REVIEW ARTICLE

The role of mycobacteria in Crohn's disease

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Introduction

Crohn's disease (CD) was first described in 1932 as a chronic inflammation of the terminal ileum. CD affects primarily young adults and the elderly, but can occur at any age.¹ It is now recognised that although involvement of the ileocaecal area is most frequent (50%), the disease can effect any area of the gastrointestinal tract from mouth to anus,¹ including the nasal cavity.² In children and adolescents there is often upper gastrointestinal tract involvement.³ Symptoms include fever, diarrhoea, cramping pain in the abdomen, nausea, vomiting, anaemia and weight loss.¹ During the past 30 years, several rare complications of CD have been described that can be life threatening.⁴

CD is one of a group of inflammatory bowel diseases (IBD) that also includes ulcerative colitis (UC). The clinical signs of CD and UC are similar; consequently mis-diagnosis occurs in an estimated 20% of cases.¹ UC affects primarily the mucosa which is congested with diffuse mononuclear cell infiltration of the lamina propria. There is also marked goblet cell depletion and gland infiltration by neutrophils. In contrast, both gland and goblet cells are preserved in CD. It is a granulomatous disease affecting the submucosa which is characterised by infiltration of inflammatory cells. CD is an invasive disease of the bowel wall, thus sampling by biopsy may be ineffective and can impede diagnosis.⁵

Understanding of the epidemiology of CD is confused because many of the symptoms of the disease are shared by other IBD. There is a lack of uniformity in the criteria used and the emphasis they are given in aetiological investigations. Population studies have indicated that CD is more frequent in the developed than in the developing world, where it is seldom reported.⁶ It is prevalent particularly in Scandinavia, the USA and the UK and also in Jewish populations.^{7,8}

Population studies of different ethnic groups, migrants and families have been used to investigate whether environmental, dietary or genetic links are implicated in the aetiology of CD.⁹ Although some studies suggest a hereditary predisposition,¹⁰ CD is thought to have a multifactorial aetiology involving genetic, environmental, microbial and immunological factors.

A specific aetiological agent of CD has not been identified. The inflammatory nature of the disease has led to studies focusing on imbalance of the immune system, resulting from either an inherited disorder or as a response to microbial antigens in the gut. A wide variety of gastrointestinal conditions, such as entero-colonic infections, can mimic CD and so confuse these investigations.⁵ Researchers testing the microbial hypothesis have sought evidence from culture, immunology, chemotherapy and animal models. The specificity and sensitivity of bacterial detection has improved recently with the application of molecular biological techniques. Despite considerable effort, progress in understanding CD has been slow. The results generated often have been inconclusive and do not clearly indicate whether the agent under investigation is causal or consequential.

Other diseases in man, animals and birds have symptoms similar to CD. They include various tuberculous-type diseases associated with the genus *Mycobacterium*. Mycobacteria can be divided crudely into specific and opportunist pathogens. The latter are free-living saprophytes commonly found in the environment¹¹ and include the *M. avium-intracellulare* group, referred to as the MAI complex. They have been associated with opportunist infections in AIDS patients,^{12,13} including a terminal ileitis which resembles CD clinically.¹⁴

Studies based on phenotype¹⁵ and genotype^{16–20} have shown that *M. paratuberculosis* clusters with the MAI complex. This species colonises the intestinal mucosa of ruminants causing Johne's disease (JD).²¹ CD and JD share clinical symptoms. Both are chronic granulomatous diseases of the gut affecting nutrient absorption and frequently affecting the young. Consequently, it has been proposed that *M. paratuberculosis*

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is the causative agent of CD.^{6,22} Interpretation of results from studies on this group of organisms is compounded by several factors. Compared to other organisms in faecal samples they are present in low numbers.^{6,23} A decontamination procedure is required to eliminate most other organisms present in faecal samples. In practice, the decontamination protocol differs between laboratories¹¹ and the regimen adopted can bias the isolation of mycobacterial species.²⁴ Because of the ubiquity of these organisms, care has to be taken to guarantee that the bacteria represent true isolates from a sample and not environmental contaminants.⁶ The purpose of this review is to evaluate the evidence linking *M. paratuberculosis* to CD.

Epidemiology of Crohn's disease

Population studies

CD occurs most frequently in the USA, UK and Scandinavia.^{25,26} It is less frequent in Central Europe and rarely reported in Asia, Africa and South America.^{27,28} Conversely, where CD is rare, other mycobacterial diseases, such as tuberculosis and leprosy, are common.¹ An increased incidence of CD was reported during the 1960s in areas where it was prevalent.^{29,30} During the 1970s there was a tendency towards a plateau in these regions.^{29,31,32} Recent data from specific locations within these areas suggest that the incidence may be falling.^{33,34} In the USA, the decrease in tuberculosis in the black population was accompanied by a corresponding increase in CD.³⁵ Currently, the incidence of CD is declining in the USA and the incidence of tuberculosis is again increasing.³⁶

Population studies show that equal numbers of males and females are affected by CD.³⁷ Peak ages for CD are 15–25 years and 50–80 years.³² There is no clear delineation of risk between urban and rural dwellers,³² but there have been reports of a higher prevalence in urban areas in Alaska,³⁸ and also in Ashkenazi Jews, in whom the incidence appeared to rise with migration to an urban population.³⁹ Epidemiology of CD has been studied in selected cases from small groups rather than large populations; consequently it is a difficult and muddled area.²⁶

Studies of Jewish populations provide evidence for both genetic and environmental factors in the aetiology of CD. Genetic factors are implied from the seven-fold increase of CD among Jews^{40–42} compared to the non-Jewish population living in the same locality.⁴³ There has been an increased incidence of CD in the Jewish populations of Tel Aviv and Beer Sheva over the past 20 years.⁴⁴ In contrast, CD is rare among Bedouin Arabs living in Beer Sheva.⁴⁵ The incidence of CD in these Bedouin Arabs, a rural population, has not increased in recent years and is similar to that of urban populations of Arabs in Kuwait and Egypt.²⁷ Arabs with chronic intestinal disease are more likely to have tuberculosis than CD.⁴⁶ The incidence of CD among Jews in Beer Sheva varied

with their origin, i.e., Israeli *versus* USA- or European-born.⁴⁷ This trend has emerged from several studies of subgroups living in, or originating from, different geographic locations. The disease is less common in non-Ashkenazi, Israeli-born Jews, than in Ashkenazi Jews, although the incidence in first degree relatives of cases was similar in Ashkenazi and non-Ashkenazi Jews.⁴⁸

It was proposed that the increased incidence of CD in Ashkenazi Jews, who eat more meat, milk and eggs, may be linked to diet but investigations showed that the prevalence of CD cannot be explained by diet alone, as the incidence in this group is lower in Tel Aviv than in the USA.⁴⁹ Further studies substantiate a multifactorial aetiology for CD. Jewish CD patients in Los Angeles showed a positive family history of 27.5%.⁴² In a follow-up study of Ashkenazi Jews with CD in California, the country of origin of the grandparents was included in the analysis.⁵⁰ A parallel study was performed in Israel on Ashkenazi and American-born Jews with CD.⁵¹ Both found that a significant majority of Ashkenazi Jews originated from "middle" Europe, suggesting a genetic link. However, in a study of Jews in Baltimore, intermarriage was not associated with a greater risk of CD, arguing against a genetic link.²⁷ In summary, the epidemiological data for the Jewish population provides evidence for and against a hereditary predisposition and environmental factors.

Familial studies

The observations from studies of the incidence of CD in the Jewish population coincide with those from familial epidemiological studies, which indicate that the aetiology of CD involves a polygenic inheritance with environmental influences.^{25,26,51} The geographic distribution of CD implies an environmental aetiology²⁵ but the few reports of IBD in patients' spouses^{52–55} and adopted children⁵⁶ suggest that environmental factors may be less important than inheritance. The polygenic nature of CD has been challenged recently; a modelling study of epidemiological data has implicated a recessive gene in preference to polygenic inheritance.⁵⁷ Until a suitable marker gene is found for CD, this cannot be substantiated.

An increased family prevalence in CD has been reported from several specialist referral centres^{10,58,59} and from population studies.^{55,60} A genetic predisposition in first degree relatives 13 times that of the control population was found in Cardiff.⁵⁵ A similar study in Sweden reported a prevalence in first degree relatives of 21 times the expected level.⁶ Corroborative evidence for an inherited predisposition was provided in the Swedish study from monozygotic and dizygotic twins. Concordance for CD occurred in four of six pairs of monozygotic twins, but in no dizygotic twins.⁶ This was consonant with data from a Swedish twin register, where eight of 18 monozygotic twins were

concordant for CD compared to one of 26 pairs of dizygotic twins.⁶

Genetic linkage

The epidemiological association of CD with countries, races and more than one family member⁶¹ has resulted in a quest for a genetic marker. It has been proposed that a genetic defect may prevent the patient from mounting a controlled and effective immune response to the causative agent.⁶² No evidence was found in familial studies,⁶³ or in a study of Jewish patients,⁶⁴ for linkage with HLA type. Clinical patterns in siblings indicated that a genetic factor not linked to the HLA system may be involved.⁶⁵ However, extensive analysis of all the available data for HLA has shown an increased but not constant association of HLA-2 with CD in caucasians.⁶⁶ Similarly, IBD patients in the Japanese and Israeli Jewish population with restricted genetic heterogeneity have definite, but different, HLA phenotypes.⁶² Chromosome 14, which regulates the immunoglobulin heavy chain complex, may be implicated in CD. There is evidence for⁶⁷ and against⁶⁸ the association of certain heavy chain allotypes with CD.

