Causation of Crohn's disease by *Mycobacterium avium* subspecies *paratuberculosis*

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J Hermon-Taylor, TJ Bull, JM Sheridan, J Cheng, ML Stellakis, N Sumar. Causation of Crohn's disease by *Mycobacterium avium* subspecies *paratuberculosis*. Can J Gastroenterol 2000;14(6):521-539. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is a member of the M *avium complex* (MAC). It differs genetically from other MAC in having 14 to 18 copies of IS900 and a single cassette of DNA involved in the biosynthesis of surface carbohydrate. Unlike other MAC, MAP is a specific cause of chronic inflammation of the intestine in many animal species, including primates. The disease ranges from pluribacillary to paucimicrobial, with chronic granulomatous inflammation like leprosy in humans. MAP infection can persist for years without causing clinical disease. The herd prevalence of MAP infection in Western Europe and North America is reported in the range 21% to 54%. These subclinically infected animals shed MAP in their milk and onto pastures. MAP is more robust than tuberculosis, and the risk that is conveyed to human populations in retail milk and in domestic water supplies is high. MAP is harboured in the ileocolonic mucosa of a proportion of normal people and can be detected in a high proportion of full thickness samples of inflamed Crohn's disease gut by improved culture systems and IS900 polymerase chain reaction if the correct methods are used. MAP in Crohn's disease is present in a protease-resistant nonbacillary form, can evade immune recognition and probably causes an immune dysregulation. As with other MAC, MAP is resistant to most standard antituberculous drugs. Treatment of Crohn's disease with combinations of drugs more active against MAC such as rifabutin and clarithromycin can bring about a profound improvement and, in a few cases, apparent disease eradication. New drugs as well as effective MAP vaccines for animals and humans are needed. The problems caused by MAP constitute a public health issue of tragic proportions for which a range of remedial measures are urgently needed.

Key Words: *Antimicrobial chemotherapy; Crohn's disease; Food safety; Johne's disease;* Mycobacterium avium *subspecies* paratuberculosis; *Polymerase chain reaction; Potable water; vaccine*

La maladie de Crohn causée par *Mycobacterium avium* sous-espèce *paratuberculosis*

RÉSUMÉ : Mycobacterium avium sous-espèce *paratuberculosis* (MAP) appartient au complexe M. *avium* (MAC). Elle diffère génétiquement des autres MAC en possédant 14 à 18 copies de la SI900 et un seul fragment d'ADN mobile impliqué dans la biosynthèse de glucides de surface. À l'inverse des autres MAC, MAP est une cause spécifique d'inflammation intestinale chez de nombreuses espèces animales, y compris les primates. La maladie va de pluribacillaire à paucibacillaire, avec une inflammation granulomateuse chronique comme la lèpre chez les humains. L'infection à MAP peut persister pendant des années sans causer de maladie clinique. On fait état d'un taux de prévalence de l'infection à MAP dans les troupeaux en Europe de l'Ouest et en Amérique du Nord compris entre 21 % et 54 %. Ces animaux porteurs d'une infection subclinique disséminent MAP dans leur lait et dans les pâturages. MAP est plus robuste que M. *tuberculosis* et le risque qu'elle soit transmise aux populations humaines par le lait vendu au détail et les systèmes de distribution d'eau domestique est élevé. MAP se loge dans la muqueuse de l'iléon et du colon chez une proportion d'individus sains et peut être décelée dans une proportion élevée d'échantillons entiers d'intestin enflammé par la maladie de Crohn au moyen de techniques de culture améliorées et par amplification en chaîne par polymérase de la SI900 si l'on utilise des méthodes appropriées. Dans la maladie de Crohn, MAP se présente sous une forme non bacillaire résistante aux protéases, peut esquiver une reconnaissance immunologique et cause probablement un dérèglement immunitaire. Tout comme les autres MAC, MAP est résistante à la plupart des traitements antituberculeux habituels. Le traitement de la maladie de Crohn avec des combinaisons de médicaments plus actives contre MAC comme la rifabutine et la clarithromycine peut apporter une importante amélioration et, dans certains cas, entraîner une éradication de la maladie. Il est nécessaire de développer de nouveaux médicaments de même que des vaccins efficaces contre MAP destinés aux animaux et aux humains. Les problèmes causés par MAP constituent une question de santé publique d'une envergure dramatique pour laquelle un ensemble de mesures curatives s'imposent d'urgence.

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Mycobacterium avium subspecies *paratuberculosis* (MAP), originally called Johne's bacillus, was first identified in 1895 as the cause of a chronic inflammatory disease of the intestine in a German cow (1). The perceptive proposition that this organism might also be involved in causing chronic inflammation of the intestine in humans was first published in 1913 (2). As hundreds of thousands of people in the developed societies of the temperate latitudes in the northern and southern hemispheres struggle with chronic inflammation of the intestine of the Crohn's disease type, much uncertainty obscures a clear understanding of the relationship between this pathogen and human disease. The purpose of the present analysis is to illuminate this issue and clarify the true nature of the threat to human populations posed by this pathogen.

GENETIC AND PHENOTYPIC DEFINITION OF MAP

MAP (3) is a member of the M *avium* complex (MAC). DNA sequence analysis of 16S rDNA, used to distinguish these organisms (4), demonstrates that MAP is very closely related to other MAC and that its rDNA does not differ by a single base pair from multiple serovars of other MAC organisms and *Mycobacterium intracellulare* (5-7). Similarly, DNA sequence analysis of the approximately 280 base pair internal transcribed spacer between 16S and 23S rDNA from four MAP isolates from bovine, primate and human sources showed identity between them and 17 strains of MAC (8). These other MAC are almost ubiquitous in the environment and in the intestines of healthy animals and humans (9,10), and do not usually cause disease unless the host is debilitated or immunocompromised. By contrast, MAP is a specific pathogen and is able to cause disease in apparently healthy animals. A detailed understanding of the molecular basis for pathogenicity and of how the genome of MAP differs from that of nonpathogenic MAC will only begin to accelerate when the whole genome sequence of at least one strain of MAP is available. At the present state of knowledge, however, three major genetic differences distinguish MAP from nonpathogenic MAC – the presence at conserved genomic loci in MAP of 14 to 18 copies of the DNA insertion element IS900 (11), a single copy of a low percentage guanine plus cytosine genetic element designated 'GS' (12) and a third genomic region incorporating a gene designated *hsp*X (13). IS900 and GS can be envisaged as foreign DNA that at some time in the past has 'hit in' to background M *avium* species and contributed to the evolutionary development of MAP and to the acquisition of the pathogenic phenotype (14).

IS900 is a 1451 to 1453 base pair repetitive element and was the first DNA insertion sequence to be identified in mycobacteria. It belongs to a family of closely related elements that includes IS110 and IS116 in *Streptomyces* species (15,16) IS901 and IS902 the same element identified independently (17,18) in pathogenic M *avium* subspecies *silvaticum* (13,19) and IS1110 in M *avium* subspecies *avium* (20). Another member of the IS900 family, designated IS1613, present in six to eight copies in some M *avium* isolates from pigs and from humans both infected and not infected with human immunodeficiency virus (HIV), has recently been characterized (21,22). IS900 hijacks the genetic machinery of the host mycobacterium by specifically entering a consensus insertion sequence between the ribosomal binding site and the start codon of 14 to 18 specific genes in MAP (23,24). This process is likely to affect the expression of these target genes and contribute to phenotypic differences from other MAC. IS900 encodes a 43 kDa DNA binding putative transposase p43 on its positive strand. This protein has been shown by Western blotting and reverse transcription polymerase chain reaction (PCR) to be expressed by MAP cultured in vitro (25), as well as in the diseased intestine of humans with inflammatory bowel disease in vivo (26). IS900 has turned out to be uniquely specific for MAP and a convenient multicopy genomic target for the PCR detection and DNA 'fingerprinting' of this difficult organism.

