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6

The *Mycobacterium avium* subspecies *paratuberculosis* (MAP) problem and its relation to the causation of Crohn's disease

J.Hermon-Taylor and F.A.K. El-Zaatari

6.1 MAP

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a member of the *M.avium* Complex MAC. A genotypic definition of MAP and its distinction from other MAC is given in Chapter xxx. MAP is, however, also defined phenotypically by its specific ability to cause chronic inflammation of the intestine in many species. MAP is a small mycobacterium of about 0.5μm x 1-2μm and is an obligate intracellular pathogen. Bovine strains of MAP, which can usually be isolated in laboratory culture, grow much more slowly than other

MAC. They may take an initial 16 weeks to produce visible colonies on primary cultures, but can take much longer. MAP also requires exogenous mycobactin an iron-transport protein for in vitro growth. On solid media such as Middlebrook 7H11, MAP colonies appear rough and translucent, whereas on Herrold's media containing egg yolk (HEYM) they are smooth and opaque. As the cultures become older and the medium dries the colonies take on a crumbly appearance. Culture conditions have a substantial effect on MAP phenotype and resistance (Sung and Collins 2003). In liquid media MAP grows in characteristic tight clumps. In laboratory culture most of the microbial cells stain red by the Ziehl-Neelsen (ZN) reagent. However, this classical mycobacterial image is not the only form these pathogens can adopt. MAP is phenotypically versatile and can switch to a tough ZN-negative form in which it is invisible by ordinary light microscopy in infected tissues. Furthermore, as with some other mycobacteria, MAP can shut down into latency in which state it differs both functionally and in its physical properties from activated MAP cells, especially in its resistance to lysis and the subtleties of its interaction with the immune system. MAP is historically difficult to isolate, and strains from sheep or humans may require months or years of incubation before its gradual emergence becomes visible. Many strains of MAP cannot be grown at all. Conventional laboratory culture is not therefore, a consistently reliable method for detecting or assessing the viability of these difficult pathogens.

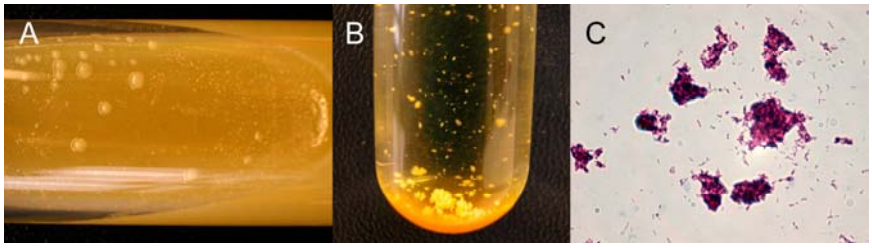

Figure 6.1. A. Smooth colonies of a bovine strain of MAP after 10 weeks of culture on an HEYM slope in a sealed tube. B. Bovine MAP after 10 weeks of culture in MGIT liquid medium (Becton Dickinson) showing characteristic clumping. C. Microscopic appearance of a bovine MAP strain from liquid medium showing the red acid-alcohol fast Ziehl-Neelsen staining typical of mycobacteria in bacillary form.

6.2 MAP INFECTION AND JOHNE'S DISEASE IN DOMESTIC LIVESTOCK

MAP was first identified in Germany in 1895 at the Veterinary Pathology Institute in Dresden, by Professor Johne and his young Bostonian research fellow Dr Frothingham (Johne and Frothingham 1895). The organism was causing chronic inflammation of the intestine in a cow. The condition became known as Johne's disease (JD). Detailed descriptions of the clinicopathological features of JD and of MAP infection in animals are presented in several reviews (Doyle 1956; Buergelt *et al*. 1978; Riemann and Abbas 1983; Chiodini *et al.*1984; Cocito *et al.* 1994; Clarke 1997; Beth Harris and Barletta 2001; Manning and Collins 2001).

Clinical JD in dairy cattle usually presents with loss of condition, a reduction in milk yield, weight loss and diarrhoea. Diarrhoea is not however, a constant feature particularly in small ruminants such as sheep and goats. There is no treatment and the disease is invariably fatal. JD is not just a disease of ruminants. Many species including monogastrics such as dogs and pigs, horses, chickens, and primates are all affected. MAP shows a distinct tissue tropism and causes chronic inflammation of the intestine even if administered subcutaneously or intravenously. The regions of the gastro-intestinal tract usually affected are the terminal ileum and colon with segmental lesions as well as rectal involvement. The gut wall is thickened, the mucosa is swollen with occasional ulcers and the regional mesenteric lymph nodes are enlarged. Microscopically, MAP disease in animals shows a broad range of histopathological characteristics in the gut wall, from pluribacillary disease with abundant Ziehl-Neelsen (ZN) positive acid-fast bacilli in intestinal macrophages, to an extreme paucimicrobial form with no visible acid-fast organisms and florid chronic granulomatous inflammation. The pluribacillary to paucimicrobial range of MAP disease in animals, thus closely resembles the range in the appearances of leprosy from the lepromatous to tuberculoid forms, in humans. The pluribacillary picture is the common form seen in naturally as well as in experimentally infected animals.

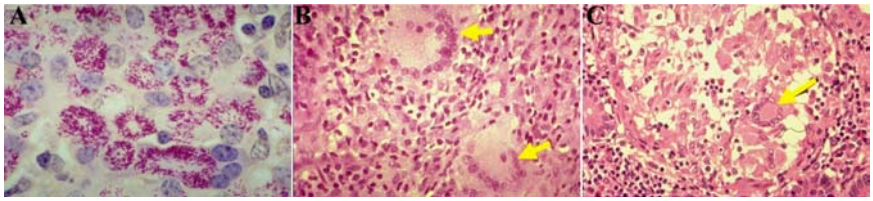

Figure 6.2. A. Microscopic appearance of the gut wall in pluribacillary Johne's disease (JD) showing macrophages containing abundant Ziehl-Neelsen positive MAP organisms in their classical mycobacterial phenotype. B. The contrasted appearance of the gut wall in paucimicrobial JD showing no ZN-staining MAP and florid granulomatous inflammatory disease with prominent giant cells (arrow). C. The chronic granulomatous inflammation of the gut wall of Crohn's disease in humans showing giant cell (arrow).

Both naturally and experimentally MAP-infected animals develop an enteric neuritis with inflammatory cells surrounding autonomic nerve fibres in the gut wall (Gwordz *et al*. 2001). The paucimicrobial form of JD closely resembles Crohn's disease in humans. MAP in JD is a systemic infection; the organisms traffic widely in macrophages and parasitize the reproductive organs of both males and females. Granulomatous lesions are seen microscopically in the spleen and liver as well as widely in lymphoid tissue.