The inclusion of patients spanning different clinical conditions of CD, i.e., mild *versus* chronic, and those with and without remission, may obscure direct relationships with a genetic marker. It has been proposed that, because of differing responses to therapy, a subclassification of CD is required.⁶⁹ Cluster analysis has indicated that CD patients fall into two groups—those who achieve remission within 3 years and those in whom disease persists after 3 years.⁷⁰ The immunological data for CD do not suggest a simple inherited disorder, but rather a multifactorial disorder.⁶²

The role of diet in CD

Food antigens have been considered as aggravating factors in CD.⁷¹ There is some evidence for immunity against food antigens,^{72,73} but whether this is a primary or secondary response remains unclear. Specific sensitisation to cow's milk proteins has been demonstrated.⁷⁴ Corroborating this was the reported ability of β -lactoglobulin to activate both peripheral and intestinal mononuclear cells.⁷⁵ However, an examination of hybridoma supernates prepared from active B cells of mesenteric lymph nodes of IBD patients found no evidence for increased antibodies to milk, cabbage or wheat.⁷⁶ The differing results may be a consequence of examining different immunological cell types.

An international study investigating childhood factors, including cereal consumption and frequency of breast feeding, found no significant differences between CD patients and controls,⁷⁷ although it has been noted that CD patients were over-represented among those with no or little breast feeding.⁷⁸ Lack of dietary

fibre^{77,79} and an increased refined carbohydrate consumption,⁸⁰ in particular refined sugar,⁸¹ have also been postulated to play a role. The levels of volatile fatty acids in the gut are increased with the consumption of dietary fibre. Corroborating evidence for the role of dietary fibre is the report that increased volatile fatty acids may be protective in CD.⁸² Special diets,⁸³ including high calorie diets,⁸⁴ have been reported to cause remission in CD. However, no clear clinical benefit emerged when two groups of CD patients were given a diet containing either a high fibre-low refined carbohydrate or a low fibre-high refined carbohydrate content.⁸⁵ The role of diet in CD remains speculative.

Bacteria as the aetiological agents in CD

The elusive aetiology of CD has led researchers to speculate that intestinal bacteria, both indigenous and pathogenic, may play a causal role in the disease. Comparative investigations of the gross intestinal flora between control groups and CD patients have been performed. Evidence for specific bacterial involvement has been sought from various microbiological and immunological techniques. However, there is little consistency with respect to the bacterial species implicated in these studies. The disparity reflects the different techniques and experimental approaches applied which obscures whether certain bacterial species play a causal or consequential role.

Microbiological investigations. A study of gross intestinal flora reported that CD patients with clinically active disease had significantly more aerobes in faecal samples than patients with quiescent disease, UC or controls. The increase in aerobes did not correlate with any of the CD clinical indices⁸⁶ indicating that the changes were non-specific.⁸⁷ Microbiological and histological examination of biopsy specimens from IBD patients showed *Klebsiella* spp. and *Chlamydia* spp. in CD patients, but these organisms were not confined to CD patients and occurred in other patient groups.⁸⁸ Similarly, intestinal bacteria and potential pathogens were isolated from the mesenteric lymph nodes and ileal serosa, respectively, in a higher percentage of CD patients than controls. This suggests that leakage from the lumen occurs in a high proportion of CD patients.⁸⁹

Immunological studies. Higher levels of agglutinins to *Eubacterium* and *Peptostreptococcus* spp., but not to *Clostridium colinum*, *Citrobacter freundii*, *Campylobacter sputorum*, mycobacteria and chlamydiae were found in the sera of CD patients than in the sera of controls; this was reported for two separate geographic areas—Rotterdam⁹⁰⁻⁹² and Cardiff.⁹³ To ascertain the significance of these findings, the occurrence of agglutinins to *Eubacterium* and *Peptostreptococcus* spp. in the sera of CD patients was compared to patients in North-East India with acute or chronic diarrhoea. The study aimed to establish if the incidence of circulating agglutinin was a consequence of increased exposure to these organisms caused by tissue

damage. Agglutinins were found less frequently in the patient groups from India with acute and chronic diarrhoea (11% and 17% respectively) than those with CD from Cardiff (44%), indicating that these organisms may play a causal role.⁹⁴ However, relative numbers of these organisms in the intestinal flora of the two populations was not determined; therefore, the significance of these results cannot be evaluated.

Serum antibody titres to *Bacteroides fragilis*, and to a lesser extent *Enterococcus faecalis*, were reported to correlate with the severity of disease in patients with CD and UC.⁹⁵ These are common intestinal commensals and increased circulating antibody probably reflects exposure to these antigens and so a secondary rather than a primary response.

Quantitative investigation of serum antibodies to several enteric pathogens has not correlated with the severity of the disease. Increased antibody titres were reported for some *Escherichia coli* serotypes,^{96,97} and to *Camp. jejuni*, *Camp. fetus*, *Listeria monocytogenes*, *Brucella abortus*, *Yersinia pseudotuberculosis* and *Y. enterocolitica* in complement fixation tests (CFT) with commercially prepared antigens, but these results proved statistically insignificant. The presence of raised serum antibody to all seven enteric pathogens examined indicated that this was probably a consequence of polyclonal B-cell stimulation during periods of active inflammation and was non-specific. The levels of serum antibodies showed no correlation with the severity of disease.⁹⁸ A study of 18 patients found no microbiological or serological evidence for the association of *Y. enterocolitica* with CD.⁹⁹

Infection with several organisms produces clinical symptoms similar to CD and can confound its diagnosis.¹⁰⁰ These include *Actinomyces israelii*,¹⁰¹ *Salmonella* spp.,¹⁰² *Giardia lamblia*,¹⁰³ *Camp. jejuni/coli*¹⁰⁴ and *Y. enterocolitica*.¹⁰⁵⁻¹⁰⁷ *Chl. trachomatis* causes proctitis which has been reported to be indistinguishable histologically from CD.¹⁰⁸ Antibodies to *Chl. trachomatis* have been demonstrated more frequently in CD cases than controls in some studies,¹⁰⁹⁻¹¹¹ but not in others.¹¹²⁻¹¹⁴ Different methodology may account for these contradictory findings. An alternative approach with PCR amplification of a *Chl. trachomatis* plasmid was used that was capable of detecting one infected cell in 10⁴ cells. The study also investigated the presence of serum antibodies to *Chl. trachomatis*. No evidence was found for an association between *Chl. trachomatis* and CD.¹¹⁵ The chlamydial data do not demonstrate clearly a causal or consequential role for these organisms in CD, and the reported high levels of these organisms in some studies may be a misdiagnosis of proctitis as CD.

The epidemiological investigations of CD have implicated geographic areas, races and diet. The evidence is circumstantial and the contribution of any one factor to the development of CD remains unclear. Similarly, the role of both the indigenous flora and bacterial pathogens in the aetiology of the disease is circumstantial and serological evidence for particular

organisms may be due to leakage of antigens from the lumen. The presence of pathogens can also confound the diagnosis of CD. The role of mycobacteria in CD is considered later.

Diseases related to CD and associated with mycobacteria in man and animals

Gut disorders associated with mycobacteria in man

In man, intestinal tuberculosis (IT) has often been confused with CD. Although they are now considered to be distinct diseases, they share certain similarities.¹¹⁶⁻¹¹⁸ Person-to-person transmission of *M. tuberculosis* is rare in IT.¹¹⁹ Three categories of IT have been defined based on clinical symptoms^{120,121} that vary depending on the degree of the host's response and the organism's virulence. The ulcerative type is the most common; it is associated with intestinal infection with *M. tuberculosis* and there is pulmonary involvement. A second type, ulcerohypertrophic, can be a consequence of pulmonary or intestinal infection and results in ulcers healing with fibrosis and stenosis of the lumen. The third, the hypertrophic form, referred to as pseudotuberculosis, is rare and is caused by primary infection of the gut; it is characterised by intense fibroblastic reactions in the submucosal and serosal layers of the bowel.¹²²

Intestinal tuberculosis must satisfy at least one of the following criteria: positive culture or the development of disease in guinea-pigs after inoculation; microscopic demonstration of acid-fast bacilli; or presence of tubercles with caseation in diseased tissue or granulomata in draining lymph nodes. The hypertrophic form seldom satisfies all of these criteria, but if one of them is met, a positive diagnosis is made.¹²³ The diagnosis of the hypertrophic form differs in the site of the caseous necrosis; it occurs in the draining lymph nodes rather than in the intestinal tissue and culture of *M. tuberculosis* was achieved in only one-third of the cases examined.^{124,125} Caseous necrosis was rarely observed in the culture-positive cases. In hypertrophic IT, the inconsistency of demonstrating *M. tuberculosis* when there is caseous necrosis and *vice versa* questions the validity of the diagnosis and whether CD and hypertrophic IT should be considered distinct pathological diseases—caseous necrosis and the presence of acid-fast bacilli forming the basis for the distinction between CD and IT.⁶ It would be informative to establish which *Mycobacterium* spp. are associated with the three types of IT, and in particular whether the hypertrophic condition represents a disease caused by *M. tuberculosis* or another *Mycobacterium* sp., or is CD.