GS in MAP (Genbank accession numbers AJ223833 and AJ223832) was discovered by subtracting the DNA of a nonpathogenic M *avium* from MAP by using representational differential analysis (12). GS has some of the characteristics seen in 'pathogenicity islands' in other bacteria (27). It occurs at the same genetic locus in all MAP isolates that the present authors have examined so far and is flanked downstream by the daunorubicin resistance operon *drr*, located at Rv2936-Rv2938 in *Mycobacterium tuberculosis* (28) and upstream by a GDP glucose dehydrogenase. GS is 6496 base pairs long and is flanked by the inverted repeat sequence GGCCAATCGA. GS contains six genes, *gsa*, *gsb*A, *gsb*B, *gsc*, *gsd* and *mpa*. Available bioinformatics programs that search for membrane, secretory signal and other protein localization signals predict that *gsa*, *gsbA*, *gsbB* and *gsc* are located within the cytoplasm of MAP. By analysis of *mpa*, 10 transmembrane regions are predicted, indicating that this protein would be tightly embedded in the microbial plasma membrane. By analysis of *gsd*, an *N*-terminal secretory signal sequence and a lipid attachment site that may cause *gsd* to be secreted out of or anchored to the microbial plasma membrane are predicted. From bioinformatics, it is predicted that *gsbA* and *gsbB* synthesize guanosine 5 -diphosphate-L-fucose (GDP-L-fucose). This fucose moiety is used by glycosyltransferases to attach fucose to other sugar units in growing oligosaccharide chains. The sequence of *gsd* indicates that a functional glycosyltransferase that may transfer GDP-fucose and *gsa* has a truncated and thus probably nonfunctional glycosyltransferase sequence. The sequence of *gsc* is homologous to sugar *O*-methylases, and *mpa* encodes an acetyltransferase sequence homologous to similar enzymes that *O*-acetylate sugars, including fucose. Homologues of *mpa* are closely linked to virulence in *Salmonella typhimurium* (29) and *Shigella flexnerii* (30), while the acquisition of a homologue to *gsc* by *Vibrio cholerae* was associated with its transition from an endemic to an epidemic strain (31,32). Homologues of all GS genes except *mpa* occur in M *tuberculosis* rearranged in two loci at Rv1511-1514 and Rv 2956-2957. The first of these loci, containing homologues of *gsa*,

*gsb*A, *gsb*B and *gsc*, lies within the RD4 region deleted in nonpathogenic *Mycobacterium bovis* Bacille Calmette-Guerin (33,34). Overall these data suggest a relationship between the presence of GS and the pathogenic phenotype in MAP.

These data are consistent with the predicted function of GS in the biosynthesis and modification of fucose, and its attachment to the terminal oligosaccharide moiety of surface glycopeptidolipid. The function of such a genetic element in simple terms can be envisaged as providing MAP with a surface 'Teflon' coat, related to its ability to survive inside the host cell and avoid immune recognition.

Our understanding of these apparent functions for GS in MAP was reinforced when the DNA sequence of the *ser2* gene cluster in pathogenic *M avium* serotype 2 became available late in 1998 and early 1999, and it was clear that this genomic region also contained GS genes (Genbank database accession numbers AF060183 and AF125999). The *ser2* region, which spans 22 to 27 kilobase pairs, contains a section with genes that are 99% homologous to GS genes but are rearranged at a genomic location different from that in MAP. In some strains of *M avium* serotype 2, the *ser2* gene cluster is flanked by an IS21-like insertion element, IS1612, which is absent from MAP. In other strains of *M avium* serotype 2 and in *M avium* subspecies *silvaticum*, which is less pathogenic than MAP, one copy of IS1612 is inserted within the *mpa* gene, probably disrupting its transcription. In further *M avium* serotype 2 isolates, *mpa* and the copy of IS*1612* it contains are deleted altogether. The *ser2* region in pathogenic *M avium* serotype 2 has been shown to function in the synthesis of glycopeptidolipid (35-38). Genomic deletions of *ser2* genes result in the loss of glycopeptidolipid expression and the permanent conversion from pathogenic smooth transparent colonies to the rough nonpathogenic phenotype (39). These, therefore, are some of the defining characteristics of MAP that distinguish it from other MAC and contribute to its ability to cause disease in animals and humans. The pace of this research has been painfully slow, and there is much more that we need to know.

DIFFICULTIES IN THE LABORATORY CULTURE OF MAP

Until recently, knowledge of microbiology was essentially limited to organisms that could be grown in the laboratory. The advent of molecular methods for detecting and characterizing microorganisms has shown that there are vastly more bacteria in natural ecosystems than those that are culturable by standard techniques (40,41). Progress in our understanding of MAP has been considerably retarded by the substantial difficulties in culturing this organism. Its ability to be cultured in vitro occupies a range intermediate between that of *M tuberculosis* and *Mycobacterium leprae*. It was seventeen years after its original description before Twort and Ingram (42) in 1912 first reported that MAP from infected cattle could be grown in the laboratory in cultures enriched with egg yolk and in the presence of extracts of *M tuberculosis* or the Timothy grass bacillus *Mycobacterium phlei*. Even then, MAP grew very slowly and the cultures were often overgrown by other organisms in the sample – difficulties that persist in the laboratory culture of MAP to this day. Although improvements have come from better methods of sample decontamination (43) and the addition of mycobactin J, reliable detection of MAP using conventional culture to the recognizable bacillary form remains lengthy and uncertain. Such conventional cultures are of little practical use for the study of MAP in the environment or in foods at risk. Veterinary diagnosis of MAP infection by conventional fecal culture requires up to 24 weeks of incubation, and results are falsely negative in about 20% of infected cattle and up to 80% of infected sheep. The introduction of commercially available BACTEC and MGIT liquid culture systems (Becton Dickinson, Franklin Lakes, New Jersey), together with the application of IS900 PCR to these cultures, has resulted in substantial improvements in the ability to detect subclinical MAP infection in ruminants, particularly sheep (44,45).

EVOLUTION AND STRAIN DIVERSITY IN MAP

In laboratory culture, as well as in environmental ecosystems and in the infected host, bacteria can undergo a high rate of mutation, with the emergence of new strains and a divergent phenotype (46-49). These adaptations can occur quite rapidly (50) and result both from the lateral transfer of DNA between organisms and from mutation or loss of pre-existing genes within organisms (51,52). Such changes have influenced the evolution of diseases such as cholera (53) and bacterial meningitis in humans (54). With the opportunity to amplify in the efficient but intensive farming of developed societies for over 100 years, MAP has probably undergone a similar but slower adaptive radiation and has made the intestine of animals and humans one of its natural habitats, acquiring an intermediate status between an environmental organism and a low grade pathogen.