In a herd with one or two clinically diseased animals, up to 50% of the other apparently healthy animals in the same herd will be subclinically infected. Infection is transmitted from cow to calf in colostrum and in milk, and from animal to animal in crowded, contaminated farm environments. MAP can persist in the intestinal tract of subclinically infected animals for years without causing clinical disease. The emergence of clinical JD can be triggered by physical or psychological stress such as calving or overcrowding. Animals are most susceptible when infected at an early age, but there is a long lead time of months or years before clinical disease, if it is going to develop, eventually emerges. There are marked genetic influences in the susceptibility of animals to MAP infection and JD (Koets *et al.*2000). Guinea pigs and rats are particularly resistant whereas young deer and goats are highly susceptible. Within bovines, Channel Island cattle, Limousin and specialist breeds such as Welsh Blacks are particularly susceptible.

The principal diagnostic tests for MAP infection in animals are individual or pooled faecal culture, ELISA, and IFNγ release from activated white cells in response to MAP antigens. PCR diagnostics have also been introduced. Faecal culture remains the gold standard and its performance has been improved by the commercial availability of BACTEC and MGIT media (Becton Dickinson) and the application of IS*900* PCR to the incubate (Whittington *et al* 2000a; Kalis *et al* 2000; Eamens *et al* 2000). To date, commercially available ELISAs lack the sensitivity and specificity to diagnose subclinical MAP infection at an early stage. The performance of these ELISAs may improve through the progressive introduction of MAP antigens of greater specificity. ELISAs are however, cheap and convenient screening tests. Their practical usefulness can be enhanced mathematically and by the derivation of likelihood ratios (Beyerbach

et al 2001; Collins 2002). IFNγ tests of cell mediated immunity to MAP antigens detect sub clinical MAP infection at an earlier stage than ELISAs.

The use in recent years of all these diagnostic procedures has revealed the widespread nature of MAP infection in domestic livestock throughout Western Europe and North America. Results from Austria, Denmark, Belgium, the Netherlands and the UK have ranged from individual animal infection rates of 1.9%-9%, and herd prevalences (1-2 test-positive animals per herd) in the range 0.8%-86%. The highest herd prevalences have been reported in the Netherlands and Denmark (Cetinkaya *et al*. 1996; Gasteiner *et al*. 1999; Gasteiner *et al.* 2000; Nielsen *et al*. 2000; Jakobsen *et al*. 2000; Boelaert *et al.* 2000; Muskens *et* al. 2000). Results from dairy cattle in the USA and Canada have been similar showing individual animal infection rates in the range 1.8%-7.29% and herd prevalences in the range 16.7%-54% (NcNab *et al*. 1991; Collins *et al*. 1994; Wells *et al.* 1996; Johnson-Ifearulundu and Kaneene 1999; Van Leeuwen *et al*. 2001). Infection rates reported in beef herds have been much lower (Dargatz *et al*. 2001; Waldner *et a*l. 2002).

6.3 DIFFERENT STRAINS OF MAP

With the opportunity to amplify in domestic livestock exposed to increasingly intensive farming practises over the course of more than a century, MAP has almost certainly done what other pathogens have done, and has undergone an adaptive radiation (Colwell 1996). Studies of strain diversity between different MAP isolates have used restriction endonuclease analysis, IS*900* RFLP, and pulse-field gel electrophoresis PFGE (Collins *et al* 1990; Whipple *et al* 1990; Bauerfeind *et al* 1996; Feizabadi *et al* 1997; Pavlik *et al* 1999; Cousins *et al* 2000; Stevenson *et al* 2002). More than 30 different MAP strains have been identified. Typing of over a thousand MAP isolates obtained from all over the world has demonstrated differences between ovine strains (S-type or type I) and bovine strains (C-type or type II), suggesting an adaptation to their respective preferred hosts. Although phenotypic and genotypic differences are found between ovine strains, and between bovine strains, they none-the-less share substantial intra-species commonality. The major differences are inter-species. Studies in Iceland and the Netherlands have shown that sheep strains of MAP can infect cattle, and cattle strains of MAP can give rise to long standing subclinical infection in sheep grazing the same pastures (Fridriksdottir *et al* 2000; Muskens *et al* 2001). Bovine species* however, have a much broader host range. IS*900* RFLP typing of MAP isolates from humans with Crohn's disease has so far demonstrated that they are all based on the cattle C-type, type II, background (Francois *et al* 1997; Pavlik *et al* 1999; Whittington *et al* 2000b).

A unique 12bp tandem repeat sequence present in sheep strains and absent from bovine strains enables these strains to be distinguished by a single specific PCR (Collins *et al.* 2002). The use of representational difference analysis has further identified an 11bp fragment present in sheep strains, which was absent from bovine strains tested (Dohmann *et al* 2003). Differences in MAP strains from cattle and from sheep have been demonstrated between Argentina and Europe (Moreira *et al* 1999), and between Australia and Iceland (Whittington *et al* 2001). Typing of IS*1311* polymorphisms from MAP isolates obtained from 9 bison in Montana, USA showed consistent variation at base position 223 compared with 13 C-type isolates from cattle and goats in the USA. The finding that bison strains of MAP (designated B-strain) differed from ambient cattle strains suggested that the epidemiology of paratuberculosis in bison in Montana may be distinct from that found in farmed livestock in other regions (Whittington *et al* 2001). Taken together, the findings are consistent with predictable geographical differences in MAP isolates between continents and different regions. Diversification of MAP strains is a continuing dynamic process, and human MAP strains with type specific features can be expected.

RFLP and PFGE are methods which limit the typing of MAP to those strains which can be cultured. Given the very slow growing and in some cases unculturable nature of these organisms, PCR-based typing procedures for these difficult pathogens are highly desirable. The methods developed so far include random amplified polymorphic DNA patterns (Scheibl and Gerlach 1997; Pillai *et al* 2001), and a multiplex PCR typing procedure (MPIL). MPIL utilises a common IS*900* primer together with a locus-specific primer directed at either side of 14 of the highly conserved insertion sites, thus reporting the presence or absence or potential rearrangement of the element at each locus (Bull *et al* 2000). PCR typing of *M. tuberculosis* based upon mycobacterial interspersed repetitive units MIRU (Supply *et al* 2000), has been adapted for MAP (Bull *et al* 2003a). PCR typing based upon 6 MIRU loci, distinguishes MAP from other MAC. This maybe useful in demonstrating that a liquid culture of MAP isolated from a sample, does not also contain other MAC organisms.

