

## 6

# The *Mycobacterium avium* subspecies *paratuberculosis* problem and its relation to the causation of Crohn disease

---

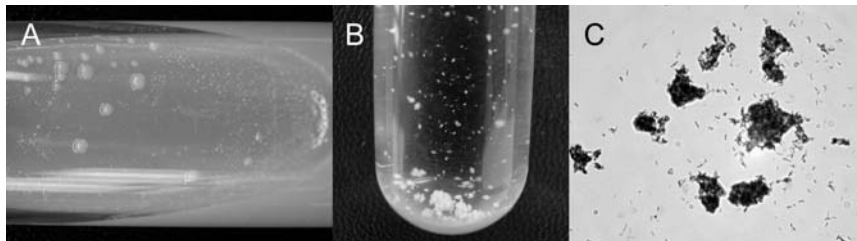
*J. Hermon-Taylor and F.A.K. El-Zaatari*

### **6.1 MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS**

MAP is a member of the *M. avium* complex. A genotypic definition of MAP and its distinction from other MAC is given in Chapter 4. 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 0.5 µm by 1-2 µm and is an obligate intracellular pathogen. Bovine strains of MAP, which can usually be isolated in laboratory culture, grow much more

© 2004 World Health Organization. *Pathogenic Mycobacteria in Water: A Guide to Public Health Consequences, Monitoring and Management*. Edited by S. Pedley, J. Bartram, G. Rees, A. Dufour and J. Cotruvo. ISBN: 1 84339 059 0. Published by IWA Publishing, London, UK.

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; on Herrold's media containing egg yolk they are smooth and opaque (Fig 6.1A). 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 & Collins 2003). In liquid media MAP grows in characteristic tight clumps (Fig 6.1B). In laboratory culture most of the microbial cells stain red by the ZN reagent (Fig 6.1C). 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 their 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.



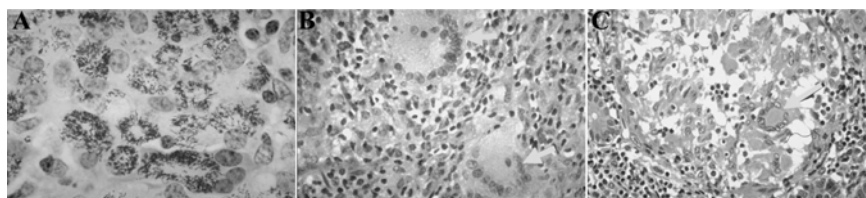
**Fig. 6.1** A: Smooth colonies of a bovine strain of MAP after 10 weeks of culture on a Herrold's media containing egg yolk slope in a sealed tube. B: Bovine MAP after 10 weeks of culture in MGIT liquid medium (Becton Dickinson) showing characteristic clumping. C: Microscopic appearance of a bovine MAP strain from liquid medium showing the red acid alcohol-fast ZN staining typical of mycobacteria in bacillary form. (See also colour plate section between pages 82 and 83).

## 6.2 MAP INFECTION AND JOHNE DISEASE IN DOMESTIC LIVESTOCK

MAP was first identified in Germany in 1895 at the Veterinary Pathology Institute in Dresden by Professor Johne and Dr Frothingham (Johne & Frothingham 1895).

The organism was causing chronic inflammation of the intestine in a cow. The condition became known as Johne Disease. 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 & Abbas 1983; Chiodini *et al.* 1984a; Cocito *et al.* 1994; Clarke 1997; Beth Harris & Barletta 2001; Manning & 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, pigs, horses, chickens and primates are 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 ZN-positive acid-fast bacilli in intestinal macrophages, to an extreme paucimicrobial form with no visible acid-fast organisms and florid chronic granulomatous inflammation (Fig 6.2). The pluribacillary to paucimicrobial range of MAP disease in animals thus closely resembles the range in the appearances of leprosy in humans: the lepromatous to tuberculoid forms. The pluribacillary picture is the common form seen in naturally as well as in experimentally infected animals.



**Fig. 6.2** A: Microscopic appearance of the gut wall in pluribacillary JD showing macrophages containing abundant ZN-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 CD in humans showing giant cell (arrow). (See also colour plate section between pages 82 and 83).