Restriction endonuclease analysis, pulsed-field gel electrophoresis and IS900 restriction fragment length polymorphisms (RFLPs) have clearly distinguished among some cattle and sheep isolates of MAP with additional geographical differences (55-59). An IS900 RFLP comparison of four human Crohn's disease and nine Johne's disease isolates of MAP in France demonstrated both similarities and differences between the human strains of MAP and those isolated from cattle and goats (60). An extensive study by Pavlik (61) in the Czech Republic of 1008 cultures of MAP isolated from many species around the world and including environmental and milk isolates demonstrated 28 different IS900 RFLP types. Strain differentiation by PCR has the obvious advantage, particularly in the case of MAP, of being independent of the need for culture. Tim Bull (personal communication) has developed a multiplex PCR system using a common IS900 primer with a locus-specific primer that reports the presence or absence of the element at each of 14 loci. This system distinguishes between some bovine and ovine strains and suggests the possible emergence of a 'human' type that lacks the insertion of IS900 at a specific

genomic locus. Other PCR methods have been based on random amplified polymorphic DNA patterns (62) and on the identification of polymorphisms in amplification products of IS1311 (63). Further advances in the strain differentiation of MAP are needed and will require collaborative studies among laboratories in different countries on a scale recently reported for *M tuberculosis* (64). These studies suggest, however, that MAP exists in multiple forms and that both it and the diseases that it causes in animals and humans are likely to be in a state of dynamic change and evolution.

MAP DISEASE IN ANIMALS AND THE PREVALENCE OF SUBCLINICAL INFECTION

MAP is a specific cause of chronic inflammation of the intestine in many different ruminants, including rare species (65-69), monogastrics such as dogs and pigs (70-72) and, so far, four different types of subhuman primates – macaques (73), baboons, gibbon and cotton-top tamarins (M Collins, personal communication); MAP shows a marked tissue tropism and causes chronic inflammation of the intestine, even if administered subcutaneously or intravenously. This has so far been demonstrated experimentally in adult cattle, rabbits (65), chickens (74), horses (75) and calves (76). MAP may persist in the gastrointestinal tract and other tissues of animals for years without causing clinical disease (66,70,77). MAP causes systemic infection and traffics widely in macrophages in both subclinically and clinically infected animals (70,78,79). The organism parasitizes the reproductive organs of both males and females (80-83) and can cross the placenta to enter the fetus (84,85). Subclinically infected animals may develop clinical Johne's disease if stressed.

MAP disease in animals exhibits a broad range of histopathological characteristics extending from pluribacillary disease with abundant Ziehl-Neelsen-positive acid-fast bacilli visible microscopically in the intestine, to the Ziehl-Neelsen-negative paucimicrobial form of the disease with chronic granulomatous inflammation like leprosy in humans (77,86-88). In paucimicrobial disease, the standard veterinary serological tests for MAP infection are unreliable or negative (89). The pathology of the disease varies considerably among animal species and among different organs in the same infected animal, so that granulomatous lesions in the liver showing no visible acid-fast MAP microscopically may coexist with pluribacillary disease in the intestine (90-92). The regions of the gastrointestinal tract usually affected are the terminal ileum and adjacent colon, but segmental lesions more proximally in the gut, as well as colonic and rectal involvement, are frequently seen. The gut wall is thickened with occasional mucosal ulcers and enlargement of the regional lymph nodes. Animals with Johne's disease die of their infection, and although perforation, stricture and fistula formation are not usually seen, these features are known to occur in regional ileitis and colitis in dogs and pigs (93,94). Wasting and protein loss are almost invariable features of clinical paratuberculosis in animals, but diarrhea is by no means constant, particularly in small ruminants such as sheep and goats (66,69,77). MAP infection of domestic livestock is widespread in Western Europe and appears to be spreading east into countries such as the Czech Republic, and south to the sheep flocks of Sardinia and Morocco, where a recent study reported that 30% of the animals tested were positive by fecal culture (95). A serological survey of 98 dairy herds in Belgium carried out between December 1997 and March 1998 reported a herd prevalence of subclinical MAP infection of 32% (96). In another study, fecal culture performed at six-month intervals over two years on pooled fecal samples from 100 dairy herds in the northern provinces of The Netherlands recently reported a herd prevalence of subclinical MAP infection of 40%, in the absence of any previous evidence of clinical paratuberculosis in these herds or of a history of animals imported into the herds over the past five years (97). The experimental seroprevalence of MAP infection in sheep and goats in the Madrid region of Spain was recently found to be 11.7%, but given the low sensitivity of the test, the true seroprevalence was estimated to be up to 44% (98). Paratuberculosis appears to be emerging in Ireland (99). An IS900 PCR study of intestinal and other tissues of 1553 cull cows coming to abattoirs in southwest England in 1994 reported a subclinical infection rate for individual animals of 3.5% (100). Because of advances that have since occurred, particularly in sample processing, the results of this important study are likely to be substantially underestimated, and the true prevalence of subclinical MAP infection in Britain remains unknown. In the United States and Canada, MAP infection is known to be endemic in domestic livestock, particularly cattle (101-105). A survey carried out in the United States in 1996 by the National Animal Health Monitoring System covering 20 states representing 79.4% of American dairy cows found that the herd prevalence of MAP infection was 21.6% (106). The prevalence of seropositive subclinically infected dairy herds in Michigan was recently reported to be 54% (107). In the same study, 6.9% of 3886 individual animals tested were serologically positive for MAP. In Ontario from 1986 to 1989 (103), it was shown that 5.5% of 400 cull cows were culture-positive for MAP, and the individual animal seropositivity rate among 14,923 dairy cattle from 304 herds was 6.1%. The risk to public health lies in the extent of subclinical MAP infection in domestic livestock.

TRANSMISSION OF MAP TO HUMANS IN RETAIL COWS' MILK

It has long been known that MAP can be cultured from the milk of clinically infected cows with Johne's disease (108-110). More recent work has shown that MAP can also be cultured from the milk of apparently healthy subclinically infected cows. Sweeney et al (111) from the University of Pennsylvania, Philadelphia, Pennsylvania, cultured MAP from the milk of 19% of healthy cows that were heavy fecal shedders of MAP and from the milk of 5% of healthy cows that were intermediate or light shedders of the organism. Streeter et al (112) from Ohio State University, Columbus, Ohio, cultured MAP from the colostrum and milk of 30% of fecal culture-positive, clinically normal animals. Relying as

they do on the ability of the MAP from these animals to survive decontamination by overnight incubation in 0.75% hexadecylpyridinium chloride and then be culturable, such studies inevitably underestimate the true prevalence of these difficult to culture pathogens.

Work carried out in Dallas, Texas more than 35 years ago found that faster growing, nontuberculous mycobacteria could be cultured from 34% of samples of raw milk taken from tank trucks arriving at processing plants between November 1962 and September 1963 (113). The same researchers also reported to the American Thoracic Society Meeting in Houston on May 28, 1968 that they had cultured nontuberculous, acid-fast mycobacteria from 13 of 458 (2.8%) samples of homogenized pasteurized cows milk (at the time cited as usually 85°C [186°F] for 15 s) taken from pint or quart cartons destined for delivery to consumers (114). Pasteurized milk and dairy products are well known to be a potential vehicle for the transmission of other less robust pathogens such as *Listeria monocytogenes*, *Salmonella* species and *Campylobacter* species to human populations (115,116). Given the high prevalence of MAP in the dairy herds and domestic livestock of Western Europe and North America, it is inevitable that MAP will from time to time be present in bulk tank milk being brought to pasteurization plants throughout both continents. The only thing that stands between these live chronic enteric pathogens and their consumption by humans is the commercial pasteurization process, which is variably practised, but is commonly 72°C for 15 s. The critical question becomes, does this treatment consistently ensure the destruction of all viable MAP?