6.4 MAP IN WILDLIFE AND IN THE ENVIRONMENT

Clinically and subclinically infected farm animals, particularly those with the common pluribacillary form of disease shown in Figure 2A, can shed huge numbers of MAP onto pastures. MAP infection and JD are endemic in Western Europe and North America. Taking North East Scotland as an example, studies beginning in 1994 demonstrated MAP infection in 8-53% of wild rabbits culled

from farms reporting clinical JD in cattle and sheep. MAP infection was also found in a smaller proportion of rabbits obtained from farms without clinical JD (Greig *et al* 1999). Typing of the isolates demonstrated that they were all of the bovine type. MAP-infected wild rabbits shed the pathogen in their faecal pellets which are consumed by grazing cattle. Experimental paratuberculosis has been demonstrated in calves following infection with a rabbit MAP isolate (Daniels *et al* 2001; Beard *et al* 2001a). Thus a cycle of infection is established comprising MAP amplified in domestic animals, environmental contamination, infection of rabbits, and re-infection of domestic livestock. In the same group of studies, MAP infection was also found in a high proportion of rabbit predators such as stoat, weasel and fox, as well as in the carrion birds crow, rook and jackdaw. Rat, wood mouse, hare and badger were found to harbour MAP (Beard *et al* 2001b). MAP infection in wildlife has been extensively reported in other regions as exemplified by MAP infected red deer and ibex from the European Alps, bison and elk in North America, and wild ruminants as well as insects and earthworms in the Czech Republic (Nebbia *et al* 2000; Ferroglio *et al* 2000; Buergelt *et al* 2000; Pavlik *et al* 2000; Fischer *et al* 2001; Fischer *et al.* 2003). MAP in domestic animals and wildlife thus constitutes a reservoir of these pathogens capable of being disseminated over substantial distances.

The survival of *Mycobacterium bovis* in the environment is thought to be limited to hours or days. By contrast MAP, which is physically much more robust than *M.bovis,* is known to survive for months and perhaps for years since no upper limit on the environmental survival and persistence of MAP has been established. Geographical regions characterised by acid soils rich in humic and fulvic acids, boreal forests and areas with a high rainfall and water table, may favour the accumulation of MAP in the environment (Kopecky 1977; Kazda *et al.* 1990; Johnson-Ifearulundu and Kaneene 1997; Iivanainen *et al* 1999; Kirschner *et al.* 1999; Reviriego *et al.* 2000;). Although investigations of MAP in the environment and in surface waters are currently in progress, there are to date no published studies to give us a detailed understanding of the ecology and fate of MAP in the environment and the potential cycling of these pathogens through human populations. From what is known about other MAC and organisms such as *Legionella* sp. it is highly likely that environmental MAP are taken up into protozoa (Barker and Brown 1994; Falkinham 1996; Ford 1999; Hermon-Taylor *et al.* 2000). Intracellular adaptation of MAP within protozoa in the environment and in biofilm communities may profoundly influence microbial survival, phenotype and virulence. *M. avium* grown in vacuoles in *Acanthamoeba castellanii* has been shown to develop an increased capacity to infect other amoebae, macrophages and human colonic epithelial cells, as well as an enhanced virulence in a beige mouse model of infection (Cirillo *et al.* 1997). *M.avium* can survive for long periods within encysted forms of

Acanthamoeba polyphaga (Steinert *et al.* 1998). The environmental exposure of MAP and its cycling through unicellular organisms, as well as through animal and human populations, has the potential to have a profound effect on the evolution of these organisms and the development of strains with enhanced pathogenicity. Much research remains to be done in this area.

6.5 TRANSMISSION OF MAP FROM ANIMALS TO HUMANS

It is unlikely that crowded human populations sharing the same geographical regions as their widely MAP-infected domestic animals shedding huge numbers of organisms into the environment would be excluded from any exposure to these robust and versatile pathogens.

6.5.1 In food

It has long been known, and has more been recently confirmed, that infected animals secrete MAP in their milk (Doyle 1954; Taylor *et al* 1981; Sweeney *et al* 1992; Streeter *et al* 1995). Faecal contamination in the milking parlour is another source of MAP in milk. MAP is more thermotolerant than *M.bovis*. Much work was carried out in several laboratories over the period 1993-2002 to determine whether exposure to 72^{o}C for 15 seconds, conditions commonly used in commercial pasteurisation, would ensure the destruction of all viable MAP (Chiodini and Hermon-Taylor 1993; Grant *et al* 1996; Meylan *et al* 1996; Stabel *et al* 1997; Sung *et al* 1998; Keswani *et al* 1998; Grant *et al* 1999; Pearce *et al* 2001; Grant *et al* 2002a; Gao *et al.* 2002). Sources of error in some of these studies and their interpretation, have come from the use of spiking with laboratory strains of MAP, the disabling effect of freeze-thaw and sonication on MAP prior to heat-shock, suboptimal culture conditions, and the unjustified conclusion that the inability after heat-shock to grow colonies of MAP in conventional cultures, meant that all the organisms were dead. Despite this, the substantial balance of experimental evidence strongly predicted that pasteurisation at 72^{o}C for 15 or 25 seconds, while reducing the number of viable organisms, would not ensure the destruction of all MAP.

Field studies using IS*900* PCR to screen retail pasteurised cows' milk for MAP in the UK, while unable to distinguish between live and dead organisms, indicated a high risk of the transmission of MAP to humans by this route (Millar *et al* 1996). Further work from the Department of Food Science, Queen's University Belfast, using optimised decontamination protocols and

immunomagnetic capture, found that 11.8% of 567 samples of retail pasteurised cows' milk in the UK tested MAP-positive by PCR, and that 1.8% of samples were MAP-positive by culture (Dundee *et al* 2001; Grant *et al* 2002). For Britain alone therefore, it is known that people are from time to time drinking live MAP in the milk supply. The finding in Switzerland that 19.7% of 1384 samples of bulk-tank milk tested IS*900* PCR positive emphasises the risk that this may be happening elsewhere (Corti and Stephan 2002). More data are required from other countries where MAP infection in dairy herds is endemic. Exploitation of specific peptide-mediated capture of MAP from milk will advance the sensitivity of detection (Stratmann *et al*. 2002). Quantitative RT-PCR and sensitive methods including culture of MAP within cell lines, and the use of susceptible C57/BL6 or immune deficient mice, may improve on conventional culture in their ability to reveal residual viable MAP, and assist in the selection of new industrially applicable processes to eliminate these pathogens from the food chain. Procedures already tested on milk include filtration, cold shock, hydrostatic pressure, and pulsed electric fields (Miller *et al.*2000; O'Reilly *et al.*2000; Rowan *et al.*2001). Specialist cheeses derived from raw milk need to come from certified MAP-free animals.

In many countries existing legislation still permits clinically diseased JD cattle or sheep, in which MAP are widely present in liver, lymph nodes and other tissue, to be sent for slaughter in abbatoirs, and the meat and offal passed for human consumption. These animals contain huge numbers of MAP with a high risk of dissemination in the abbatoir environment, and surface contamination of other meat being processed. Vegetables are at risk where MAP-infected slurry is applied to market gardens or agricultural land as a fertiliser.