Only limited success has been achieved with animal models of IT with *M. tuberculosis*, suggesting that this is not the preferred site of infection with this organism.⁶ This contrasts with *M. paratuberculosis* infection and JD in animals, in which animal models mimicking the disease have been established.¹²⁶

Ruminants and Johne's disease

JD is a granulomatous disease of ruminants caused by colonisation of the intestinal mucosa by *M. paratuberculosis*. Young ruminants, particularly those < 30 days old, are susceptible to infection,^{127, 128} thereafter, an age-dependent resistance develops.^{129, 130} Clinical disease developed in sheep inoculated orally with 10^9 but not 10^3 *M. paratuberculosis* organisms.¹³¹ Although the infectious dose remains unknown, investigators have inferred from morbidity that the ingestion of relatively few organisms may cause infection.^{132, 133} A long incubation period precedes the clinical disease,¹³⁴ but is probably dose related.⁵⁸ JD can take up to 15 years to develop,¹³⁵ but occurs most frequently 3–5 years after infection with *M. paratuberculosis*. Infection of ruminants with *M. paratuberculosis* does not always result in JD. When adults become infected, the lesions are less pronounced and contain fewer bacilli, and often the organism is eliminated.¹³⁶ There are also reports of animals infected with *M. paratuberculosis* that never develop clinical disease, but become carriers and shed the organism throughout their lives. Susceptibility to infection varies with different cattle breeds.¹³⁷ Within a chronically infected herd, animals are classified as clinically ill, subclinically infected and infected. The clinical symptoms and pathological lesions differ with ruminant species.

The immunological response in JD reflects the stage of the disease. The initial cell-mediated response is replaced with a humoral response as JD progresses. This in turn is superseded by anergy as the disease enters a chronic phase.¹³⁸

M. paratuberculosis can be detected in JD by culture, a CFT and an enzyme-linked immunosorbent assay (ELISA). The accepted diagnosis for JD in cattle was based on the culture of *M. paratuberculosis* from faecal or tissue samples.¹³⁹ The problems and length of time associated with culture of *M. paratuberculosis* have led to a search for alternative immunological techniques. Although none of these tests offers 100% reliability, *M. paratuberculosis* is detected in a high proportion of samples. For example, in a study of 26 cows with advanced JD, the CFT was positive in 81% of serum samples and *M. paratuberculosis* was isolated from 76% of faecal specimens.¹⁴⁰ A recent PCR method based on the 16S rRNA gene showed 100% correlation with culture positive samples from cattle with clinical and subclinical JD.¹⁴¹

A review of the available protocols noted the need for more sensitive and specific serological tests to facilitate early detection of JD in cattle.¹³⁸ An ELISA based on lipoarabinomannan was described¹⁴² and evaluated in cattle. The ELISA was better at predicting faecal shedding than tissue infection but was less discriminatory (49%) than culture (87%).¹⁴³ Recent improvements to this ELISA with purified antigen resulted in 100% detection in sheep¹⁴⁴ and 83% in cattle.¹⁴⁵ A reliable immunological method for the

diagnosis of subclinically infected cattle is still needed. In asymptomatic cows, ELISA results gave 100% specificity and 70% sensitivity. In an attempt to detect only positive results in cattle with subclinical JD, the authors chose a high ELISA antibody index thus decreasing the sensitivity.¹⁴⁶ If *M. paratuberculosis* produces a similar disease, i.e., CD, in man, the diagnostic methods applied and results from immunological studies should broadly mimic those found in JD.

Other animal diseases associated with mycobacteria

Strains of atypical *M. avium* were isolated from wood-pigeons with tuberculous infection.¹⁴⁷ Subsequently, similar strains which were distinct from *M. avium* were isolated from granulomatous lesions.^{148, 149} The MAI complex has also been associated with granulomatous colitis in a horse.¹⁵⁰ *M. paratuberculosis* is grouped phylogenetically with the MAI complex. Identification of the species within this group by phenotype is difficult, especially for atypical isolates. Consequently, reports based on phenotypic identification can be unreliable. The taxonomic details of the MAI complex are considered in a later section.

Strains identified as *M. paratuberculosis* were isolated from a colony of Stumptail Macaques with clinical and pathological features resembling JD in ruminants and *M. avium* infection in primates. This was the first report of *M. paratuberculosis* infecting non-human primates.¹⁵¹ The criteria used to differentiate these strains from the other members of the MAI complex were based on 5S rRNA ribotyping developed by Chiodini and co-workers.¹⁵² The ribotype obtained from strains isolated in this study and strains isolated from adolescent CD patients gave identical profiles.^{132, 133, 152}

Presence of mycobacteria in food

Although members of the MAI complex are ubiquitous in the environment, a comprehensive epidemiological study in Germany found no evidence to suggest that the environment provided the reservoir for human disease.¹⁵³ This is consistent with previous reports in which soil and human isolates had different biochemical profiles. Unless the biochemical profiles change in the human gut, it seems unlikely that environmental strains provide the source for human infection.^{24, 154} However, if *M. paratuberculosis* or organisms of the MAI complex cause CD, food is a likely vehicle for transmission.

Raw milk has been considered a source of mycobacterial infection.¹⁵⁵ Strains of the MAI complex have been isolated in the USA from raw and pasteurised milk,¹⁵⁶ and from raw milk, but not pasteurised milk in the USA¹⁵⁷ and Australia.¹⁵⁸ *M. paratuberculosis* was found in the milk of nine of 26 cows

with advanced clinical symptoms of JD.¹⁴⁰ This is consonant with reports of *M. leprae* in the milk of mothers with leprosy.¹⁵⁹ A study of asymptomatic cows showed a direct relationship between numbers of *M. paratuberculosis* in supramammary lymph nodes and milk, and faecal shedding. The frequency of *M. paratuberculosis* was less than occurred in symptomatic cows.¹⁶⁰ Strains belonging to the MAI complex have been found in oysters,¹⁵⁷ beef and pork.^{161, 162} The MAI complex have been shown to be more heat resistant than *M. bovis* in meat products;¹⁶³ naturally-occurring isolates are reputedly more susceptible to heat than laboratory strains.¹⁶⁴ In countries where *M. avium* infection in chickens is frequent and laying hens are kept longer, there is a corresponding increase of this group of organisms in human infections.¹⁵³ It is unlikely that cooking processes commonly used for eggs, except for hard-boiling, would destroy these organisms.¹⁶⁵

If the aetiology of CD and JD are comparable, the ingestion of a few organisms could cause disease.¹³⁸ In contrast, if the situation parallels that of IT in man, the size of inoculum causing disease would be larger as results, for example, from the ingestion of milk heavily contaminated with *M. bovis*.¹²⁴ The effectiveness of pasteurisation or cooking procedures and the numbers of surviving organisms would be important if CD had a food-borne mycobacterial origin. There are few studies documenting the thermoresistance of the MAI complex organisms, but it has been demonstrated that they are more heat resistant than *M. bovis*.^{164, 166} MAI complex organisms in liquid media were more thermoresistant than *M. bovis*, but virulent serovars 2 and 3 were less heat resistant than avirulent serovars.¹⁶⁶ The results from survival studies indicate that current pasteurisation processes eliminate viable MAI complex organisms.

The role for mycobacteria in CD: culture, taxonomy and molecular biological studies

Culture of mycobacteria from patients with CD

Mycobacteria are a heterogeneous group of acid-fast bacilli. In 1932 Crohn designated CD as a disease distinct from IT because of the absence of tubercle bacilli.¹⁶⁷ However, interest in their role as an aetiological candidate for CD has continued to be investigated. The first successful culture of mycobacteria from patients with IBD was in 1952 by Van Patten.¹⁶⁸ Seven different media containing extracts from IBD lymph nodes and normal intestine were employed; five supported the growth of *M. paratuberculosis*. A total of 1043 samples from 43 patients were cultured over 12–15 months. Colonies of acid-fast bacteria were isolated from clinical specimens of three patients, but their subculture was not accomplished and so they were never formally identified. Cultivation of mesenteric lymph nodes from 27 patients with CD yielded one strain of *M. kansasii*. The presence of cell-wall-

deficient acid-fast bacteria was confirmed by electron-microscopy in 22 of the patients, but these remained non-cultivable and did not revert to forms with cell walls, preventing formal identification. Spheroplasts were also observed in six of 13 UC and one of 11 control samples, respectively.¹⁶⁹ The occurrence of cell-wall-deficient forms of acid-fast bacteria has been described in sarcoidosis and tuberculosis. Mycobacteriophages isolated from stool and tissue samples converted mycobacteria to a lysogenic state producing "mutants" that could not be cultured, with altered appearance, drug susceptibility and staining properties.¹⁷⁰ There are no reports of mycobacteriophages and CD. There have been several independent reports of the isolation of slow growing mycobacteria from resected intestinal tissue of CD patients^{132, 133, 152, 171–173} and from biopsy samples.^{23, 174} Because of the invasive nature of the disease, they have been found predominantly in resected tissue specimens rather than biopsy samples.⁵