Animals that are naturally or experimentally infected with MAP 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 CD 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 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 IFN $\gamma$  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 IS900 PCR to the culture (Eamens *et al.* 2000; Kalis *et al.* 2000; Whittington *et al.* 2000). To date, commercially available ELISA kits lack the sensitivity and specificity to diagnose subclinical MAP infection at an early stage. The performance of these ELISA tests may improve through the progressive introduction of MAP antigens of greater specificity. ELISA is, however, a cheap and convenient screening test. Their practical usefulness can be enhanced mathematically and by the derivation of likelihood ratios (Beyerbach *et al.* 2001; Collins 2002). IFN $\gamma$  tests of cell-mediated immunity to MAP antigens detect subclinical 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 United Kingdom have ranged from individual animal infection rates of 1.9-9% and herd prevalences (1 to 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, 2000;

Boelaert *et al.* 2000; Jakobsen *et al.* 2000; Muskens *et al.* 2000; Nielsen *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% (McNab *et al.* 1991; Collins *et al.* 1994; Wells *et al.* 1996; Johnson-Ifearulundu & Kaneene 1999; Van Leeuwen *et al.* 2001). Infection rates reported in beef herds have been much lower (Dargatz *et al.* 2001; Waldner *et al.* 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). More than 30 different MAP strains have been identified using methods such as restriction endonuclease analysis, IS900 RFLP, and 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). 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 nonetheless 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 strains, however, have a much broader host range. IS900 RFLP typing of MAP isolates from humans with CD has so far demonstrated that they are all based on the cattle C-type, type II background (François *et al.* 1997; Pavlik *et al.* 1999; Whittington *et al.* 2000a).

A unique 12 bp 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 11 bp fragment present in sheep strains which was absent from bovine strains tested (Dohmann *et al.* 2003). Differences in MAP strains from cattle and sheep have been demonstrated between Argentina and Europe (Moreira *et al.* 1999), and between Australia and Iceland (Whittington *et al.* 2001b). Typing of IS1311 polymorphisms from MAP isolates obtained from nine bison in Montana, USA showed consistent variation at base position 223 compared with 13 C-type isolates from cattle and goats in the United States. 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.* 2001a). 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 growth, 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, and discussed in Chapter 5, include random amplified polymorphic DNA patterns (Scheibl & Gerlach 1997; Pillai *et al.* 2001) and a multiplex PCR typing procedure which utilizes a common IS900 primer together with a locus-specific primer (Bull *et al.* 2000). PCR typing of *M. tuberculosis* based upon mycobacterial interspersed repetitive units (Supply *et al.* 2000) has been adapted for MAP (Bull *et al.* 2003a). PCR typing based upon six mycobacterial interspersed repetitive units 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 6.2 (A), 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 (Beard *et al.* 2001a; Daniels *et al.* 2001). 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.* 2001). 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 (Burgelt *et al.* 2000; Ferroglia *et al.* 2000; Nebbia *et al.* 2000; Pavlik *et al.* 2000; Fischer *et al.* 2001, 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, the physically more robust MAP is known to survive for months and perhaps years since no upper limit on the environmental survival and persistence has been established. Geographical regions characterized 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-Ifeorulundu & Kaneene 1997; Iivanainen *et al.* 1999b; 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 & 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 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 recently been 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*. Studies carried out in several laboratories have sought to determine whether exposure to 72 °C for 15 seconds, conditions commonly used in commercial pasteurization, would ensure the destruction of all viable MAP (Chiodini & Hermon-Taylor 1993; Grant *et al.* 1996, 1999, 2002a; Meylan *et al.* 1996; Stabel *et al.* 1997; Keswani & Frank 1998; Sung & Collins 1998; Pearce *et al.* 2001; Gao *et al.* 2002). Despite some potential limitations in these studies, the substantial balance of experimental evidence strongly predicted that pasteurization at 72°C for 15 or 25 seconds, while reducing the number of viable organisms, would not ensure the destruction of all MAP.

Field studies in the United Kingdom using IS900 PCR to screen retail pasteurised cows' milk for MAP, 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 optimized decontamination protocols and immunomagnetic capture, found that 11.8% of 567 samples of retail pasteurized 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 IS900 PCR positive emphasizes the risk that this may be happening elsewhere (Corti & 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 (reverse transcription 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 and the meat and offal passed for human

consumption. These animals contain huge numbers of MAP with a high risk of dissemination in the abattoir 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 fertilizer.