6.5.2 In water supplies and aerosols

Although work is currently in progress, there are at the end of 2003 no detailed published studies, using molecular and other methods of established validity, which reliably inform us about MAP contamination of waters close to population centres, or of those sourced for domestic supply. However the information available for other robust zoonotic pathogens which can survive in the environment (Szewzyk *et al.* 2000; Le Dantec *et al.* 2002), would suggest that the risk that MAP may from time to time be transmitted to people in drinking water or by aerosols, is high (Hermon-Taylor *et al*. 2000). MAP in lakes and rivers contaminated by run-off from heavily grazed pastures, will be present in planktonic form, within protozoa, or more likely both. If these adhere to particles of suspended solid, then the MAP content of native water abstracted for domestic supply will be depleted by subsequent treatments, such as counter-

current dissolved air floatation filtration (COCUDAFF). CT values (disinfectant concentration ppm x t min. to reach 3 log units of cell death) for the effect of chlorine on MAP have been estimated to be up to 580 to 2,300 times greater than those for *E.coli* (Taylor *et al.* 2000; Whan *et al.* 2001). MAP getting through the stage in water treatment plants of removal of suspended solids, is therefore unlikely to be destroyed by subsequent chlorination. These pathogens arriving at domestic outlets in high dilution may accumulate in biofilms present in household cold and hot water storage and delivery systems. If research tells us that this is indeed happening, we may need to consider exploiting the susceptibility of MAP to UV irradiation (Miyamoto *et al.* 2000) using additional industrially applicable treatments in flow-through units.

While we wait for reliable scientific data, it is worth revisiting two published studies where exposure to waters whose catchments included heavily grazed pastures, was associated with conspicuous clusters of Crohn's disease. The first of these involved the village of Blockley, a rural community of about 2,000 people in Gloucestershire, UK, in which 12 people developed Crohn's disease between 1960 and 1983, an increase of observed over expected (for that time) of 6.7 fold and equating with a CD incidence of $28/10^5$/year (Allan et al. 1996). The village, which had its own water supply from local springs, lay in a hollow surrounded by upland pastures grazed by cattle in which clinical Johne's disease was evident (R.N. Allan personal communication 1992). The second CD cluster occurred in the town of Mankato, Minnesota, USA and involved the occurrence of 7 cases of CD amongst 285 graduates of Mankato West High School class of 1980. All 7 had been swimming in local ponds and lakes. The school also lay close to the Minnesota River, just downstream from the entry of the Blue Earth River whose catchment included rich agricultural grazing land sloping towards the river. High faecal coliform counts in Blue Earth river water, monitored over the period, indicated extensive contamination with faecal run-off. Seventy-five percent of the water supply to Mankato was reported to be drawn from beneath the Blue Earth river (Van Kruiningen and Freda 2001). Although these authors do not mention it, it is highly likely that these waters were from time to time, heavily contaminated with MAP. There are data also which implicate domestic hot water systems. Two case control epidemiological studies carried out independently in the UK, each unexpectedly identified the availability of fixed hot water supplies in the early childhood home as a significant risk factor for the subsequent development of CD, but not for ulcerative colitis UC (Gent *et al.* 1994; Duggan *et al.* 1998).

Mycobacteria are known to occur in aerosols in whose droplets the organisms may achieve a concentration far higher than that in the water from which they arose (Blanchard and Syzdek 1972; Wendt *et al.* 1980;). Cardiff is a city on the

coastal plain of South Wales in the UK, beside the sea. North of the city lie the Brecon Hills, steep upland pastures that are grazed by sheep and cattle in whom MAP infection is endemic. Heavy rains from the Atlantic wash off these pastures into spate rivers. One of these rivers, the Taff, runs through the middle of Cardiff. Epidemiological research carried out in Cardiff during the 1970s demonstrated a highly significantly increased incidence of CD (p=<0.001), but not of UC, in 11 of the local electoral city wards (Mayberry and Hitchens 1978). Of these high incidence wards, 8 directly bordered the river Taff and the 3 that did not were immediately adjacent to the North and East. This is the direction in which aerosols would be carried by the prevailing South Westerly winds (Hermon-Taylor 1993). Inflammatory involvement of the trachea and bronchi with abnormal lung function tests are demonstrable in a significant proportion of people with CD, and CD in children can present with chronic granulomatous tracheo-bronchitis (Heatley *et al.* 1982; Bonniere *et al.* 1986; Herrlinger *et al.* 2002; Dierkes-Globisch and Mohr 2002; Calder *et al.* 1993). Much research is needed on MAP in the environment, in surface and ground waters, and in aerosols.

6.6 CROHN'S DISEASE

6.6.1 Definition

Crohn's disease (CD) is a systemic disorder whose principal clinicopathological manifestation is chronic inflammation of the intestine. Any part of the gastrointestinal tract from mouth to anus may be involved in the chronic granulomatous process, but the terminal ileum and colon are the regions most frequently affected.

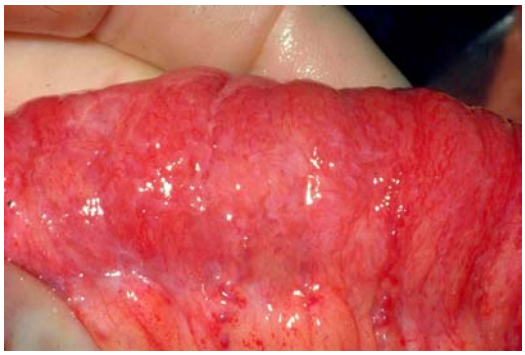

Figure 6.3. Typical appearance of an inflamed terminal ileum in a person with active Crohn's disease.

CD usually presents with abdominal pain, feeling unwell, loss of energy, and loss of weight, night sweats, mouth ulcers and joint pains. It sometimes presents as an abdominal emergency with peritonitis, perforation of the terminal ileum, or mimicking acute appendicitis. As in animals, onset of clinical disease may be triggered by physical and psychological stress. About 60% of patients have diarrhoea which may contain pus and blood. The tissues around the anus and perineum may become ulcerated or chronically inflamed with sinuses discharging pus and faecal material. In children, growth and sexual maturation is retarded or arrested. The mucosa lining the gut first becomes leaky, then ulcerated with long serpiginous fissures. Mucosa surviving between the ulcers is swollen, inflamed, and oedematous and frequently goes on to form inflammatory pseudopolyps. The chronic inflammatory process and inflammatory cell infiltrate extends deep into, and often right across the gut wall. Granulomata, consisting principally of clusters of activated macrophages with conspicuous multinucleate giant cells, are seen microscopically in only about half of CD cases. As in naturally and experimentally MAP-infected animals (Gwordz *et al.*2001), humans with CD demonstrate abnormalities of the enteric nervous system, with neuronal and axonal hyperplasia, axonal damage, and periaxonal inflammatory cell cuffing, associated with MHC class II expression on enteric glial cells (Geboes and Collins 1998; Geboes *et al* 1992).