Biovariants of *M. paratuberculosis* or a new *Mycobacterium* species were isolated from teenage CD patients by Chiodini.^{132, 133, 152} These isolates—Linda, Ben and Dominic—have been used extensively in subsequent studies, becoming the prototype strains for mycobacteria associated with CD.¹³³ In a further study, mycobacteria were isolated from 16 of 26 CD patients, but not from 26 patients with UC or other bowel disorders. Seven of 10 spheroplasts isolated from CD patients were identified as *M. paratuberculosis* by agglutination with specific antisera raised against strain Dominic.¹⁵² In a study of 105 patients comprising UC, CD and non-IBD controls, mycobacteria and spheroplasts were isolated from all patient groups,²³ but only a single isolate of *M. paratuberculosis*, biochemically similar to strain Linda, was obtained from a UC biopsy. The authenticity of this identification was later challenged by the results of molecular typing studies.⁶ Slow growing mycobacteria and spheroplasts were present in the clinical specimens, but sparse growth on subculture precluded their identification. They did not appear similar to strain Linda.²³ Spheroplasts were detected in only 27% of resected samples from CD patients and occurred more frequently in resected tissue than in biopsies. It was concluded from these results that mycobacteria were associated with diseased tissue and that they did not exclude a mycobacterial aetiology for CD.²³ With improved media for culture and spheroplast reversion,¹⁷⁵ the same research group obtained 15, 10 and 19 mycobacterial isolates from 74 CD, 10 UC and 38 non-IBD patients, respectively, and 21 spheroplasts from all three patient groups. This substantiated the conclusions of their earlier study that mycobacteria were associated with diseased tissue and that the association was not restricted to CD patients. Only one isolate was biochemically similar to the CD *M. paratuberculosis* isolate—strain Linda. The other isolates were biochemically distinct and were not speciated.¹⁷⁶

Mycobacteria have been isolated in other studies of IBD. Four mycobacterial strains were isolated from resected tissue of 27 CD patients and one from 55 controls by the culture techniques used by Chiodini. Two isolates were identified by biochemical techniques as *M. chelonae* subsp. *abscessus* and *M. paratuberculosis*.¹⁷⁷ In a separate study, acid-fast bacilli were isolated from 11 of 32 CD patients, but not from 17 IBD patients (including UC, and non-specific IBD). Two of the isolates from patients with active CD were identified from cultural, physiological and biochemical data as *M. chelonae*.¹⁷³ The isolation of mycobacteria other than *M. paratuberculosis* supports the findings of Graham *et al.* and contradicts those of Chiodini; in contrast, the absence of mycobacteria in other IBD patient groups supports Chiodini's data, and contradicts Graham's data.

The studies of Chiodini and Graham and their associates are not directly comparable because the treatment of the specimens differed. Graham's group cultured homogenates of the resected tissue, whereas Chiodini's group cultured the mucosa and submucosa from the resected tissue separately. The disinfection procedures employed by the two groups also differed. The isolation of organisms of the MAI complex other than *M. paratuberculosis* from IBD, control, UC and CD samples by Graham and his associates has been attributed to a lack of stringency in their decontamination process. Chiodini considered that the regimen used by Graham precluded the isolation of *M. paratuberculosis* and that their mycobacterial isolates were environmental mycobacteria, which occur at similar levels.⁶ However, the decontamination procedure of Graham did enable the isolation of strain Linda and a similar strain.²³ Other groups used the Chiodini methodology and obtained mycobacterial isolates other than *M. paratuberculosis* from CD tissue.^{174, 177}

In summary, the data on the isolation and identification of mycobacteria, and in particular *M. paratuberculosis*, from CD and IBD patients are conflicting and confusing. The disarray stems from the different techniques and stringencies applied by different groups. The problem is further exacerbated by the occurrence of spheroplasts. The role of mycobacteriophage in their formation remains to be investigated.

Taxonomy of the mycobacteria implicated in CD

The discrepancy over the mycobacterial species implicated in CD arises from the taxonomic methods employed. Originally, biochemical techniques were used for speciation, but this approach is unreliable, particularly for species of the MAI complex.¹⁷⁸ Mycobactin auxotrophy is the principal criterion for ascribing isolates to the taxon *M. paratuberculosis* in preference to the MAI complex.¹⁷⁹ There are reports of atypical animal isolates of *M. avium* that are mycobactin dependent.¹⁸⁰⁻¹⁸² It has been reported recently that the demonstration of mycobactin dependence

requires rigorous experimental conditions;¹⁷⁹ these may not have been practised in earlier studies.

A more precise identification of mycobacterial isolates from CD patients was achieved by restriction fragment length polymorphism (RFLP) with probes to the 5S rRNA of *E. coli*. Identical restriction patterns were obtained with 11 restriction endonucleases (RE) for *M. paratuberculosis* ATCC19698 and three CD isolates (Dominic, Ben and Linda) and also, with spheroplast and cell-wall-producing revertants of these three strains. The RFLP patterns were distinct from those obtained with strains of the MAI complex serovars 2 and 4 and *M. kansasii*.¹⁵²

Southern hybridisation studies in which the 5S rRNA probe was replaced with genomic clones of strain Ben also gave identical profiles for the *M. paratuberculosis* and CD isolates Ben, Dominic and Linda. The RFLP patterns obtained with these strains were again distinct from those obtained with strains of *M. phlei*, *M. kansasii* and the MAI complex serovars 2 and 5. One genomic clone distinguished CD isolates from strains of the MAI complex serovars 2 and 5.¹⁸³ A second series of three clones derived from the same genetic library produced similar, but not identical RFLP patterns with three *M. paratuberculosis* strains—ATCC19698, a JD isolate and strain 18 (isolated and identified by biochemical criteria by Merkal¹⁸⁴). RFLP analysis of strain 18 gave banding patterns identical to *M. avium* serovar 2. Subsequent studies have confirmed the designation of strain 18 as *M. avium*.¹⁸⁵⁻¹⁸⁷ Strain 18 has been used in several immunological studies to assess the humoral response in CD patients (see *Immunology* section).

The studies by Chiodini's group offered improved speciation of the CD isolates, but were limited to a few strains of *M. paratuberculosis*. Two comprehensive studies of > 100 strains examined the genetic variation among isolates of *M. paratuberculosis*. A probe directed against a repetitive DNA sequence specific to *M. paratuberculosis* was used in the RFLP analysis. Cattle isolates of *M. paratuberculosis* from diverse origins showed remarkable RE conservation, and the patterns obtained were generally distinct from sheep and goat isolates.¹⁸⁵ A second study confirmed the heterogeneity among isolates of *M. paratuberculosis* and included strain Linda, the CD isolate, which had a pattern identical to an Australian cattle isolate.¹⁸⁸ In contrast, a separate study with IS900, the repetitive DNA sequence specific for *M. paratuberculosis* which differentiated *M. paratuberculosis* and CD isolates from two serovars of *M. avium*,¹⁸⁹ was unable to distinguish between ovine and bovine strains within *M. paratuberculosis* in the same RE digests. Four distinct patterns within strains of *M. paratuberculosis* were produced.¹⁸⁶

The heterogeneity of *M. paratuberculosis* strains indicated from Southern blot analysis with insertion sequences as probes^{185, 186, 188} is corroborated by similar studies with a probe directed against the 16S rRNA; negative results were obtained with some field isolates

of *M. paratuberculosis*.²⁰ This was unexpected and suggests either mis-identification, or that *M. paratuberculosis* comprises of a heterogeneous clade and so questions the authenticity of defining *M. paratuberculosis* as a single species. Partial 16S rRNA sequence analysis over the most variable regions of the molecule showed that *M. paratuberculosis* was indistinguishable from *M. intracellulare* serovars 4, 5, 6, 8, 9, 10 and 11 and differed in one position from *M. avium*, suggesting that these should be assigned to *M. avium*.¹⁹⁰ The close phylogenetic relationship of *M. avium* and *M. paratuberculosis* is indicated by the inability of the 16S rRNA probe to distinguish between some strains of *M. avium* and *M. paratuberculosis*.²⁰ DNA homology studies suggest that *M. paratuberculosis* should be considered a subspecies of *M. avium*.^{16, 17, 149} Immunodiffusion¹⁹¹ and cellular fatty acid analysis¹⁷ also indicate that *M. paratuberculosis* and *M. avium* share a common line of descent and diverged recently.¹⁹

The atypical wood-pigeon isolates¹⁹² did not hybridise with the repetitive element specific for *M. paratuberculosis*,¹⁸⁷ suggesting that they may form a clade distinct from *M. paratuberculosis*. This corroborates evidence from immunodiffusion studies of two variants of *M. avium* that the atypical wood-pigeon isolates belonged to Group A, whereas *M. paratuberculosis*, with the exception of two strains, belonged to Group B.¹⁹¹ It has been proposed from DNA homology studies that *M. avium* be subdivided into three subspecies, *avium*, *paratuberculosis* and *columbae*.¹⁶ The latter group contains the wood-pigeon strains.