### 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 that can survive in the environment (Szewzyk *et al.* 2000; Le Dantec *et al.* 2002a) would suggest that there is a high risk that MAP may from time to time be transmitted to people in drinking-water or by aerosols (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. CT values for the effect of chlorine on MAP have been estimated to be up to 580 to 2300 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 CD. The first of these involved the village of Blockley, a rural community of about 2000 people in Gloucestershire, England in which 12 people developed CD between 1960 and 1983, an increase of observed over expected (for that time) of 6.7-fold and equating with a CD incidence of  $28/10^5$  per year (Allan *et al.* 1986). 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 JD 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 the Mankato West High School class of 1980.

All seven 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. High faecal coliform counts in the Blue Earth River, 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 & Freda 2001). Although not discussed by these authors, it is highly likely that these waters were from time to time heavily contaminated with MAP. There are also data which implicate domestic hot water systems. Two case control epidemiological studies carried out independently in the United Kingdom, 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 (Gent *et al.* 1994; Duggan *et al.* 1998).

Mycobacteria are known to occur in aerosols, and to concentrate to high levels in the water droplets (Blanchard & Syzdek 1972; Wendt *et al.* 1980; refer also to Chapter 3). Cardiff is a city on the coastal plain of South Wales in the United Kingdom, 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. Research carried out in Cardiff during the 1970s demonstrated a highly significant increased incidence of CD ( $p < 0.001$ ), but not of ulcerative colitis, in 11 of the local electoral city wards (Mayberry & Hitchens 1978). Of these high incidence wards, eight directly bordered the river Taff and the three 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; Calder *et al.* 1993; Dierkes-Globisch & Mohr 2002; Herrlinger *et al.* 2002). Much research is needed on MAP in the environment, in surface and groundwaters and in aerosols.

## 6.6 CROHN DISEASE

### 6.6.1 Definition

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 (Fig 6.3).



**Fig. 6.3** Typical appearance of an inflamed terminal ileum in a person with active CD. (See also colour plate section between pages 82 and 83).

CD usually presents with abdominal pain, feeling unwell, loss of energy and 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 *et al.* 1992; Geboes & Collins 1998).

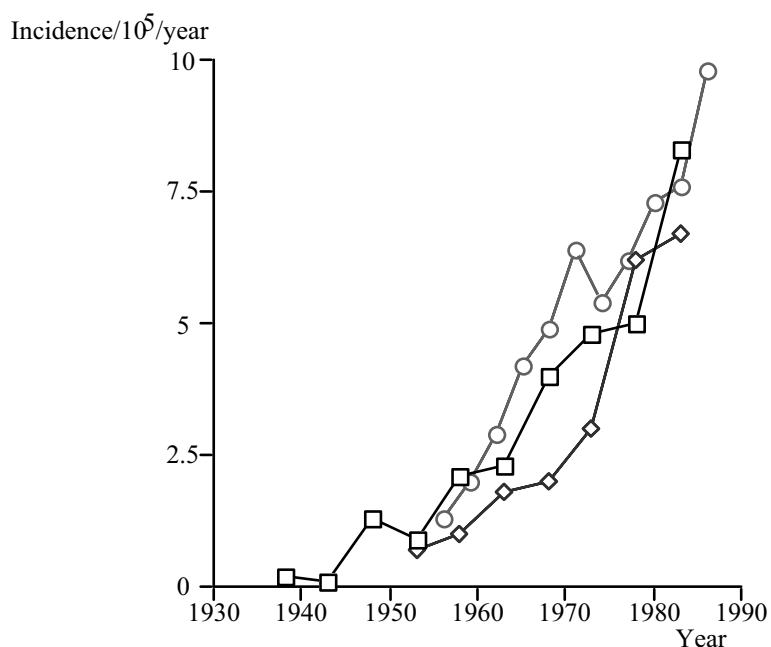
Treatment of CD has been limited to the suppression or modulation of the inflammatory process. This can sometimes achieve and maintain remission over prolonged periods. Relapse frequently occurs and is often triggered by physical and psychological stress. Surgery is required if the disease gets out of 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 as 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 characterized 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 first appearing in developed societies in temperate regions with intensive farming. From a low background level of sporadic cases recorded over many years (Combe 1813; Moschowitz & Wilensky 1923), chronic inflammation of the intestine of the CD type began to emerge perceptibly about a third of the way into the 20<sup>th</sup> century (Crohn *et al.* 1932). Thereafter, with plateaus at times in some regions, the incidence and prevalence of CD have continued to climb (Fig 6.4).

