; 5-HETE: eosinophils, neutrophils, fibroblasts; PAF: eosinophils; IL-1: leukocytes; and TNFα: leukocytes), factors that cause inflammatory cell activation (IL-6: T-cell; GM-CSF: neutrophils, eosinophils; IL-8: neutrophils; IL-1, G-CSF, and TNFα: neutrophils, eosinophils, macrophages) and factors that cause the proliferation of fibroblasts and keratinocytes and are instrumental in the wound healing process (12-(R)HETE, 5-(S)HETE, LTB_4, IL-1, and TGF).

Given this sequence of events, cercarial penetration should cause an intense inflammatory reaction. During skin penetration the stratum corneum should be disrupted thereby releasing stored IL-1. In addition, penetration and migration of _S. mansoni_ causes extensive tissue destruction, thus further IL-1 should be released. Finally, cercarie and schistosomulae synthesize both PGE_2 and LTB_4, in vitro, both of which should activate keratinocytes. To further complicate the matter, schistosomulae have been shown to be killed, in vitro, by macrophages activated by IFNγ and TNF, and platelets stimulated by TNF. Also, the schistosomulae itself is a source of foreign antigen and should activate the skin SALT (skin associated lymphoid tissue) system (predominately Langerhans's cells in humans) which, again, should induce an inflammatory response resulting in schisto-
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