The position of the taxon *M. paratuberculosis* within the MAI complex requires further clarification to elucidate if there is a putative role for *M. paratuberculosis* alone, or in combination with MAI complex species, in CD and in granulomatous disease in animals. In this respect, paratuberculosis has been reported as indistinguishable from infection with *M. avium*.¹⁹³

Molecular typing of mycobacterial isolates in CD

A search for mycobacterial species in CD tissue by use of molecular techniques instead of culture has produced inconclusive results. DNA sequences homologous to mycobacterial sequences from strain Linda were detected in resected tissue in 10 of 19 CD patients, two of six UC patients and one of six control non-IBD samples. Measurement of the melting point of the hybrids indicated that the related sequences detected were of mycobacterial origin, but were not identical to each other nor to strain Linda. The genetic relatedness of strain Linda to the type strain *M. paratuberculosis* ATCC19698 and *M. avium* was in good agreement with other studies.^{17, 194} In-situ hybridisation studies revealed that most of the homologous sequences in CD patients occurred in the muscle layer, which is consistent with the pathology of CD.¹⁹⁵ Mycobacterial

DNA was not detected in total DNA prepared from mesenteric lymph nodes of 21 CD patients,¹⁹⁶ or CD tissue from resected samples¹⁹⁷ when probed with genomic clones of *M. paratuberculosis* strain Ben. The sensitivity of detection was calculated from tissue samples spiked with *M. avium* as 2×10^5 bacilli—equivalent to 1 ng of mycobacterial DNA in 10 µg of human DNA or one mycobacterial genome/10 human cells. Three probes were employed in this study, permitting the detection of several mycobacterial species including *M. paratuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. bovis* and *M. tuberculosis* if present.¹⁹⁷ Mycobacterial DNA was still not detected when the sensitivity was increased 10-fold by utilising *IS900* from *M. paratuberculosis*, estimated at more than 10 copies/genome.¹⁹⁸

Several diseases with a mycobacterial aetiology, in which culture had proved unreliable or unsuccessful, have now been diagnosed by PCR. Probes directed against the 65-kDa antigen proved more sensitive for *M. tuberculosis* than traditional culture in sputum samples.¹⁹⁹ Sarcoidosis, a granulomatous disease, has been suspected of having a mycobacterial aetiology. Culture for *M. tuberculosis* has been unsuccessful, but homologous DNA was detected by PCR^{200–202} except in one study.²⁰³ PCR inhibition occurred with some tissue samples, but was removed by further sample purification.²⁰⁰ Published results from a more comprehensive study of sarcoidosis proved inconclusive; DNA homologous to *M. tuberculosis* occurred in only some cases of sarcoidosis suggesting that, if it was present in all samples, the PCR test was insufficiently sensitive.²⁰⁴ A PCR protocol has been developed that permits the detection of *M. leprae* in skin biopsies.²⁰⁵

Improved sensitivity for the MAI complex has been achieved with PCR for the detection of a mycobacterial heat shock protein,^{206–209} and has been used in conjunction with reverse dot hybridisation.²¹⁰ PCR regimens specific for *M. paratuberculosis*²¹¹ and *M. avium* subsp. *silvaticum*,²¹² (the wood-pigeon isolates)¹⁴⁹ have been developed. Amplification primers are directed against the insertion sequences *IS900*¹⁹⁸ and *IS902*,²¹² specific for *M. paratuberculosis* and *M. avium* subsp. *silvaticum*, respectively. (The original clone pMB22, encoding *IS900*, hybridised to genomic DNA from *M. avium* serovars 2 and 5,¹⁸³ but the specificity for *M. paratuberculosis* was realised by increasing the stringency of hybridisation and targeting a 400-bp fragment.) The detection limits of the method were estimated at one *M. paratuberculosis* genome/2500 human cells.²¹¹ When the PCR method based on amplification of *IS900* was applied to mycobacterial cultures from CD tissue, DNA homologous to *M. paratuberculosis* was detected in six CD cultures and one control, but in no UC cultures; DNA homologous to *M. avium* subsp. *silvaticum* was detected in two CD cultures, two UC cultures and two non-IBD cultures. *M. paratuberculosis* DNA was present in 65% of CD, 4.3% of UC and 12.5% of non-IBD tissue examined. However, its presence was 10^5 - and 10^7 -fold lower than

in JD and the intensity of the PCR signals bore no relation to visible spheroplastic or bacillary mycobacterial growth.²¹³

The application of PCR to CD tissue failed to detect DNA homologous to a 65-kDa antigen from *M. tuberculosis*, *M. kansasii*, *M. avium* or *M. paratuberculosis*. The sensitivity of detection was estimated at 30 mycobacterial genomes, equivalent to one bacteria in 670 human cells. Sample inhibition, which interfered with the ability to detect mycobacteria in sarcoidosis,²⁰⁰ may explain why mycobacteria were not detected in CD tissue.²¹⁴ Although *M. tuberculosis* was detected in similar samples, the specificity and sensitivity for other mycobacterial species may not be comparable. The inclusion of CD tissue spiked with *M. paratuberculosis* DNA would resolve whether tissue inhibition of PCR reaction is occurring. This approach was used to avoid false negative results; DNA homologous to *M. paratuberculosis* IS900 was detected in six of 28 cultures from CD patients.²¹⁵

Culture studies failed to resolve whether *M. paratuberculosis* or MAI complex organisms are present in CD or groups of CD patients. Unlike other mycobacterioses, except for tuberculoid leprosy and tuberculosis, culture of these organisms from clinical samples is inconsistent. Several important questions remain outstanding with respect to this hypothesis. Firstly, if CD is the equivalent human disease to JD and *M. paratuberculosis* is the causative organism, why are large numbers of bacilli not shed during chronic disease enabling consistent detection of *M. paratuberculosis* from a group or subgroup of CD patients? Secondly, with sensitive PCR techniques, why is *M. paratuberculosis* detected only in some CD tissue? Is the inability to demonstrate *M. paratuberculosis* because it is present at such low levels, and could the mycobacteriophage be responsible for this by transforming them into unrecognisable forms resistant to detection? Could such an event explain why the pathogenesis of *M. paratuberculosis* is different in CD from JD? Alternatively, does CD represent a group of diseases in which *M. paratuberculosis* or the MAI complex are the causative agents in some groups? The resolution of this hypothesis requires that taxonomic groupings within the MAI complex, in particular the genealogical heterogeneity within *M. paratuberculosis*, are clarified; and that very specific probes capable of differentiating between these taxa are employed to establish which organism either alone or in combination cause CD. Finally, CD patients should be grouped by age and other available criteria in analysing results.

The role of mycobacteria in CD: immunological data

Detection of specific antibodies for CD

In severely inflamed areas IgG-producing B cells are found in the mucosal inflammatory filtrate.⁶² Although

a correlation has been demonstrated between both mycobacterial culture and serum antibody titre in JD,¹⁴⁶ this has not been demonstrated for CD. There are conflicting reports of elevated mycobacterial antibodies in serum; the controversy has been exacerbated by the absence of specific *M. paratuberculosis* antibody or antigen.

Antibody responses to *M. kansasii* were higher in CD patients than in controls, but were not increased for 16 other mycobacterial species.¹⁶⁹ Similar findings were also reported elsewhere²¹⁶ but *M. paratuberculosis* was not included in either of these studies. Data suggesting increased serological response to *M. paratuberculosis* in CD patients was reported by collaborators of Chiodini,²¹⁷ but these results have not been repeated and *M. paratuberculosis* strain 18—now reclassified as *M. avium* serovar 2—was used. Increased titres of IgA and IgM, but not IgG, to soluble antigens of *M. tuberculosis* were observed in CD patients.²¹⁸ Later studies failed to demonstrate greater seroreactivity to mycobacterial antigens in CD patients than in controls.^{219–221} Agglutination studies with *M. paratuberculosis* and *M. avium* proved inconclusive with sera from equal numbers of CD patients and controls.⁹³ Agglutination of mycobacterial isolates obtained from CD material was demonstrated only partially with antisera prepared to the CD isolate, strain Dominic.¹⁵² There are several possible explanations for the conflicting results. The choice of microbial constituent against which the antibody is directed is important for sensitivity and specificity. Cross-reaction between immunogens of mycobacteria has been reported^{222–224} and between mycobacterial antigens and monoclonal antibodies prepared to various micro-organisms and to host tissue.²²⁵

In early studies, serum antibody responses in CD patients were measured with crude mycobacterial sonicates and were often directed against cell-wall components common to mycobacteria, e.g., lipoarabinomannan.²²⁶ The significance of the antibody responses detected in these studies is difficult to assess. Firstly, antibodies to mycobacterial sonicates may be high in human serum because of the environmental ubiquity of some mycobacterial species, particularly the MAI complex.^{24, 153, 227} Therefore, the detection of antibody reacting to common mycobacterial antigens is probably not specific for CD. Corroborating this is the report that the seroreactivity to arabinomannan bore no relationship to the duration of illness and was the same for CD patients as for controls.⁹⁸ A further criticism of the early serological studies on mycobacteria and CD is that testing for cell-wall antigens may be inappropriate. Spheroplasts, observed in CD tissue, are bacterial cells which have lost their cell wall. Therefore, circulating antibody to cell-wall components may not be present.