Where the required endpoint for food safety is microbial death, but where the methodological endpoint in conventional tests for process control is limited to culturability, this is not an easy question to answer experimentally for MAP (117,118). Since 1993, seven studies have shown that bacillary-form MAP prepared in in vitro cultures, spiked into whole cows' milk at a range of microbial concentrations and then treated with experimental pasteurization, remained culturable from some samples after exposure to 65°C for 30 mins (the standard holder method) or 72°C for 15 s (the high temperature, short time method) (119-125). These studies have been criticized principally on the grounds that experimental pasteurization does not accurately reproduce the conditions such as turbulent flow that occur in commercial pasteurization units (126). Two other studies (127,128) reported the complete loss of culturability of MAP spiked into milk and heated to 72°C for 15 s in either a laboratory scale pasteurizer unit representing a miniature version of industrial pasteurizers or in capillary tubes submerged in a circulating water bath. The validity of the first of these studies is undermined because the MAP, known to be disabled by freezing and thawing, was frozen and thawed as well as sonicated beforehand, both of which treatments may have increased the susceptibility of MAP to heat shock. The heat-shocked organisms were then diluted 10-fold and resonicated before culture, which was on solid media only and limited to an incubation period of 12 weeks (126). These authors concluded that "treatment of raw milk at 72°C for 15 s effectively killed all *Mycobacterium paratuberculosis*", whereas a preferred interpretation would be that they could not culture frozen/thawed, sonicated and heat-treated MAP from milk following the methods that they used. Their further conclusion that their results indicated that "transmission of viable *M paratuberculosis* from animals to humans via pasteurized dairy products is unlikely", is, therefore, wholly unsafe. Keswani and Frank (128) diluted the experimentally pasteurized milk samples 100-fold before culturing on solid media.

An extensive survey carried out in England and Wales from 1990 to 1994 found that an overall 7% of cartons and bottles of retail whole pasteurized cows' milk tested positive for MAP by IS900 PCR (129). The sensitivity of the test at that time was not great because of the early stage of development of sample processing procedures. There was, however, a conspicuous seasonality in the occurrence of cartons testing positive, reminiscent of that described earlier for other nontuberculous mycobacteria in pasteurized milk from Dallas, Texas, by Chapman and Speight (114). The distribution of positive PCR signals in centrifugal cream and pellet fractions of retail milk was consistent with the presence of intact MAP. Liquid cultures inoculated with MAP-positive samples of cream or pellet subsequently demonstrated the microscopic presence of sparse clumps of acid-fast mycobacteria when examined within four to 12 weeks of incubation. These cultures in multiple flasks were strongly positive by IS900 PCR and suggested the presence of residual viable MAP. These cultures invariably went on to become overgrown by other organisms, and proof of live MAP by subculture onto solid media was not obtained. However, 50% of PCR-positive and 16% of PCR-negative cartons of retail milk subsequently gave rise to long term liquid cultures whose centrifugal pellets were strongly IS900 PCR-positive, sometimes in multiple flasks in a manner that was not explicable on the basis of carryover of naked DNA or dead organisms from the original milk fractions. Although they fall short of proof, these findings are consistent with the residual presence of a very slowly replicating population of MAP in retail pasteurized milk in the United Kingdom and a high risk of human exposure to these pathogens.

Subsequent research by Irene Grant (130) and her colleagues at the Queen's University Belfast, Northern Ireland, funded by the United Kingdom Ministry of Agriculture, using raw milk spiked at 10^6 colony-forming units/mL, confirmed the ability of MAP to survive pasteurization conditions at 72°C for 15 s, as well as demonstrated a considerable range in the heat tolerance of different strains of MAP right up to residual culturability after 90°C for 15 s. However, none of the strains investigated remained culturable after exposure to 72°C for 25 s, suggesting that extension of the holding time is more likely to achieve complete inactivation of MAP in milk (130). Ongoing work in the same laboratory, using improved sample processing procedures such as immunomagnetic capture of MAP (131) and optimized decontamination before culture and IS900 PCR, is demonstrating

MAP in about 10% of samples of retail pasteurized cows' milk widely obtained in the United Kingdom. Acid-fast organisms visible microscopically in IS900 PCR-positive liquid cultures from these retail milk samples, and the occurrence of very small, slow growing colonies on solid media with the appearances of MAP that are also IS900 PCR-positive, strongly suggest the residual presence of viable MAP in retail pasteurized milk in the United Kingdom.

The issue of residual viable MAP in retail pasteurized milk is critical to public health and to the dairy industry. When assessing this risk, it is essential to retain a clear understanding of the limitations of the experimental methods that have so far been applied and to ensure that the results of tests on milk are meticulously interpreted. The outcome of spiking experiments in other systems is influenced by a varying microbial thermotolerance depending on how test organisms are prepared (132,133), as well as by the methods used in their recovery (134). The phenotype of endogenous MAP in natural raw milk may differ substantially from the phenotype of in vitro cultured MAP used in spiking experiments. There are also problems of sublethal injury (135), the ability of bacteria to adopt the viable but nonculturable state (136) and the demonstration that pathogens such as *V cholerae* may revert to a viable state in the human intestine (137). Compounding these uncertainties is the historic difficulty of accurately detecting the presence of viable MAP, particularly in low abundance, using conventional culture. The inability of Rahn et al (138) to culture MAP from unpasteurized bulk tank milk samples collected from 1224 dairy farms in Ontario led these authors to reassure health authorities and consumers that the risk of exposure to MAP from milk in Ontario is "extremely low". There is a high risk that such reassurance does not reflect what is actually happening, particularly given the high prevalence of subclinical MAP infection in dairy herds in North America, and in Ontario in particular (103). Molecular methods for detecting MAP and new procedures for assessing microbial viability and food safety need to be developed and applied (139).

Unfortunately, there is more. Until it is proved otherwise, ultrahigh temperature treatment of milk at 132°C for 1 s, which kills dispersed vegetative bacteria and confers long life properties on the retail product, cannot be assumed to ensure the destruction of all viable MAP that is characteristically present in protective clumps (140). Nor can it be assumed by regulatory authorities that, because MAP could not be cultured from experimentally pasteurized milk (72°C for 15 s) previously spiked with 10 colony forming units/mL or less (125), the enteric pathogens had all been killed and that retail pasteurized milk containing MAP at or below this abundance exposed to current pasteurization conditions is, therefore, safe. In 1997, people in Britain consumed an average of 2.23 L of liquid milk/head/week (141). Studies from 1990 to 1994 (129) estimated the detection limit of the test applied to retail milk at a level of about 200 MAP/mL. This estimate is likely to be rather inaccurate, but even if the true abundance of viable MAP were overestimated by 20-fold, it would still equate with an individual consumption of about 90,000 of these robust, versatile mycobacteria each month during peak periods of spring and autumn. These organisms are specifically taken up by the terminal ileum and other regions of the intestine in animals, where they may remain for years without necessarily causing clinical disease (142,143). The acquisition of a resident population of MAP in the intestine of humans is cumulative and may subsequently result in the development of chronic inflammatory disease in people with an inherited or acquired susceptibility. There is a clear need to increase the volume and intensity of research into the presence of MAP in dairy products and other food items at risk, using contemporary molecular methods. In the meantime, taking into account the information available (124,130,144), it would be prudent to stop the sale of raw milk (currently permitted in the United Kingdom) from source regions in which subclinical infection with MAP is widespread in dairy herds and to implement an increased stringency of milk pasteurization.

MAP IN THE ENVIRONMENT AND DOMESTIC WATER SUPPLIES

In the first half of the 20th century, dairy cows and domestic livestock were extensively infected with *M bovis* (145). The organism was conveyed to human populations in milk supplies. The problem was overcome by tuberculin testing of herds and introducing milk pasteurization using conditions known to destroy these well recognized pathogens (146). Subclinically and clinically infected livestock are now shedding abundant MAP onto pastures. Unlike *M bovis*, MAP can survive in the environment for prolonged periods (70,147,148). A further contribution to the environmental contamination by MAP is made by wildlife reservoirs such as infected deer and rabbits (149). Microorganisms with recognized zoonotic potential such as *Escherichia coli* 0157, *Campylobacter* species, *L monocytogenes* and *Cryptosporidium* species, which also survive in the environment, are known to access human populations in water supplies (150-153). Other MAC organisms, widely distributed in the environment and in natural waters, act as a source of nontuberculous mycobacterial disease in humans where infection is acquired, not by person to person transmission but by environmental exposure (154-156). Drinking water acts as a source of *M avium* superinfections in humans with acquired immunodeficiency syndrome and primates with simian immunodeficiency virus (157-159).