Treatment of CD has been limited to the suppression or modulation of the inflammatory process. This can sometimes achieve and maintain remission over prolonged periods. Relapse frequently occurs, and again is often triggered by physical and psychological stress. Surgery is required if the disease gets out of control, or if specific complications develop. These take the form of obstruction of the gut due to stricturing, abdominal abscesses, perforation of the gut, or fistulous connections leading to discharge of intestinal content from other organs such the bladder or vagina. About 40% of people with colonic CD will end up having to have their whole colon removed, and an abdominal bag collecting intestinal effluent from an ileostomy. CD characterised by cycles of disease remission followed by activity, with its physical, emotional, sexual, social and family morbidities, involves a lifetime of medical care and huge economic cost (Sandler *et al.* 2002)

6.6.2 Epidemiology, environmental factors, and inherited susceptibility to CD.

CD is a 'new' disease which began by appearing in developed societies in temperate regions of the globe, with intensive farming. From a low background level of sporadic cases recorded over many years (Combe 1813; Moschcowitz and Wilensky 1923), chronic inflammation of the intestine of the Crohn's disease type began to emerge perceptibly about a third of the way into the 20th century (Crohn *et al* 1932). Thereafter, with plateaus at times in some regions, the incidence and prevalence of CD have continued to climb.

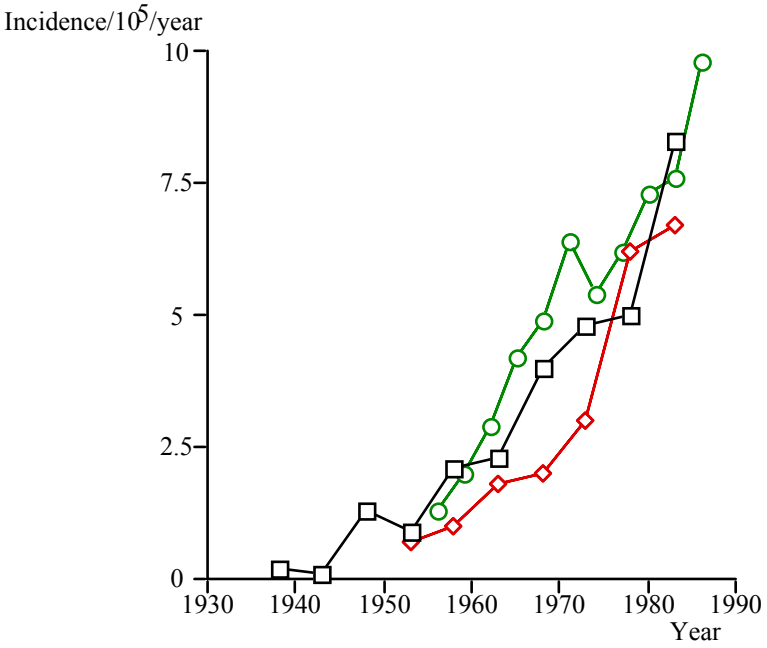

Figure 6.4. The incidence of Crohn's disease in three regions of the United Kingdom, South Wales □---□ (Rose *et al.* 1988), the Midlands ◊---◊ (Fellows *et al.* 1990), and Northeastern Scotland ○---○ (Kyle 1992), over the 50 year period 1940 to 1990.

Comparable increases in the incidence of CD are recorded in North America and continental Europe (Loftus *et al.* 1998; Munkholm *et al* 1992). In the UK in recent years, increases in CD have particularly affected children (Cosgrove *et al.* 1996; Armitage *et al.* 2001; Sawczenko *et al.* 2001). Continents in the Northern hemisphere demonstrate a North-South gradient in the incidence of CD

(Sonnenberg *et al.* 1991; Shivananda *et al.* 1996). In Europe, while CD remains uncommon in Greece (Tsianos *et al.* 1994), there is evidence that a higher incidence is spreading South to the Iberian peninsular (Ruiz 1989; Veloso *et al.* 1989; Cebolla *et al.* 1991; Lopes Miguel *et al.* 1999) and East to European countries like Hungary and Croatia (Lakatos *et al.* 2002; Mijandrusic Sincic *et al.* 2002). CD also appears to be rising in countries formerly presumed to have a low incidence such Iran (Merat *et al.* 2002), India (Pai and Khandige 2000), and Brazil (Gaburri *et al.* 1998), as well as in China and Japan which have substantially increased production and consumption of dairy products (Yao *et al.* 2000).

The highest overall incidence (15.6/100,000/yr) and prevalence (198.5/100,000 population) of CD so far reported in the world is in Manitoba Canada (Bernstein *et al.* 1999; Blanchard *et al.* 2001). Individual incidence rates for CD across the 52 postal regions in these Manitoba studies ranged from 1 to 26/100,000/yr, making the province a fruitful region for further environmental research into CD causation. In the absence for the most part, of national population-based data, the overall scale of the CD problem in human populations at the present time can only be estimated. Best guess for the USA would be about 600,000 CD sufferers (Loftus *et al.* 2002) though it may be as high as one million, for Western Europe 300,000-500,000, and for Britain about 100,000 (Rubin *et al.* 2000). In Northern Stockholm County, Sweden, the incidence of CD in children under 16 increased from 1.7/100,000/yr in 1990-92 to 8.4/100,000/yr in 1999-2001 (Hildebrand *et al* 2003). At the other end of the planet in Victoria, Australia, the CD incidence in children rose from 0.128 to 2.0/100,000/yr over the period 1971-2001 (Phavichitr *et al* 2003). In each case, this represents an alarming average 5 fold increase in CD in children per decade. There are a lack of recent data for the incidence and prevalence of CD in adults in Australia, New Zealand, and South Africa, and a need for the relevant epidemiological research to be carried out.

CD occurring in spouses and their children sharing common environments, and CD increasing to that of the host population in migrants moving from low to high incidence areas, clearly indicate the involvement of one or more environmental factors in CD causation (Laharie *et al.* 2001; Montgomery *et al.* 1999). Exposure to MAP with its ability to cause chronic inflammation of the intestine in so many species including primates is a strong candidate environmental factor. The familial occurrence of CD (Orholm *et al.* 1991; Peeters *et al.* 1996), the higher incidence in some races such as Jewish people (Yang *et al.* 1993), and the concordance rate of CD of 58% in monozygotic twins and of 0% in dizygotic twins (Orholm *et al.* 2000), also show that a susceptibility to CD can be inherited. Recognition of one molecular basis for

this, came with the discovery of missence variants and frameshift mutations affecting the *CARD15(NOD2)* gene on human chromosome 16 (Hugot *et al.* 2001; Ogura *et al.* 2001; Hampe *et al.* 2001). This gene encodes a transmembrane receptor for bacterial products like lipopolysaccharide (LPS) expressed on monocytes, and related to the Apaf-1 family of apoptosis regulators. Several polymorphisms are associated with CD susceptibility (Lesage *et al.* 2002; Cuthbert *et al.* 2002) the strongest being an insertional mutation in exon 10 resulting in truncation of the leucine-rich carboxyterminus of the protein, and a reduction in cellular response to LPS activation. However, while the linkage between *CARD15(NOD2)* mutations and CD has been confirmed for Europeans, North Americans, Australians and Jewish people (Brant *et al.* 1998; Cavanaugh *et al.* 1998; Vermeire *et al.* 2002; Cavanaugh 2001; Zhou *et al.* 2002), it does not occur in Japanese, Korean or Chinese patients with CD (Inoue *et al.* 2002; Yamazaki *et al.* 2002; Croucher *et al.* 2003). An Ala893/Thr polymorphism in the multidrug resistance gene on chromosome 7q is also associated with an increased risk of IBD (Brant *et al* 2003), and a number of other loci have been implicated on chromosomes 1,3,4,5,6,7,12, and 14 (for reviews: Watts and Satsangi 2002; Duerr 2002; Bonen and Cho 2003). These genetic loci may influence susceptibility to CD……. they do not cause it.