Serological studies were performed with the cell-membrane glycolipid specific for *M. paratuberculosis* as antigen²²⁸ and the presence of mycobacterial anti-

body was measured with an ELISA in which antigens were prepared from crude cell sonicates or purified glycolipid of *M. paratuberculosis* strain 18. No significant differences in antibody titres were observed between CD and control groups.²²⁹ The suitability of the choice of antigen was questioned because it has not been detected in animals naturally infected with *M. paratuberculosis*⁶, nor was it expressed in all clinical isolates of *M. paratuberculosis*.²²⁸ The antigen prepared to the glycolipid also cross-reacted with MAI serovar 2.²³⁰ Two further studies have been conducted with a protoplasmic antigen from *M. paratuberculosis* strain 18. This preparation comprised a mixture of polypeptides. Although there was a wide variation in titre of antibody in CD patients to a commercial preparation of strain 18 antigen in an ELISA test, the results were not significantly different from those obtained with UC patients and controls. Immunoblotting demonstrated a strong reaction to a 45-kDa antigen in the commercial preparation in four of 10 and a weak reaction in two of 10 CD patients, but also occurred in four of 15 UC patients.²³¹ A negative correlation was also obtained between the same commercial antigen preparation and serum from 108 CD patients.²²³ Subsequent taxonomic studies based on genotype have designated *M. paratuberculosis* strain 18 as *M. avium* serovar 2. These studies were not evaluating the humoral response to *M. paratuberculosis* in CD.

Antigen A60, a membrane glycoprotein, was prepared from strains of *M. paratuberculosis*, *M. bovis* and *M. avium*. Sera from patients with tuberculosis gave a strong reaction to A60 preparations from *M. avium* and *M. bovis* in an ELISA, but were not tested against A60 preparations from *M. paratuberculosis*. In contrast to the results from the tuberculosis patients, sera from controls and CD patients did not react with any of the A60 preparations. This particular antigen is expressed at high levels during the exponential growth phase in *M. bovis*. Because of the slow growth of MAI complex organisms, the choice of antigen may have been inappropriate.²³² In a carefully conceived study, neither antigenic preparations of lipoarabinomannan, characteristic of mycobacterial spheroplasts, nor protoplasm antigens specific for the CD isolate strain Linda, detected significantly higher antibody titres for IgA, IgG or IgM in any subsets of CD patients compared to controls. The subsets analysed included age, sex, CD activity index, location of CD and duration of disease.²³³

Mycobacterial stress proteins are targets for the immune system in tuberculosis and leprosy and may play a role in auto-immunity. The occurrence of antibodies to ATP-binding stress proteins from the CD isolate strain Linda and from human material in the sera of CD patients was investigated. There was no significant difference in pattern or frequency of antibodies against any single protein or combinations of proteins, making it unlikely that these mycobacterial stress proteins were implicated in the pathogenesis of

CD.²³⁴ Cell extracts from human cell lines failed to detect auto-antibodies in CD patients.²³⁵ Specific antibodies secreted by hybridomas were generated from activated B cells of mesenteric lymph nodes of nine patients with IBD. The hybridoma supernates seldom bound to food antigens, but bound to one or more bacterial genus, most frequently to mycobacteria including CD isolates. Mycobacteria are not considered normal gut flora; therefore, this result was unexpected and supports a mycobacterial role in IBD.⁷⁶

Serological studies implicating agents other than mycobacteria in CD have been reported. Increased antibody titres have been reported for colonic mucosal extracts,²³⁶ but a subsequent study of different patient groups showed that the increased titres of antibody to colonic tissue were not specific for CD.²³⁷ Increased levels of complement-dependent lymphocytotoxic antibody have been reported for CD patients.²³⁸ High levels of antibody to pancreatic juice (PAB) occurred in 31 % of 222 CD patients. Although the positive sera could not be correlated with subgroups of CD, PAB was shown to be highly specific for CD when tested against sera of patients with various chronic inflammatory disorders including UC. It was proteinaceous, stable and belonged to the IgG class. This is consonant with the reports of increased production of IgG-containing cells²³⁹ and supports data suggesting that pancreatitis may be an extra-intestinal manifestation of CD.^{240, 241}

Antigen detection. In immunohistochemical examination of CD tissue, antibodies prepared to *M. paratuberculosis*, *M. tuberculosis* and lipoarabinomannan detected various species including *M. tuberculosis*, *M. kansasii*, *M. fortuitum*, *M. chelonae*, *M. paratuberculosis* and both spheroplast and cell wall forms of the MAI complex. Mycobacterial antigens were not detected in 67 specimens from 30 CD patients examined, but sparsely distributed cells of *M. tuberculosis* and *M. kansasii* were detected in clinical samples.²⁴² Of four monoclonal antibodies (MAbs) raised to *M. avium*, two showed a positive reaction with a CD mycobacterial isolate. The MAbs reacted with the gut wall of CD patients and controls, but the location of the reaction differed in the two groups. In samples from CD patients, binding occurred in the submucosa and subserosa, whereas in control groups it was limited to the lamina propria.²⁴³ These results are consistent with the clinical description of CD and provide circumstantial evidence for mycobacterial involvement with CD.

Despite enhancing the specificity of the antigens and linking data to subgroups in CD, the serological data generated do not support a mycobacterial aetiology for CD. The situation is compounded by the absence of a specific antibody for *M. paratuberculosis* that distinguishes it from closely related mycobacteria that may be environmental contaminants. Ironically, in the only study in which increased sero-reactivity to *M. paratuberculosis* was reported, a strain of *M. avium*

was used for the antigen preparation.²¹⁷ The humoral response in CD is not demonstrated readily and parallels that of localised granulomatous diseases, e.g., sarcoidosis and tuberculoid leprosy.²⁰⁴ In JD, the mycobacterial disease that most closely resembles CD, antibody to *M. paratuberculosis* can be detected in the advanced stage of the disease, but not always in subclinical disease. Antibodies to *M. paratuberculosis* were detected in the sera of 84% of Stumptail Macaques with disease that both clinically and pathologically resembled paratuberculosis in ruminants.¹⁵¹ Serum antibodies may not always provide a reliable indication of mycobacterial disease. In CD this may reflect an impairment of secondary immune response which has been reported with some antigens.²⁴⁴

Evidence for cell-mediated immunity

In supporting a mycobacterial origin for CD, it has been suggested that because of the chronic nature of CD, a correlation between antibody titres and development of the disease is not always demonstrable, as in the case of tuberculoid leprosy and tuberculoid-type paratuberculosis in cattle,⁶ and that the most useful immunological data are from assays based on cell-mediated immunity (CMI) or delayed-type hypersensitivity (DTH). The immune response in mycobacterial infection is primarily CMI and there are increased numbers of T cells in CD, particularly associated with granulomas in the submucosa.⁶²

An increased proportion of positive skin tests with standard purified protein derivatives (PPD) or bacterial antigens derived from atypical mycobacteria belonging to Runyon Groups I, II or III were not observed in CD patients.^{245, 246} Similar results were obtained with PPD in a later study,²⁴⁷ which also failed to find evidence for the high incidence of anergy reported in CD patients.²⁴⁸ Positive skin tests with *M. kansasii* preparation were reported for CD samples,¹⁶⁹ but in a later study the same group found no significant difference between CD patients and controls.²¹⁹

Sonicates of *M. paratuberculosis* had an inhibitory effect in a lymphocyte proliferation assay in five of six CD patients but not in other IBD patients.²⁴⁹ In contrast, no significant difference was observed between CD, UC and control subjects in T cell responses with peripheral blood lymphocytes (PBL) in a macrophage migration assay with sonicates of *M. tuberculosis*, *M. avium*, *M. kansasii* and *M. paratuberculosis*.²⁵⁰

M. leprae contains antigenic components that can either suppress or stimulate lymphocyte proliferation, depending on the form of disease, i.e., tuberculoid or lepromatous.^{251, 252} IBD patients and controls were studied to determine if a parallel situation occurred in CD. Proliferation of PBL from normal individuals in response to *M. paratuberculosis* antigen was reduced by depletion of CD4 T cells. In CD patients, PBL proliferation was lower than that of controls in the presence of *M. paratuberculosis* antigen and the

proliferative response did not increase with depletion of CD8 T cells. However, the proliferative response was reduced in the presence of other mycobacterial and candida antigens, indicating that the effect was non-specific.²⁵³ The second part of the study compared the ability of CD patients and control groups to develop suppressor cell activity—measured by the ability of *M. paratuberculosis* antigen to suppress concanavalin A-induced proliferation of PBL. The ability to develop suppressor cell activity was reduced with the removal of CD8 T cells; suppression was observed with PBL from non-IBD groups and controls, but it was more marked in CD and UC patients, and in particular, in CD patients with active disease. It was concluded that *M. paratuberculosis* may contribute to an immunosuppressive state in CD patients. This effect was not observed with candida antigens, indicating an element of specificity; other bacterial antigens were not examined.²⁵³ As a note of caution, the mycobacterial antigen used in this study came from the laboratory²²⁹ where the antigen supposedly specific for *M. paratuberculosis* later proved to be from *M. avium*.⁶