Comparable increases in the incidence of CD were recorded in North America and continental Europe (Loftus *et al.* 1998; Munkholm *et al.* 1992). In the United Kingdom 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; Lopez Miguel *et al.* 1999) and east to European countries such as 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 as Iran (Merat *et al.* 2002), India (Pai & 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).



**Fig. 6.4** The incidence of CD in three regions of the United Kingdom: South Wales □---□ (Rose *et al.* 1988); the Midlands ◇---◇ (Fellows *et al.* 1990); and north-eastern Scotland ○---○ (Kyle 1992) over the 50 year period 1940 to 1990. (See also colour plate section between pages 82 and 83).

The highest overall incidence (15.6/100 000 per year) 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 per year, 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. Loftus *et al.* (2002) estimated the number of CD sufferers in the United States to be 600 000 although it could 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 year in 1990-92 to 8.4/100 000 per year in 1999-2001 (Hildebrand *et al.* 2003). In Victoria, Australia the CD incidence in children rose from 0.128 to 2.0/100 000 per year over the period 1971-2001 (Phavichitr *et al.* 2003). In each case, this represents an average five-fold increase in CD in children per decade. There is 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 (Montgomery *et al.* 1999; Laharie *et al.* 2001). 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.* 1993a), 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 missense variants and frameshift mutations affecting the *CARD15(NOD2)* gene on human chromosome 16 (Hampe *et al.* 2001; Hugot *et al.* 2001; Ogura *et al.* 2001). This gene encodes a transmembrane receptor for bacterial products like lipopolysaccharide expressed on monocytes, and related to the Apaf-1 family of apoptosis regulators. Several polymorphisms are associated with CD susceptibility (Cuthbert *et al.* 2002; Lesage *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 lipopolysaccharide 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; Cavanaugh 2001; Vermeire *et al.* 2002; 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 inflammatory bowel disease (Brant *et al.* 2003), and a number of other loci have been implicated on chromosomes 1, 3, 4, 5, 6, 7, 12 and 14 (Duerr 2002; Watts & Satsangi 2002; Bonen & 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. However, the picture is blurred by the putative dispersal of MAP in food products, water and 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<sup>2</sup> 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 population. About 60% of the people live in the capital Reykjavik. There are three hospitals, the main one in Reykjavik, and centralized 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 five 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 would not grow in culture; a characteristic of sheep MAP strains. The organism from these cattle was later confirmed as the sheep strain of MAP by IS1311 restriction endonuclease analysis (Whittington *et al.* 2001b). Slowly the infection spread so that by the late 1950s the disease was epidemic with about 30% of sheep farms affected and huge annual losses. The mean incidence of CD (number of cases/10<sup>5</sup> per year) 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 five-year period being 8.2 in 1992. Young people were particularly affected (Bjornsson 1989; Bjornsson *et al.* 1998; Bjornsson & Johannsson 2000).

Apart from an increase in the sale of cigarettes during World War II, no nutritional or environmental risk factors were found to 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 exactly what would be expected if the major environmental factor causing CD was MAP.

## 6.7 MAP CAUSING CROHN DISEASE

In 1988 a previously healthy seven year-old-boy living in a village outside Cambridge, England developed NTM 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 IS900 PCR. Drug treatment was continued for almost two more years during which he was disease free. About two years after stopping the drugs, the CD recurred in the ileum next to the anastomotic site, despite his having been off all British milk products. His CD again responded to rifabutin and clarithromycin.

The value of this isolated case lies in the way in which it illustrates the relationship between MAP and CD. MAP infection of the cervical lymph glands in this boy was probably acquired from British milk just as with *M. bovis* before pasteurization. The ingested MAP pathogens would also have colonized 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 colonization 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 & Bishai 2000; zu Bentrup & 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 Crohn disease

The proposition that MAP (Johne's bacillus) could cause chronic inflammation of the intestine in humans as well as 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 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 R. Chiodini in the United States during the mid-1980s when he and his co-workers, using optimized cultures and incubation times of months or years, isolated an unclassified *Mycobacterium* sp. from the inflamed gut of three people with CD (Chiodini *et al.* 1984a, 1986; Chiodini 1989). These isolates caused chronic inflammation of