6.6.3 The isolated case of Iceland

Does the distribution of MAP infection in animals match the distribution of CD in humans? The answer to this on a continental basis is yes. A more focussed picture is blurred by the putative dispersal of MAP in food products, in water, and in the environment, happening across regional, national and international boundaries, as well as by the potential exposure to MAP during international travel. Our understanding is also limited by our lack of knowledge of MAP in the environment, in different habitats and phenotypes, and also because the necessary epidemiological research to detail the comparative incidence and prevalence of MAP infection in animals and in humans has not been carried out.

It is, however, worth taking a closer look at the isolated community of Iceland, an island of 103,000 km^2 in the North Atlantic. The population was 229,187 in 1980 rising to 266,006 in 1994, with a low migration rate and ethnically homogeneous Nordic. About 60% of the people are centred in the capital Reykjavik. There are 3 hospitals the main one being in Reykjavik, and centralised registration of health information. Farming involves principally the 480,000 Icelandic breed of hill sheep, with some dairy and beef cattle.

Prior to 1930 MAP infection and JD in Iceland were virtually unknown. Then in 1933, 20 Karakul sheep were imported from Germany and, after

quarantine, were distributed to 14 farms (Fridriksdottir *et al.* 2000). Although apparently healthy, some of the Karakul sheep were subclinically infected with MAP. They transmitted MAP to the Icelandic sheep population though they never developed disease themselves. By 1938 clinical JD appeared in Icelandic sheep on 5 of the original farms. By about 1945, clinical JD was in the cattle on the same farms, although infection in the cattle was difficult to diagnose as the organisms, characteristically for sheep MAP strains, would not grow in culture. The bug from these cattle was later confirmed as the sheep strain of MAP by IS*1311* restriction endonuclease analysis (Whittington *et al.* 2001a). Slowly the infection spread so that by the late 1950's the disease was epidemic with about 30% of sheep farms affected and huge annual losses. The mean incidence of CD (number of cases/10^5/yr) in the human population was 0.4 from 1950-59, 0.45 from 1960-69, 0.9 from 1970-79, 3.1 from 1980-89 and 5.6 from 1990-94 inclusive, the highest annual figure over this last 5 year period being 8.2 in 1992. Young people were particularly affected (Bjornsson 1989; Bjornsson *et al.* 1998; Bjornsson *et al.* 2000).

Apart from an increase in the sale of cigarettes during World War II, no nutritional or environmental risk factors were found to parallel and perhaps explain the magnitude of this increase in CD. Although causation is not proven, with the slow growth of MAP, the need for the pathogen to adapt to each new host (Woodhouse *et al.* 2001), and the long lead time to the emergence of clinical disease (if it is going to occur) in both animals and humans, the sequential picture of JD then CD observed in Iceland over 50 years, is just exactly what would be expected if the major environmental factor causing CD was MAP.

6.7 MAP CAUSING CROHN'S DISEASE

In 1988 a previously healthy 7 year old boy living in a village outside Cambridge U.K., developed non-tuberculous mycobacterial cervical lymphadenitis which was later shown to be caused by MAP (Hermon-Taylor *et al* 1998). After failing to respond to standard anti-TB treatment, the enlarged lymph glands were removed. Five years later he developed severe CD of the terminal ileum and adjacent colon. This healed completely after a year's treatment with anti-MAP drugs rifabutin and clarithromycin leaving a dense fibrous ileal scar with narrowing of the gut and impending intestinal obstruction. The scar was removed and the continuity of the gut restored. The scar tested strongly positive for MAP by IS*900* PCR. Drug treatment was continued for almost 2 more years during which he was disease free. About two years after stopping the drugs, the CD recurred in the ileum next to the anastamotic site,

despite his having been off all UK milk products. His CD again responded to rifabutin and clarithromycin.

The value of this isolated case lies in the way in which it illuminates the relationship between MAP and CD. MAP infection of the cervical lymph glands in this boy was probably acquired from UK milk just as happened with *M.bovis* before pasteurisation. The ingested MAP pathogens would also have colonised his gut at the same time, but as in animals, a lead time of several years passed before clinical disease emerged. On this occasion most of the organisms were sensitive and the disease healed on anti-MAP drug treatment, but continuing colonisation of the gut with residual MAP probably in a state of latency such as occurs with TB and *M.avium*, persisted (Bermudez *et al.* 1999; Manabe and Bishai 2000; zu Bentrup and Russell 2001). When the residual MAP reactivated the disease responded again to the same therapy. At no time was MAP either seen microscopically or isolated in conventional culture from his diseased tissues. Recognition of the true nature of the causation of the lymphadenitis and the subsequent chronic enteric infection depended entirely on the detection of MAP using appropriate molecular methods. This isolated case also shows that MAP infections are extremely difficult to eradicate.

6.7.1 MAP in the inflamed gut of people with CD.

The proposition that MAP (Johne's bacillus) could cause chronic inflammation of the intestine in humans as well as in animals, was first published by the Glasgow surgeon T.K.Dalziel in 1913 (Dalziel 1913). The uncertainty nearly 100 years later as to whether or not this proposition is true, is almost entirely due to the difficulties of reliably detecting this robust, versatile and often unculturable pathogen. A pivotal contribution was made by Dr Rod Chiodini in the USA during the mid-1980s, when he and his co-workers, using optimised cultures and incubation times of months or years, isolated an unclassified *Mycobacterium* sp. from the inflamed gut of 3 people with CD (Chiodini *et al* 1984; Chiodini *et al* 1986; Chiodini 1989). These isolates caused chronic inflammation of the intestine when administered to young goats. In half the goats no ZN-positive mycobacteria could be seen microscopically in the inflamed tissues, just like CD (Van Kruningen *et al* 1986). Other workers were able to isolate spheroplasts and acid-fast bacilli from CD, but in the absence at that time of molecular methods of sufficient specificity and sensitivity, the nature of these could not be precisely demonstrated (Markesich *et al* 1988). Similar contributions were made by other research groups (for reviews Hermon-Taylor *et al* 2000; El-Zaatari *et al* 2001; Chamberlain *et al* 2001).