What then is known about the environmental distribution and ecology of MAP? The astonishing answer is nothing at all. In the absence of any data, a model of what is likely to be happening must be constructed from what is already know to occur in the case of other pathogens and closely related mycobacteria. Research in India in the late 1970s showed that *M avium* could be taken up and replicated within trophozoites of *Acanthamoeba castellanii* (160). The mycobacteria were noted to transfer to the cytoplasm of amebic daughter cells during mitotic division. Further work in recent years has revealed an increasing number of human pathogens, including *Legionella pneumophilia, L monocyto-*

genes, V cholerae, Salmonella species, *Chlamydia pneumoniae* and other mycobacteria, which may infect and replicate within protozoa (161). Like MAP, many of these organisms are intracellular pathogens and are harboured within macrophages in the infected animal or human host. Amoebae, which are also very widely distributed, can be envisaged as environmental 'macrophages' and are known to use mechanisms for receptor recognition, phagocytosis, respiratory burst and inhibition of phagosome-lysosome fusion in their interaction with microorganisms, which are also seen in macrophages (162-165). Interaction with protozoa in the environment and in biofilm communities can profoundly influence microbial survival and virulence (166,167). *M avium* grown in vacuoles in *A castellanii* develops an increased capacity to infect other amoebae, macrophages and human HT29 colonic epithelial cells, as well as an enhanced virulence in the beige mouse model of infection (168). *M avium* can survive within the walls of the robust, encysted form of *Acanthamoeba polyphaga* (169). Similar overall changes have been demonstrated in the interaction of amoebae with other bacterial pathogens, including resuscitation of viable but nonculturable forms (170), intracellular multiplication (171), alteration of microbial surface properties (172), enhancement of invasion (173), increased resistance to antibiotics and chlorination (174,175) and resistance to heat (176). The intimacy of such prolonged interactions both between the intracellular pathogen and the host cell and between parasitized host cells and other inhabitants in bacterial biofilms can have a profound effect on the molecular ecology and pathogenicity of bacteria (177-181).

Based on this abundance of data from other systems, our concept of what is happening with MAP is as follows. Rains falling onto contaminated pastures wash plumes of MAP into ground waters and rivers. Some of the organisms are planktonic, some are in characteristic clumps, and some are harboured within protozoa abundant in soil and natural waters. Intracellular adaptation in the environmental cycle through protozoa enhances the pathogenicity and resistance of MAP. Where a heavily contaminated river runs through a population centre, aerosols from surface water expose the neighbouring residents to inhalation of MAP, a risk well characterized for other environmental mycobacteria (182,183). Pulmonary involvement is well known in Crohn's disease (184-189), but given the tissue tropism of MAP, the principal clinical manifestation that emerges eventually is chronic enteritis. This is a likely explanation for the clustering of cases of Crohn's disease along the River Taff in South Wales, United Kingdom, where it runs through the city of Cardiff (190,191). Where major abstraction of water is taken from contaminated lakes or rivers for domestic supply, MAP that is unlikely to be removed or killed by water treatment procedures (192,193) will be conveyed to consumers. In the studies on Crohn's disease reported by Mishina and colleagues (26) from New York, New York, the reference strain of '*M avium*', which later turned out to be MAP, had been isolated from a filter used to test the drinking water supply of Los Angeles, California. Mycobacteria and amoebae are known to flourish in biofilms in domestic hot water systems (194,195). These are ecological niches where waterborne MAP arriving at domestic outlets in high dilution may accumulate and amplify. Two epidemiological studies carried out independently in the United Kingdom each showed a significantly increased risk of subsequent Crohn's disease (but not of ulcerative colitis) where the early childhood home had a continuous fixed hot water supply (196,197). The involvement of water supplies in the transmission of MAP to human communities in Canada has been discussed (198). Manitoba has the highest reported incidence of Crohn's disease in the world – 14.6/100,000 population (199). In considering why the incidence should be more than double that in Olmsted County (200) only 644 km to the south, it was disclosed that, in Manitoba, there was a sixfold difference in the incidence (range four to 23 per 100,000/ population/year) in Crohn's disease between the lowest and the highest incidence postal areas throughout the Manitoba study region (201). Further epidemiological research to identify the source waters and supply pathways to these low and high incidence areas, and a laboratory-based investigation into the presence of MAP in domestic water system biofilms in these different areas using appropriate molecular methods may provide some explanation for these substantial differences. Once again, in the case of MAP, there is a general need to increase the volume and intensity of environmental research.

DETECTION OF MAP IN CROHN'S DISEASE BY CULTURE AND PCR

Given the extensive prevalence in domestic livestock of an agent able to survive in food products from these animals as well as in the environment, it is unlikely that humans in the same regions would remain isolated from any exposure to these versatile pathogens. Renewed recognition of the potential involvement of MAP in the causation of Crohn's disease-type chronic inflammation of the intestine in humans owes much to the original work of Rod Chiodini (202) in culturing these organisms from human intestinal tissues. In this context, 'culture' means the ability eventually to isolate colonies in conventional solid or liquid media with the morphological, phenotypic and biochemical characteristics of very slow growing, mycobactin-dependent, bacillary-form MAP, which could then be subcultured and maintained in the laboratory. Over a 10-year period, such MAP isolates were achieved by several research workers but only in up to 5% of people with Crohn's disease and often after incubation for many months or years (203-208). The application of IS900 PCR to long term cultures has raised the detection rate of MAP in Crohn's disease gut to about 30% (209,210). IS900 PCR, using experimental methods carefully developed and optimized over many months of preliminary work and then applied directly to DNA extracts of full thickness surgically resected gut samples, revealed the presence of MAP in about two-thirds of people with Crohn's disease (211). Since then, there have been 18 peer reviewed reports of similar studies using a wide variety of sample processing

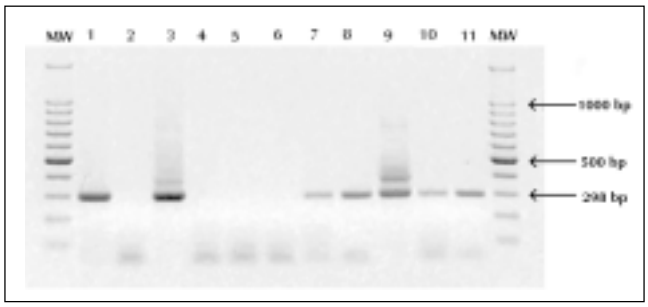

Figure 1) *Detection of* Mycobacterium avium *subspecies* paratuberculosis *(MAP) in inflamed human tissues by nested IS900 polymerase chain reaction (PCR). Tissue samples were lysed in SDS protease K. MAP DNA was released by mechanical disruption of the lysate at 6.5 m/s for 45 s in Hybaid Ribolyser (Hybaid US, Franklin, Massachusetts) in blue capped tubes. DNA was extracted by the phenol/chloroform method. Five microlitres of purified DNA amplified by nested PCR using the primers 5′-GAAGGGTGTTCGGGGCCGTCGCTTAGG-3′ with 5′-GGCGTTGAGGTCGATCGCCCACGTGAC-3′ for the first round 30 cycles and 5′-ATGTGGTTGCTGTGTTGGATGG-3′ with 5′-CCGCCGCAATCAACTCCAG-3′ for the second round 40 cycles, yielding a specific amplification product of 298 base pairs (bp). Lane 1 Positive control with IS900-containing plasmid pILD60 fewer than 50 copies; Lane 2 Negative buffer control; Lane 3 DNA extract from normal gut spiked with pILD60 fewer than 50 copies; Lanes 4,5 and 6 DNA extracts from the normal gut; Lane 7 Mesenteric lymph node from patient with Crohn's diease (CD) from Essex, United Kingdom; Lane 8 Surgical gut sample from a CD patient from the Czech Republic; Lane 9 Biopsy of oral granuloma, London, United Kingdom; Lane 10 Surgical gut sample from a CD patient, Scotland, United Kingdom; Lane 11 Surgical gut sample from a CD patient, Yorkshire, United Kingdom. MW Molecular weight ladder*