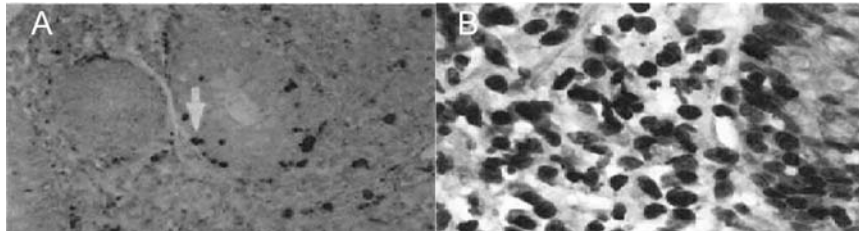
the intestine when administered to young goats. In half the goats no ZN-positive mycobacteria could be seen microscopically in the inflamed tissues, as with CD (Van Kruningen *et al.* 1986). Other workers were able to isolate spheroplasts and acid-fast bacilli from CD, but in the absence at that time 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 (Hermon-Taylor *et al.* 2000; Chamberlain *et al.* 2001; El-Zaatari *et al.* 2001).

The availability of IS900 (Green *et al.* 1989) and its use as a probe and 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.* 1987a; Moss *et al.* 1992; Wall *et al.* 1993). IS900 PCR together with DNA extraction protocols optimized using fresh surgically removed 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 showed 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 optimized 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 localization of MAP in CD tissues, such as laser capture microdissection and *in situ* hybridization. Researchers 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 six of seven (86%) people with CD (Schwartz *et al.* 2000). They also cultured MAP from the breast milk of two women with CD who had recently given birth, but not from the milk of five women who did not show the disease (Naser *et al.* 2000). Collaborative research at the Baylor College of Medicine, USA and at 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).

*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 none of 12 disease controls (Ryan *et al.* 2002). IS900 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 United States and Denmark (Collins *et al.* 2000). Research in London, England using optimized tissue processing (with mechanical disruption) and nested IS900 PCR, detected MAP in fresh ileocolonoscopy mucosal biopsies (Fig 6.5) 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.* 2003). In this study, identity with IS900 was verified in every case by amplicon sequencing. The IS900 multicopy element as defined by its entire DNA sequence is unique for MAP.



**Fig. 6.5** Demonstration of MAP in CD tissues by *in situ* hybridization on paraffin-embedded tissue sections using an IS900 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). (See also colour plate section between pages 82 and 83).

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. pneumoniae*, *N. meningitidis*, and *H. pylori*.

### 6.7.2 Serological recognition of MAP proteins in Crohn disease

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 (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 ELISA tests 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 hypothesis that CD patients are infected with MAP.

### **6.7.3 Response of Crohn disease 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 is generally resistant to natural streptomycetes antibiotics and MAP infections in animals have never been convincingly eradicated (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 (Hermon-Taylor 1998; Thomas *et al.* 1998). Drugs such as rifabutin and clarithromycin are man-made chemical modifications of natural streptomycetes 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 & 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 randomized 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: 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 (Gui *et al.* 1997; Douglass *et al.* 2001; Borody *et al.* 2002; Shafran *et al.* 2002).

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 2002).

#### 6.7.4 Pathogenic mechanisms of MAP in Crohn disease

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 minimize 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 impaired immune regulation are also well known in CD (Fiocchi 1998; Monteleone *et al.* 2002; Shanahan 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 impaired immune regulation 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 and enteric neurones which may be an early event in MAP infection of the gut (Hermon-Taylor & 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.* 2000), MAP shares some of the neuropathic properties of *M. leprae* (Rambukkana *et al.* 1997). Parasitization of the abundant and heterogeneous population of enteric glial cells by MAP would account for the MHC class II expression on enteric glial cells 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 & Collins 1998; Gwozdz *et al.* 2001). Damage to enteric glial cells 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 (Cornet *et al.* 2001; Bush 2002). Abnormalities affecting enteric glial cells 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 KEY RESEARCH ISSUES

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.

## Acknowledgements

This work was supported in part, in the UK by grants from the Medical Research Council, the Natural Environment Research Council and the charity Action Medical Research, and in the USA by the NIH grant DK63092 and the Research Service of the Department of Veterans Affairs, Houston, TX.