The availability of IS*900* (Green *et al.* 1989) and its use as a probe and as a target for PCR, confirmed the CD isolates of Chiodini as MAP, and showed that a substantial proportion of long-term CD cultures contained these pathogens (McFadden *et al.* 1987; Moss *et al.* 1992; Wall *et al.* 1993). IS*900*PCR together with DNA extraction protocols optimised using fresh surgically resected MAP-positive CD tissues, demonstrated MAP in the inflamed gut of 65% of people with CD and in 12% of uninflamed control gut samples (Sanderson *et al.* 1992). Subsequent PCR studies over the period 1994-99 were conflicting (Hermon-Taylor *et al.* 2000), though work from the University of Bari in Italy had shown that people with CD may excrete MAP in their stool (Del Prete *et al.* 1998).

Recent research has established the extraordinary resistance of MAP in human and animal tissues, and in milk and other samples, to chemical as well as enzymic lysis, and the need to incorporate an optimised mechanical disruption step in sample processing to ensure reliable access to MAP DNA for PCR detection (Hermon-Taylor *et al.* 2000; Odumeru *et al.* 2001). Recent years have also brought the commercial availability of improved media for the isolation of MAP such as the MGIT system, the result of some years of developmental work in Becton Dickinson. New methods have been applied to the localisation of MAP in CD tissues, such as laser capture microdissection and in situ hybridization. Research at the University of Central Florida and El Paso Texas cultured MAP in MGIT medium after about a year of incubation, from the inflamed gut of 6 of 7 (86%) people with CD (Schwartz *et al.* 2000). They also cultured MAP from the breast milk of 2 women with CD who had recently given birth, but not from the milk of 5 normal women (Naser *et al.* 2000). Collaborative research at the Baylor College of Medicine in the USA and the University of Oulu, Finland demonstrated MAP for the first time, in 6 of 15 (40%) granuloma-positive CD patients and in none of 22 patients without CD, using in situ hybridization (Hulten *et al*. 2001).

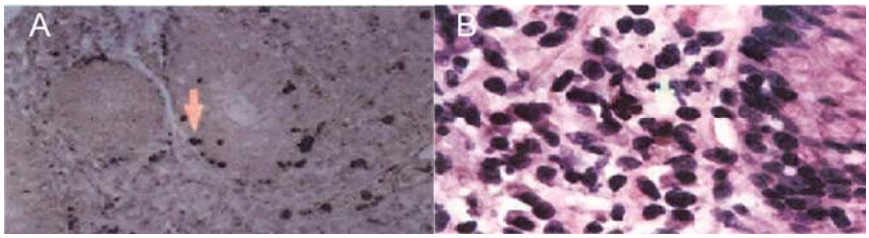

Figure 6.5 Demonstration of MAP in CD tissues by *in situ* hybridization on paraffin-embedded tissue sections using an IS*900* probe. (A) In situ hybridization with no counter stain showing MAP DNA in the lamina propria and occasionally infiltrating a

gland (x40). (B) Same as in (A) with H & E as a counter stain showing MAP as brown positive spots within macrophages in the lamina propria. (x 100).

In situ hybridization studies from the Universities of Sassari in Sardinia and Rome, demonstrated MAP in 27 of 33 (82%) CD patients with no relationship to the presence of granuloma, and in none of 40 patients without CD (Sechi *et al.* 2001). Research in Ireland using laser capture microdissection and PCR (without a mechanical disruption step) of sub-epithelial granulomas detected MAP in 6 of 15 (40%) patients with CD and in 0 of 12 disease controls (Ryan *et al.* 2002). IS*900* PCR (without a mechanical disruption step) was positive for MAP in 15 of 79 (19%) CD patients and 3 of 48 (6%) control patients from the USA and Denmark (Collins *et al.* 2000). Research in London UK using optimized tissue processing (with mechanical disruption) and nested IS*900* PCR, detected MAP in fresh ileocolonoscopic mucosal biopsies in 9 of 34 (26%) of people without clinicopathological CD, and in 34 of 37 (92%) of people with CD (odds ratio 3.47; p = 0.0002) (Bull *et al.* 2003b). In this study, identity with IS*900* was verified in every case by amplicon sequencing. The IS*900* multicopy element as defined by its entire DNA sequence is unique for MAP.

Taken together these studies show that the detection rates for MAP in CD depend critically on the validity of the methods used. When these are optimal almost everybody with CD is found to be infected with MAP. The presence of MAP colonization of the gut in a minority proportion of people without CD is consistent with widespread environmental exposure to these pathogens, as exemplified also in the population biology of *M.tuberculosis*, *S.pneumonae, N.meningitidis,* and *H.pylori*.

6.7.2 Serological recognition of MAP proteins in CD.

Sera from animal healthcare workers exposed to abundant ZN-positive bacillary-form MAP show significantly higher levels of IgG antibody binding to microtitre plates coated with the crude soluble fraction of MAP lysates, than do sera from healthy humans (Chiodini *et al.* 1996). Sera from people with CD in general show no significant difference in antibody binding to such crude MAP extracts compared with controls (reviewed in Hermon-Taylor *et al.*2000*)*. In this situation, such tests report an overall immune responsiveness to common MAC antigens.

The ZN-negative form of MAP in CD minimizes immune recognition. Significant differences in IgG and/or IgA antibody binding are, however, observed in ELISAs using selected highly purified or recombinant MAP

proteins and peptides. This has so far been demonstrated for a 24kDa MAP protein and an 18kDa bacterioferritin (Elsaghier *et al.* 1992), for the MAP-specific C-terminal recombinant peptide fragment of the 34kDa component of the MAP A36 complex (Vannuffel *et al.* 1994), for the recombinant p35 and p36 antigens from MAP (El-Zaatari *et al.* 1999; Naser *et al.* 1999; Naser *et al.* 2000), for the alkylhydroperoxide reductase AhpC and a 14kDa protein secreted by MAP (Olsen *et al.* 2001), and for the mycobacterial protein HupB (Cohavy *et al.* 1999). These serological data strongly support the demonstration that CD patients are infected with MAP.