and PCR procedures, nine of which could identify MAP in Crohn's disease some or most of the time (26,212-219), and nine of which could not (220-228). A recent study from Sweden (229), which reported MAP in three of five surgical samples from patients with Crohn's disease, used 16S rDNA PCR, which is nonspecific and indicates the presence of M *intracellulare* and other MAC. Discrepancies and experimental difficulties have surrounded the PCR detection of other bacterial pathogens, particularly in the chronically inflamed, diseased tissues of people with tuberculosis (230), Lyme disease (231), brucellosis (232) and tuberculoid leprosy (233). Apart from obvious methodological errors, there are two main reasons for the conflicting results on the PCR detection of MAP in Crohn's disease. These reasons are the low abundance of the primary specific pathogen and the tough protease-resistant phenotype of MAP in humans and in sheep tissues from animals with the paucimicrobial form of Johne's disease. MAP in Crohn's disease is not a conventional spheroplast. Work by Ann Verstocken and David Winterbourne in our laboratory (234) showed that lysis of a Crohn's disease tissue sample in SDS proteinase K or 6 M guanidine thiocyanate, which would reliably release the DNA from most other bacteria, do not do so for MAP. Optimal access to target MAP DNA required the inclusion of a mechanical disruption step by vibrating the sample lysate in a slurry of silica and ceramic particles using the Hybaid Ribolyser system (Hybaid US, Franklin, Massachusetts). Figure 1 shows the results of using these optimized methods for people with Crohn's disease coming from different parts of the United Kingdom and the Czech Republic.

Shown in Figure 1 for the first time (lane 9) is the detection of MAP in a tissue biopsy from the mouth of a young boy suffering from a condition resembling orofacial granulomatosis. The additional higher molecular weight amplification product, similar to that seen in the positive control (lane 3), is characteristic of IS900 PCR in the presence of excess target DNA. This reflects a relative abundance of MAP in this granulomatous mouth lesion at an early stage of the infective process. A similar situation characterized the MAP cervical lymphadenitis described in a seven-year-old boy that preceded the onset of terminal ileal Crohn's disease by five years (235). At later stages of the disease process, the abundance of MAP in the chronically inflamed intestine is much lower.

In our view, these studies clearly show that MAP can be detected in Crohn's disease if the correct methods are used. Dr Saleh Naser and colleagues (personal communication) at the University of Central Florida have developed a system for the detection of MAP that comprises an approximately 10-week incubation of a decontaminated tissue extract in the improved mycobacteria growth indicator tube liquid culture medium available from Becton Dickinson (Sparks, Maryland), followed by IS900 PCR on the culture. This system exploits what may be a time window of limited microbial replication of MAP in the early few weeks following isolation from the sample and has the advantage of demonstrating the organism in an activated state. The results to date have identified MAP in six of seven (86%) full thickness surgical samples of intestinal wall from patients with Crohn's disease (236). Further work with larger numbers of patients and appropriate normal control tissues is in progress. Using the same system of mycobacteria growth indicator tube liquid culture followed by IS900 PCR on the culture, researchers have also isolated MAP from the centrifugal pellets (but not the cream fractions) of two samples of human breast milk obtained from each of two mothers with Crohn's disease who had recently given birth (237). In a similar manner, *Borrelia burgdorferi* has been identified in the breast milk of women with active Lyme disease (238). This pivotal result in Crohn's disease research, if confirmed, will demonstrate that in humans, as in animals, MAP infection is systemic and that the organism pursues the same sinister biological strategy of quietly seeking out the reproductive pathway to pass from infected parent to offspring when it is most susceptible. This may account for some but not all of the familial tendency that is well known in Crohn's disease.

IMMUNOLOGICAL RESPONSES TO MAP IN HUMANS

Compared with the advances that have come from the application of molecular diagnostics and recently improved culture systems, little progress seems to have come from the application of conventional immunological methods. This in itself may reveal something. A serological study in 1980 attempted to identify agglutination of three strains of MAP by

Crohn's disease sera. No response was seen with two of the strains, and the agglutination observed with the third MAP strain showed no difference between Crohn's disease and normal sera (239). Between 1984 and 1994, five research groups in the United States, Italy, United Kingdom and Argentina used crude extracts of 'M *paratuberculosis* strain 18' in ELISAs to look for differences in antibody binding between Crohn's disease and control sera (240-244). With one exception (240), no differences were reported. In the context of human infection, specifically with MAP, these studies are of doubtful validity because 'M *paratuberculosis* strain 18' is not MAP at all but an M *avium* species (245). Despite this doubt, three of the four negative studies were interpreted as providing evidence against a causal relationship between MAP and Crohn's disease. Three further studies conducted between 1988 and 1993 used crude extracts of human MAP strain Linda or a veterinary MAP isolate coated on ELISA plates to look for differences in antibody binding between Crohn's disease and control sera; no differences were found (246-248). A recent study from Japan (249), however, reported a significant increase in immunoglobulin (Ig) G binding to a crude protoplasmic extract of MAP by Crohn's disease sera compared with normal controls ($P<0.05$). Three other research groups have tested for differences in peripheral blood or mucosal cell-mediated immune (CMI) responses to sonicates of MAP, heat-killed MAP or purified protein derivative preparations of MAP between Crohn's disease and control subjects; again no differences were found (250-252). The authors of all these negative serological and CMI studies concluded that their data do not support a mycobacterial etiology for Crohn's disease.

These studies and the interpretations that have been placed on them suffer from two important flaws. First, humans are exposed and have immunity to MAC, to which MAP belongs and is very closely related. We might not expect to detect differences in serological or CMI reactivity between patients with Crohn's disease and those without Crohn's disease with the crude antigenic preparations used. Second, interpretations of the results are based on the general assumption that MAP disease in humans is expected to be like tuberculosis, where mechanisms of inflammation involve direct immunological reactions to many components of the organism and the mycobacterial cell wall. MAP in humans and in animals with established paucimicrobial disease does not have a classical mycobacterial cell wall; the organism is present in very low abundance. The pathogenic mechanisms involved in this type of paucimicrobial MAP disease are likely to be quite different. On the other hand, animal health care workers who may be repeatedly exposed to high dose bacillary-form MAP develop direct immunological responses to these organisms. These responses can be detected using crude antigen extracts of in vitro cultured bacillary-form MAP and are significantly different from those of control subjects who do not have such a high level of exposure (253). Such demonstrable immunity to bacillary-form MAP may be one of the reasons why veterinarians and farm workers do not apparently have a conspicuously high incidence of Crohn's disease, though anecdotally, cases of Crohn's disease linked to a clear exposure to animals with Johne's disease some years earlier are encountered by clinicians in the field.