A reduction in the levels of interleukin 2 has been reported for CD patients; this was not affected by the number of CD8 cells.²⁵⁴ A role for CD4 cells is implied from the reported remission of gastrointestinal symptoms with progressive HIV infection in a CD patient.²⁵⁵ Alteration in immunoregulation relating to suppressor cell activity has been observed in CD patients. Induction of suppressor cell activity of peripheral blood mononuclear cells (PBMC) by concanavalin A was reduced with a loss of function correlating to disease activity.²⁵⁶ A deficiency of suppression of proliferation of autologous lymphocytes was noted in CD patients with active but not quiescent disease.²⁵⁷ Minor abnormalities of suppressor activity were noted in two relatives in a genetic study of T and B cells of peripheral blood in quiescent CD patients and relatives, but no differences were found for natural killer activity and proliferative responses.²⁵⁷

There are reports that PBL may not be an accurate reflection of the lymphocytes in the diseased tissue. Immunological abnormalities exhibited by PBL are secondary to CD and are related to duration, severity, nutritional status and therapy.⁶² Other studies comparing PBL with lymphocytes from a solid organ compartment, such as the intestine, show that lymphocytes from different organs often have specialised biological functions.^{258, 259} However, a study comparing responses of T lymphocyte from peripheral blood, inflamed tissue and non-inflamed tissue to microbial antigens reported that proliferative responses from inflamed tissue and peripheral blood were similar, whereas T cells from non-inflamed tissue were unresponsive.²⁶⁰ Studies of intestinal mononuclear cells (MC) are inconclusive, with reports of increased²⁶¹ and decreased²⁶² suppressor cell activity. The reaction of both PBMC and mesenteric lymph

node mononuclear cells (MLNMC) in a lymphocyte proliferation assay with various antigens, including some of mycobacterial origin showed that MLNMC responses to antigens were generally greater than PBMC responses. The study did not show an increased response to mycobacterial antigens, but found evidence for increased CMI to a range of non-mycobacterial antigens, in particular *Y. enterocolitica*, in both PBMC and MLNMC in CD and UC patients.²⁶³ A similar finding was reported in a previous study in which increased reactivity to bacterial antigens was non-specifically influenced by the disease process and could act as a secondary sensitisation process.²⁶⁴

The most compelling evidence for potential mycobacterial involvement in CD has come from phagolysosomes termed "R" bodies which have been observed in 19 cases of CD.²⁶⁵ It has been proposed that these "R" bodies comprise lipid coated bacterial fragments. The suggested species include mycoplasmas, mycobacteria and streptococci because of their requirement for certain lipids. The "R" bodies are thought to act as immunological adjuvants and appear in macrophages in active disease, but not in health.²⁶⁶ Roediger suggested that the absence of the "R" bodies in other IBD, the frequency with which CD occurs in the ileocaecal region where lipid absorption takes place, the success of remission in CD when patients are fed elemental diets which contain primarily vegetable oils that do not promote mycobacterial antigenicity, all support the "R" body hypothesis.²⁶⁵ Corroborative evidence comes from a study of the survival of *M. paratuberculosis* in monocytes and monocyte-derived macrophages. Macrophages may not only provide a favourable environment for growth of the bacilli, but also in turn may promote their growth in immature monocytes through local concentrations of cytokines.²⁶⁷ The theory is also consistent with the pathology of paratuberculosis, in which there is an association of acid-fast bacteria with macrophages during the early stages.²⁶⁸

Studies of CMI neither exclude a mycobacterial aetiology for CD nor provide conclusive evidence for a role, particularly a specific role for *M. paratuberculosis*. The immunological study of CD is hampered because of the specific effect of the disease on the intestine making conventional techniques to measure CMI inappropriate. The antigen may be present only transiently and the results of the inflammatory response may mask its presence, as implied from the presence of "R" bodies. If fragments of mycobacterial origin are involved in an adjuvant effect, the application of molecular techniques should facilitate their detection. A recent study successfully used PCR to determine the presence of *M. tuberculosis* and *M. bovis* in macrophages.²⁶⁹ This approach could be applied to test the "R" body hypothesis.

The effect of chemotherapy on CD—evidence for a mycobacterial aetiology

The isolation of *M. kansasii* from lymph nodes of CD patients¹⁶⁹ instigated a new approach to the treatment of CD. Anti-mycobacterial regimens were administered to CD patients and have met with varied success. Many of the anti-mycobacterial drugs were developed for *M. tuberculosis*. Their efficacy against *M. paratuberculosis* and species of the MAI complex is difficult to assess.

Trials of anti-mycobacterial drugs in CD patients

Rifampicin and ethambutol²⁷⁰ were used in the first trial as they were effective against *M. kansasii*, which was then considered to be a putative aetiological agent of CD.²⁷¹ The results of this double-blind study showed no difference between CD patients receiving the active drug and those receiving the placebo, as determined from clinical indicators of disease activity. There was no evidence to suggest that subgroups of patients were favourably affected by the regimen.²⁷⁰ It has been suggested that although rifampicin is effective against strains of *M. paratuberculosis*, ethambutol was a poor choice because organisms readily develop resistance to it;¹³⁸ effectively, patients received a monotherapy regimen which is inefficient in treating mycobacterial disease. However, the authors who criticised the use of monotherapy reported on the efficacy of rifabutin alone, as well as in combinations with other drugs, in the treatment of paratuberculosis in non-human primates.¹⁵¹ Other studies with rifabutin alone, or in conjunction with ethambutol or isoniazid, have failed to achieve long term remission from CD, and often the trials have been terminated prematurely because of toxic effects.^{272, 273} Disappearance of acid-fast bacilli from colon biopsies was observed during remission in a CD patient; relapse of the disease was not accompanied by the appearance of the acid-fast bacilli. The patient responded to sulphasalazine and corticosteroid therapy. The authors assumed that the acid-fast bacilli were mycobacteria and suggested that they may play a collateral role in CD.²⁷³ However, they may have represented other bacteria sensitive to the antibiotic regimen.

There have been reports of remission in CD patients with anti-mycobacterial chemotherapy.^{274–277} Quadruple therapy of rifampicin, ethambutol and isoniazid with the addition of pyrazinamide or clofazamine produced remission in 10 of 22 CD patients. At the time of the report, the patients were still in remission after 9 months and no toxic side-effects were evident.²⁷⁸ Dapsone, which is widely used in the treatment of leprosy, was administered to five CD patients. Remission occurred in two patients. Concurrent with remission was the presence of high antibody titres to *M. paratuberculosis* and not to other mycobacterial species including the MAI complex. High antibody titres to *M. tuberculosis* were reported after treatment

of tuberculosis with rifampicin.²⁷⁹ The authors concluded that *M. paratuberculosis*, or a bacterial species sharing the same antigens, may cause CD in some cases.²⁸⁰

Efficacy of anti-mycobacterial drugs against the MAI complex

The inconsistent results of anti-mycobacterial therapy suggest that a mycobacterial aetiology may be implied for some, but not all cases of CD; this would concur with the hypothesis of an heterogeneous pathology in CD.^{69, 281} It is argued that administration of anti-mycobacterial therapy to those CD patients who do not belong to the group with a mycobacterial aetiology would mask this relationship, which assumes that the therapeutic agents are specific for mycobacteria, in particular *M. paratuberculosis* or the MAI complex. The agents used in these studies were developed for *M. tuberculosis*; inhibition of the MAI complex is highly variable. Rifampicin and isoniazid inhibit 50% and 10–30% of MAI complex strains, respectively; the effect of ethambutol is highly variable and stepwise resistance often develops.²⁸² Streptomycin and isoniazid are most active against the MAI complex in log phase.²⁸³ Clofazimine is reputedly highly effective against MAI complex organisms.^{12, 284} There are no reports of the effect of pyrazinamide, but metabolically inactive tubercle bacilli are resistant to it and it is generally ineffective in long-term therapy.

In a comprehensive screen of the susceptibility of 170 mycobacterial strains representing 17 species, sulphasalazine and related compounds, including dapsone, were ineffective against 60 strains of slow growing mycobacteria which included the MAI complex.²⁸⁵ The various combinations effective against the MAI complex may offer some explanation for the different successes of these studies. The presence of plasmids in many MAI complex strains²⁸⁶ may contribute to antimicrobial resistance, especially when used for long-term therapy. In the most successful study,²⁷⁸ clofazimine, probably the most effective drug against the MAI complex, was substituted if any other proved unsuitable. The authors did not indicate the frequency with which this occurred, or the patients involved. In view of the proposed link of the MAI complex with CD, it would be interesting to know whether clofazimine was used in the patients in whom remission was reported.