6.7.3 Response of CD to treatment with anti-MAP drugs.

Clinical infections caused by MAC organisms are known to be difficult to eradicate by treatment using standard anti-TB therapy. Relapses and the development of microbial drug resistance are common. MAP are generally resistant to natural streptomyces antibiotics and MAP infections in animals have never been convincingly eradicated (reviewed in Hermon-Taylor *et al.* 2000). Other anti-mycobacterial agents such as isoniazid, ethambutol and pyrazinamide act by blocking the biosynthesis of cell wall components including mycolic acids. MAP in CD is in its ZN-negative form and does not have a conventional mycobacterial cell wall. Treatment of CD with combinations of drugs such as these would not, therefore, be predicted to confer any lasting benefit, and it does not (Thomas *et al.* 1998; Hermon-Taylor 1998). Drugs such as rifabutin and clarithromycin are man-made chemical modifications of natural streptomyces antibiotics with enhanced activity against MAC and MAP. In their inhibition of RNA polymerisation and of microbial protein synthesis at the level of the ribosome, rifabutin and clarithromycin act in synergy and may also be potentiated by the anti-leprosy drug clofazimine (Warek and Falkinham 1996; Ghebremichael *et al.* 1996; Hermon-Taylor 2002). All three agents have the additional advantage of being concentrated within macrophages where MAP in CD occurs. A double blind randomised placebo-controlled trial of rifabutin clarithromycin and clofazimine treatment in CD, based on several centres throughout Australia, is due to report in November 2004 (Selby *et al.* 2001). In the meantime, the results of four open-label clinical studies of the use of rifabutin and clarithromycin, with or without clofazimine, all say essentially the same thing (Gui *et al.* 1997; Borody *et al.* 2002; Shafran *et al.* 2002; Douglass *et al.* 2001). This is that a substantial proportion of people with active CD will get better and their inflamed gut will heal when treated with these anti-MAP agents.

Rifampicin and erythromycin, the parent compounds, will kill many ordinary gut bacteria, but they are not active against MAP, and do not heal CD. Rifabutin and clarithromycin will also kill many ordinary gut bacteria, but they are usually active against MAP, and can heal CD. This reasoning favours the conclusion that when CD heals on rifabutin and clarithromycin treatment, it is because these agents are acting against the underlying causative MAP infection (Hermon-Taylor *et al.* 2002).

6.7.4 Pathogenic mechanisms of MAP in CD.

Although there have been recent advances (Clark-Curtiss 1998; Brosch *et al.* 2001), we still do not have a complete understanding of the way in which *M.tuberculosis*, *M.leprae,* and MAC cause disease. We know little of the specific pathogenic mechanisms of MAP. How can a relatively low copy number of very slowly replicating ZN-negative intracellular MAP, able to minimise immune recognition, cause so much chronic inflammatory disease right across the gut wall in CD ? It is most unlikely to be a direct florid response to MAP 'antigens'.

Epidemiological evidence suggests that the increased gut permeability well known to occur in CD is determined by exposure to environmental factors (Soderholm *et al.* 1999). Monocyte dysfunction and immune dysregulation are also well known in CD (Fiocchi 1998; Shanahan 2002; Monteleone *et al.* 2002). *M.avium* infection perturbs immune function (Holland 2001; Wagner *et al.* 2002). A model for the way in which MAP causes CD which is consistent with all the clinicopathological and therapeutic data, is one in which parasitization of immunoregulatory cells like macrophages and cells of the lamina propria by MAP, makes the gut mucosa leaky and establishes a variable immune dysregulation throughout the gut wall, and probably elsewhere. The inflammation itself then results from a disordered immune response to entry into the gut wall of food residues and microorganisms from the gut lumen. This would be why immunosuppression or immunomodulation can make CD better, when it would make TB worse. This would be why CD can improve with elemental diets, by reducing the allergic component of the inflammatory response to food residues and altering the intestinal flora. This would be why in colonic disease, CD can improve on treatment with drugs such as ciprofloxacin and metronidazole which are active against the invasion by ordinary gut bacteria. It would also be why active CD usually returns when these treatments are stopped, because the underlying causative MAP pathogens are still there.

Other specific disease mechanisms involve MAP-induced damage to enteric glial cells (EGC) and enteric neurones which may be an early event in MAP infection of the gut (Hermon-Taylor and Bull 2002). Through ligands such as

the HupB protein (Cohavy *et al.* 1999; Shimoji *et al.* 1999) which participates with specific terminal trisaccharides in mediating initial Schwann cell adhesion by the leprosy bacillus (Ng *et al*), MAP shares some of the neuropathic properties of *M.leprae* (Rambukkana *et al.* 1997). Parasitization of the abundant and heterogeneous population of EGCs by MAP would account for the MHC class II expression on EGCs in CD (Geboes *et al.* 1992). It would also participate in establishing the enteric neuritis and the neuronal changes well known in CD, and clearly demonstrated in the gut of MAP-infected animals (Geboes and Collins 1998; Gwozdz *et al.* 2001). Damage to EGCs in a transgenic mouse model has been shown to impair gut mucosal as well as vascular integrity, and to result in inflammatory disease of the small and large intestine with pathological features reminiscent of early CD (Bush 2002; Cornet *et al.* 2001). Abnormalities affecting EGCs and enteric neurones are clearly involved in the pathophysiology of CD (Shanahan 1998; Cabarrocas *et al.* 2003) and it is probable that these are caused by MAP.

6.8 SUMMARY AND CONCLUSIONS

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a member of the *Mycobacterium avium* complex. It is a robust phenotypically versatile mycobacterium which can adopt both a ZN-positive and ZN-negative forms. It can be extremely difficult to isolate in laboratory culture. MAP causes chronic inflammation of the intestine Johne's disease (JD) of a broad range of histopathological types, affecting many species of animals including primates. At least 30 different genotypes of MAP have been characterized including distinct cattle and sheep strains. MAP can infect animals for years without causing clinical disease. MAP infection in domestic livestock is widespread in Western Europe and North America. In the UK, MAP is transmitted to humans in retail pasteurised milk. Infected farm animals shed MAP onto pastures where the organism can survive for months or years. Wildlife such as rabbits and their predators as well as other animals become infected. Grazing cattle ingesting infected rabbit droppings can establish a cycle of re-infection. MAP in run-off from contaminated pastures can access surface waters with an associated risk of transmission to human populations in aerosols or in domestic water supplies.

Crohn's disease (CD) in humans is a systemic disorder whose principal feature is chronic inflammation of the intestine. It closely resembles paucimicrobial JD in animals. CD is a new disease which emerged from a low background level in Western Europe and North America in the late 1940's. It has continued to rise in incidence and prevalence so that there are now about $6x10^5$ cases in North America and $3\text{-}5x10^5$ in Western Europe. In Iceland the

import of MAP infected animals in 1933 resulted in widespread JD by 1960, followed by a 13 fold increase in CD by 1990-94. Exposure of humans to MAP may, after a long lead time, result in CD in those with an inherited or acquired susceptibility. MAP is detected in the inflamed gut of almost everyone with CD if appropriate methods are used, and is associated with antibody recognition of selected MAP components. CD can heal on treatment with anti-MAP drugs though the MAP infection is very difficult to eradicate. MAP in CD is present in its tough ZN-negative form. The proposed disease mechanism is a MAP-induced immune dysregulation and enteric neuritis. Chronic inflammation results from the perturbed immune response to entry into the leaky gut, of microorganisms and food residues from the lumen.

Much research is needed to identify the environmental compartments, habitats and pathways of MAP, as well as the effect on MAP physiology and evolution, of intracellular trafficking through protozoa. Much research is also needed to identify the detailed distribution of MAP infection in animals and humans and to develop a range of preventative and therapeutic vaccines.

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