More recent work has focused on antibody recognition of selected proteins and peptides of MAP, some of which are highly specific for the organism. Eighty-four per cent of Crohn's disease sera were found to recognize one or more of three proteins of 38 kDa, 24 kDa and 18 kDa from MAP (254). Research in Brussels, Belgium (255,256) demonstrated significant recognition by one-third of Crohn's disease sera, of a specific B cell epitope in the carboxyterminal 13.6 kDa portion of the 34 kDa component of the A36 immunodominant complex of MAP. This target is also recognized by sera from cattle infected with MAP (255,256). Fouad El-Zaatari and colleagues (257,258) from the Baylor College of Medicine, Houston, Texas, identified relevant MAP proteins p35 and p36 by screening a genomic expression library of MAP with rabbit antisera. Either or both of these were recognized by 93% of Crohn's disease sera and 26% of normal control sera. Antibody recognition of both proteins occurred in 77% of Crohn's disease and in none of the control sera ($P<0.001$) (259,260). Our own studies have demonstrated a peptide epitope in the carboxyterminal 12 kDa portion of p43 encoded by IS900, which is recognized by IgG from Crohn's disease sera (261,262). The results of these studies indicate a significant recognition of MAP in Crohn's disease but that it is relatively weak and only visible if highly specific immunological targets are selected. Although more research is needed in this area, it appears at present as if MAP infection in humans is associated with the ability of the organism to evade immune recognition – a strategy widely used by other pathogens (263).

PATHOGENIC MECHANISMS OF MAP IN HUMANS

MAP, when ingested or inhaled, is taken up primarily into macrophages (264). Depending on whether infection begins with a large dose or a slow accumulation, there may be a transient immunological response of the type seen in animal health workers, but in this case unrecognized in the absence of clinical manifestations. As with other mycobacteria, the organisms may end up free in the cytoplasm of macrophages, in a nonacid-fast form essentially invisible to the immune system, and persist in a state of latency for many years (265,266). As in animals, the colonization of the human host may remain subclinical, with either no disease or minimal nonspecific inflammatory changes visible only on endoscopy and biopsy usually performed for some other reason, and difficult to classify. In individuals who have inherited a susceptibility, or in others who become susceptible because of psychological (267,268) or physical stress including injury or intercurrent infection, the clinical manifestations of chronic enteritis eventually emerge. A chronic disease develops that is a synthesis of both immune activation and suppression (269), and is characterized by cycles of activation and remission (270).

A simple question that is frequently asked is, 'how can so few MAP cause so much inflammation and tissue damage in Crohn's disease'? The precise answer to this question is not known, but it is most unlikely that a major component of the disease mechanism is a direct reaction of the immune system to molecules or 'antigens' produced by MAP itself. It is much more likely that MAP parasitization of immunoregulatory cells causes an immune dysregulation of variable intensity, which, together with an increase in mucosal permeability (271-273), results in an exaggerated inflammatory and allergic response to leakage into the intestinal wall, of food residues and microorganisms that are normally present in the intestinal lumen (274). Perturbation and manipulation of cell-mediated immunity and cytokine responses are broadly identified in many mycobacterial diseases, including those caused by other MAC (275-281). An extreme example is the response induced by the polyketide mycolactone from *Mycobacterium ulcerans* (282). Perturbation of immune function and cytokine regulation occurs in Crohn's disease (283-286), and a chronic enteritis dependent on the presence of resident enteric bacteria (287) is induced by genetic knock-out of many genes in animals, including interleukin (IL) -10, IL-2 and *N*-cadherin (288-290). A pathogenic mechanism based upon a MAP-induced immune dysregulation would explain why Crohn's disease can be improved by suppressing or modulating the immune response itself or by reducing the intensity of the allergic component with accompanying changes in enteric flora by treatment with elemental diets. It also explains the clinical improvement that may follow the prolonged use of general antimicrobial agents such as metronidazole and ciprofloxacin. Without killing the underlying causative organisms, however, such therapeutic approaches do not usually achieve lasting resolution of the disease.

One interesting microscopic feature of Crohn's disease that has so far escaped a causative explanation but deserves to be mentioned is the observation of structural and inflammatory changes affecting the enteric nervous system in the intestinal wall. The lesions occur particularly in Auerbach's ganglia and around nerve fibres, and consist of an infiltrate of lymphocytes, mononuclear cells and eosinophils. The neural inflammation is accompanied by the expression of major histocompatability complex class II molecules on associated glial cells (291,292). MAP may share some of the neuropathic properties of *M leprae*. If so, a nonbacillary form of MAP may bind to alpha-dystroglycan, via a laminin intermediate (293,294).

TREATMENT OF MAP INFECTION IN ANIMALS AND HUMANS

From the description by Larsen et al (295) in 1950 from Auburn, Alabama, of the use of streptomycin in the treatment of four cows with Johne's disease, there have, to our knowledge, been 14 studies of the use of conventional antituberculous and antileprosy drugs in the treatment of MAP infection in animals (296-308). The animals tested included adult cattle and calves, sheep, goats and experimentally infected rabbits. The drugs used were streptomycin, isoniazid, clofazimine, rifampicin, ethambutol, pyrazinamide and dapsone, either as single agents or in combination (309). In general, the number of animals in these studies was small and the scope of the work was limited by the cost of the drugs. Randomized, controlled trials of these agents in experimentally or naturally MAP-infected animals were not done. Overall, the results of treatment were very similar. Where single agent therapy was used, either no effect or a transient clinical improvement with a reduction in fecal shedding was seen. Clinical improvement, if it occurred, usually lasted only a few weeks and was inevitably followed by relapse, either on treatment or after stopping the drug. The clinical and microbiological responses to drugs used in combinations such as streptomycin, isoniazid and rifampicin were more marked and more prolonged than with single agent therapy, but fecal shedding of MAP and eradication of the infection were never convincingly achieved, and persistence of disease and relapse occurred in the majority of these studies.

From 1975 to 1989, there were 11 anecdotal reports and open studies of the use of antimycobacterial drugs in the treatment of Crohn's disease. In 1975, Ward and McManus (310) in Edinburgh, United Kingdom, reported a marked clinical improvement in four of six patients with Crohn's disease treated with dapsone. A more extensive study (311) from Lille, France, in 1977 reported that 40 of 52 patients with severe Crohn's disease treated with various combinations of rifampicin, isoniazid, streptomycin and ethambutol showed clinical improvement, though the disease itself could not be eradicated. A similar improvement was reported from Paris, France, in Crohn's disease patients treated with rifampicin (312). Schultz et al (313) from Atlanta, Georgia, described the complete remission of severe Crohn's disease in a 52-year-old man treated with rifampicin, isoniazid, pyrazinamide and ethambutol. The patient had begun his career as a veterinarian, with extensive contact with both farm and small animals (313). The same drug combination used in a 60-year-old man with coexisting pulmonary tuberculosis and severe Crohn's disease was followed by the cessation of diarrhea of up to six times a day for the first time in 16 years and weight gain from 43 to 51.5 kg (314). Further examples of Crohn's disease responding to antituberculous drugs came also from studies in New York, New York (315), Genoa, Italy (316), Rome, Italy (317), London, United Kingdom (318) and Orebro, Sweden (319). Taken together, the results of these case reports and open studies represent the cumulative experience of this treatment approach in 107 selected patients with Crohn's disease from 11 different centres throughout North America and Western Europe. The message, which is consistent, is that there is a very small subgroup of people with Crohn's disease who show clinical improvement that is occasionally dramatic in response to treatment with conventional antituberculous chemotherapy. With few exceptions, however, clinical improvement is not lasting, and disease eradication has not been achieved.

A significant beneficial effect of antimycobacterial drugs to a larger proportion of people with Crohn's disease has not been substantiated in most randomized, controlled trials.

Shaffer et al (320) found no subsequent difference in Crohn's disease activity index between 14 patients treated with rifampicin and ethambutol, and 13 placebo controlled patients. A study from Dublin, Ireland, of 28 patients found that clofazimine used as a single agent was ineffective in inducing remission in Crohn's disease (321). Rutgeerts et al (322) from Leuven, Belgium, reported that rifabutin and ethambutol did not prevent recurrent Crohn's disease in the neoterminal ileum after surgery for Crohn's disease. In a further study from Rome, Italy, Prantera et al (323) randomly assigned 40 patients with severe refractory steroid-dependent Crohn's disease to receive rifampicin, ethambutol, clofazimine and dapsone, or placebo. Significant improvement in biochemical and hematological parameters in the treatment group compared with controls occurred, together with a relief of symptoms. In a controlled trial of rifampicin, isoniazid and ethambutol versus placebo, Swift et al (324) reported a significant reduction in abdominal pain, well being score and the presence of abdominal mass at two months in the treated versus the control group. This apparent improvement was not, however, maintained, and no long term advantage in the course of the disease was subsequently seen (325). These controlled trials involved a cumulative total of 245 patients.

Comparison of the results of treating MAP infections in animals and Crohn's disease in humans with antimycobacterial drugs needs to be approached with care. Naturally occurring and experimental MAP infection in animals almost always represent pluribacillary disease, with the organisms having established mycobacterial cell walls. The situation in Crohn's disease is one in which MAP is present in very low abundance, and with the organisms in a nonbacillary phenotype, so that differences in drug susceptibility between the animal and human disease might be predicted. Despite this difference, there are obvious similarities in the outcomes of the treatment of MAP-infected animals and of humans with Crohn's disease using antimycobacterial drugs. The impression in both cases is that, whereas on some occasions clinicopathological improvement may follow the use of combinations of multiple agents, remission is unlikely to be sustained and disease eradication will not be achieved. This is consistent with what has long been known – that MAC in general are resistant to standard antituberculous drugs (326-328). MAC can prevent these agents from penetrating the mycobacterial cell and can rapidly develop mutations that confer drug resistance (329-333). MAC infections in immunocompetent hosts are difficult to eradicate; prolonged treatment is required, and relapse either on treatment or off treatment is common.

An advance in the treatment of MAC infections in both HIV- and non-HIV-infected patients, as well as in the availability of candidate drugs for the treatment of Crohn's disease, came with the development of a new series of therapeutic agents that are chemical modifications of natural streptomyces antibiotics. Of particular relevance was rifabutin (ansamycin), a derivative of rifamycin-S (334,335), the macrolide clarithromycin, a derivative of natural erythromycins and the azalide azithromycin. These agents were found to have markedly improved activity against MAC in vitro (336-340), both alone and in combination with other agents. They also have the particular advantage of being concentrated within macrophages and other cells (341). Furthermore, rifabutin and clarithromycin demonstrated good activity in vitro against MAP (342,343) and appear to synergize (344). Early studies of the use of rifabutin in primates (macaques) naturally infected with MAP (73) and in six patients with Crohn's disease were promising (345). A preliminary report of a controlled trial of monotherapy with clarithromycin in 15 patients with Crohn's disease demonstrated sustained remission in the treatment group (346); however, a subsequent study of clarithromycin and ethambutol failed to show any benefit in Crohn's disease (347). Monotherapy with clarithromycin may be followed by an initial 'honeymoon' response in active Crohn's disease, but it invites the development of drug resistance and should be avoided (348-352). Both rifabutin and clarithromycin target microbial protein synthesis rather than inhibition of cell wall biosynthesis and were predicted to be applicable to the nonbacillary phenotype of MAP in Crohn's disease. We began a two-year outcome analysis of the use of a combination of these drugs in 46 patients with active Crohn's disease in 1992. This study demonstrated a highly significant improvement in the disease activity index in patients after six months of treatment that was maintained at two years ($P<0.001$) and a significant improvement in inflammatory parameters (353). The efficacy of rifabutin and macrolide therapy in active Crohn's disease was further supported by work carried out independently in Sydney, Australia (354), and a randomized, controlled multicentre trial of rifabutin, clarithromycin and clofazimine in Australia was initiated in September 1999.

About one-quarter of cases of active Crohn's disease are resistant to rifabutin and clarithromycin from the outset. Side effects may be troublesome in about half the patients, and relapses on treatment and after stopping a 2.5-year period of treatment occur. Fluoroquinolones are active against MAC (355-357) but suffer from the theoretical disadvantage that they target DNA replication, and the replication rate of MAP in humans is likely to be low. New drugs are needed, and the fucosyl transferases of the GS element in MAP are promising targets.

PROSPECTS FOR PREVENTIVE AND THERAPEUTIC MAP VACCINES

The situation with which we are challenged is a persistent and widespread infection in our food animals with an organism that can survive in the environment. Cycles of reinfection with MAP in each new generation of young animals comes by direct passage from parent to offspring and by the acquisition of infection from contaminated farm environments and pastures. A substantial reduction in the burden of infection can be made by altering farm practices, but the improvement that follows these measures is temporary. An additional policy of 'diagnose and cull' will also reduce the bur-

den of infection and environmental contamination by domestic animals, but in the presence of wildlife reservoirs, especially in intensely farmed regions, continued environmental contamination and a re-emergence of infection is inevitable. The 'diagnose and cull' strategy will not solve the problem, and the requirement for its continued application in the absence of other measures would be wasteful and hugely expensive. A low cost, effective animal vaccine is needed. Vaccines for MAP infection in animals have been around for years (358-365). The preparations used have either been heat-killed MAP or live attenuated MAP that in European studies has usually been the 'Weybridge' strain. The vaccination is given when the animal is young and results in a consistent major reduction (up to 93%) in the incidence of clinical disease. There are, however, two major problems. These whole MAP vaccines interfere with the diagnosis of M *tuberculosis* infection, particularly important in dairy animals, and although fecal shedding of MAP is usually reduced, subclinical infection remains. A good example of this comes from the extensive work carried out by the Animal Health Service North-Netherlands from 1984 to 1994 (366). In this study, calves and adult cattle were vaccinated with heat-killed whole MAP and monitored by clinical examination, by repeated fecal culture and by eventual postmortem examination sampling ileum, colon and lymph nodes for histopathology, Ziehl-Neelsen staining and culture for MAP. The findings showed conclusively that vaccination using whole heat-killed organisms reduced the rate of clinical Johne's disease by about 90% but did not prevent subclinical infection. Although the number of organisms was reduced, fecal shedding was not eliminated. This approach to the problem, therefore, drives it underground. Although systemic vaccination makes the body of the animal a hostile place for these pathogens, MAP persists in its preferred ecological niche in the gastrointestinal tract. New vaccines must be able to make the gastrointestinal tract of domestic livestock a hostile place as well. Investment in research is required in order to produce effective DNA vaccines for MAP (367,368) and disabled mutant strains of MAP using gene knockout (369). Candidate vaccines can then be given to young animals intranasally or by other means that ensure the acquisition of mucosal, as well as systemic, protection. As with other chronic infections, therapeutic vaccination against MAP can be devised for humans to assist in immune-mediated microbial clearance. This vaccination will need to take advantage of the simplicity of vaccination using naked DNA, followed if necessary by boosting with recombinant protein or peptides. The need is to identify pathogenicity-associated genes relevant to the therapeutic vaccine strategy. The glycosyl transferase gsd from within the GS element is already a promising candidate.

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