The results of the sulphasalazine study suggesting that this drug is active against another bacterial agents, or that it has an anti-inflammatory effect, are interesting, as this drug is part of the mainstay therapy for CD patients.²⁸⁷ Inflammation is the consequence of CD, not the cause, but control of inflammation results in marked improvement. Bovine zinc copper superoxide dismutase (SOD), an anti-inflammatory drug, achieved remission for 8 years in 19 of 26 patients. Antimicrobial agents were not used in this study.²⁸⁸ The levels of SOD are decreased in inflamed tissue in

CD patients²⁸⁹ and these studies suggest that there is decreased endogenous protection against oxygen-derived radicals in CD. This is consistent with reports of increased levels of eicosanoids derived from arachidonic acid metabolism in CD patients.^{290–292} Sulphasalazine and its derivative 5-aminosalicylic acid affect arachidonic acid metabolism and, in particular, synthesis of prostaglandin (PG) and leukotriene (LT) which are pro-inflammatory agents.²⁹³ LT may be more important than PG in CD.²⁹⁴ In particular, levels of LT₄ are significantly higher in both UC and CD patients than in controls²⁹⁵ and are responsible for the chemotaxis of neutrophils into the inflamed tissue.²⁹⁶ High levels of free radicals may also explain the increased chromosomal breakage which occurs in lymphocytes of CD patients.²⁹⁷

Although the chemotherapy data do not exclude mycobacterial involvement in some cases of CD, neither do they provide definitive proof. Clearly their role, if any, is only contributory as demonstrated from the results of therapeutic regimens with antimicrobial and anti-inflammatory agents. The factors governing the success of these regimens in some cases, but not in others, still elude clinicians. The success of some anti-mycobacterial regimens in treating CD may indicate that CD comprises of a heterogeneous group of disease, with a mycobacterial aetiology for certain subgroups.

Animal models of CD

Studies of the factors involved in the pathogenesis of CD would be facilitated by the development of a suitable animal model. Attempts have been made to reproduce the clinical disease in animals with homogenates from CD tissue and with mycobacterial species, including both environmental isolates from the MAI complex and isolates from CD or related disease. CBA mice given homogenates of CD tissue developed granulomatous lesions confined to the bowel or mesenteric lymph nodes. The disease was simulated when homogenates were filtered through a 0.2- μ m filter, which would not preclude bacteria without a cell wall, but not when homogenates were autoclaved.²⁹⁸ Injection of *M. kansasii* into five immunologically different types of mice, including BALB/b by several unspecified routes failed to reproduce the disease.¹⁶⁹ Four strains of atypical mycobacteria isolated from wood-pigeons with granulomatous disease, which had not been agglutinated by antisera to strains of *M. avium*, were tested for pathogenicity along with two JD isolates of *M. paratuberculosis*. A clinical granulomatous disease ensued with all six strains in mice and with the four atypical wood-pigeon strains in chickens. In calves, three of the four wood-pigeon strains and *M. paratuberculosis* strains each produced clinical JD.¹⁹¹

Strains of the MAI complex from animal and environmental sources were tested for their ability to

produce intestinal lesions after injection into chickens, mice, rabbits, guinea-pigs and calves. Lesions were observed only in calves with mycobactin-dependent *M. avium* and *M. paratuberculosis*. These species and *M. intracellulare* were also recovered from lymph nodes of infected animals. However, these clinical conditions occurred only with some strains of these species and not in every calf.¹¹ *M. avium* isolates from swine lymph nodes showing granulomatous tubercloid features were pathogenic for mice, causing granulomatous lesions in the liver and spleen, but not for chickens.²⁹⁹ These studies indicate that both strain and host factors are important in the pathogenesis of paratuberculosis.

The human CD isolate, strain Linda, produced symptoms resembling CD when injected by a number of routes into BALB/c mice, but not in other laboratory species.^{132, 133} It also caused a granulomatous disease in Leghorn chickens²⁷⁴ and in infant goats fed cream with 10⁷ cfu/100 ml—this inoculum size is reported to produce JD in ruminants.³⁰⁰ Appropriate control animals did not develop symptoms of the disease nor were acid-fast bacteria cultured.^{132, 274, 300} In goats, the earliest lesion was a cluster of eosinophilic macrophages between the lymphoid nodules and the muscularis mucosa and occurred in the ileum. Giant cells were evident adjacent to the granulomas. Despite the sparsity of acid-fast bacilli in intestinal tissue, strain Linda was recovered from intestinal segments.³⁰⁰ The type strain of *M. paratuberculosis* and strain Linda produce identical RFLP profiles when probed with *E. coli* 5S rRNA.¹⁵² In contrast to strain Linda, mycobacterial isolates from CD tissue, *M. paratuberculosis* strain 410 and *M. chelonae* strain 390 did not cause JD in goats.¹⁷⁶ The designation of *M. paratuberculosis* strain 410 was achieved by phenotype, not genotype, and may not be reliable, explaining its inability to cause paratuberculosis in goats. *M. chelonae* isolates from CD patients did not simulate CD when injected into mice.¹⁷³

A wasting disease with intestinal lesions was produced in SCID mice inoculated intraperitoneally, and to a lesser extent when fed orally, with a bacterial suspension prepared from an intestinal segment of a cow with JD. The presence of granulomas and giant cells were rare in SCID mice, but acid-fast bacteria were prevalent in the liver and spleen and later were associated with MC in the intestinal submucosa.³⁰¹ Neonatal rabbits infected orally with *M. paratuberculosis* developed granulomatous enteritis. Although the organisms were cultured from tissue, complement fixation and delayed-type hypersensitivity tests failed to detect infection.³⁰² Disease with similar, but not identical, clinical and pathological symptoms to paratuberculosis has been described in several animal hosts when MAI complex isolates have been administered orally or intraperitoneally. The variable symptoms and susceptibilities of hosts, for instance with breeds of mice, indicate that immune status, host species and mycobacterial strain are all important.

Therefore, although the pathogenesis of CD differs in several respects from paratuberculosis in animals, studies of animals models do not exclude a mycobacterial aetiology.

Conclusions

Epidemiological studies have provided insight into putative aetiological factors for CD. The increased incidence of CD in certain geographic areas and the variation of incidence within high risk races consuming different diets suggest an environmental aetiology. The increased incidence in what are considered shared environments, e.g., in populations and in families, the infrequent occurrence of CD in spouses, in migrants and in races of different origin but now in the same geographic location, and the higher incidence in monozygotic twins than in dizygotic twins, lends credence to the hypothesis of an inherited aetiology. Epidemiological evidence for CD supports both environmental and inherited factors, i.e., a multifactorial aetiology.

The potential role of bacteria as aetiological agents has been investigated. The evidence for a mycobacterial aetiology is more compelling than that obtained from studies of the indigenous flora and other bacterial pathogens. However, a synergic effect involving several bacterial species cannot be excluded. The main evidence for an aetiological role for bacteria comes from the quantitative rather than qualitative antibody studies. The presence, or elevated levels, of serum antibodies may be a consequence of patients with CD having greater exposure to the indigenous flora as a result of the inflammation, or being at greater risk from, or more susceptible to, bacterial pathogens. Without prospective studies, the significance of these factors is difficult to assess.

The evidence for a mycobacterial aetiology is more substantial. Although there are many inconsistencies in isolation and culture from different studies, mycobacteria with and without cell walls have been isolated from CD tissue. These isolates have most frequently belonged to the MAI complex, but precise speciation of the isolates as *M. paratuberculosis* was lacking. Arguably, the high DNA homology, the identical antigenicity and the production of a granulomatous disease, which shares some pathological features with CD, when either *M. avium* or *M. paratuberculosis* is administered to neonatal animals suggests that either species is a possible aetiological candidate for CD. However, despite their high genomic identity, strains of these species differed in their ability to cause granulomatous disease and often were host specific.

The diagnosis of CD does not parallel the situations in other mycobacterial disease. Serum antibodies are found in tuberculosis and leprosy, and in JD are detected in the advanced stages, but not always in the subclinical disease. Despite considerable effort, antibody to *M. paratuberculosis* has not been detected in CD patients. There is some circumstantial evidence for

a mycobacterial aetiology from CMI studies. Positive culture of *M. paratuberculosis* still provides the most reliable diagnosis for JD, but has been inconsistent in CD. However, new diagnostic techniques based on PCR have been developed for mycobacterial diseases such as leprosy and sarcoidosis where culture of the organisms has met with little success. *M. paratuberculosis* and *M. avium* DNA has been detected in some but not all PCR studies of cultured CD tissue samples—albeit at 10^5 -fold lower concentration than occur in JD. In some mycobacterial disease, there are reports of mycobacteriophages which produce non-culturable atypical forms. This has not been investigated in CD and may provide an explanation why mycobacteria, if present, remain elusive to culture. Further studies applying molecular biology techniques and the specific probes developed for *M. paratuberculosis* and the MAI complex should establish whether both, or either of these species are commonly present in CD tissue and clarify their putative aetiological role in CD. They may also provide information on the classification of IT, particularly with respect to the hypertrophic form of IT and its relationship with CD. However, confirmation of presence, especially at the low levels at which *M. paratuberculosis* has been detected in CD tissue, does not provide irrefutable evidence for an aetiological role in CD. These organisms have been detected in control colonic tissue, albeit less frequently. A systematic investigation of the prevalence of these organisms in

different locations of the gut is needed. A molecular approach is also required to elucidate whether mycobacterial fragments reside inside MNC and act as adjuvants.

It is important that future studies are both comprehensive and strategically directed. Few studies have correlated results with clinical details of the patients that might enable the association of mycobacteria with a particular subgroup. For example, if a parallel is to be drawn between JD and CD, it may be expected that CD and mycobacterial infection would be more common among the adolescent group of CD sufferers. The existence of subgroups within CD is suggested from studies of anti-mycobacterial therapy, if it can be assumed that the effects were specific. In this respect it would be interesting to know whether the patients who responded to SOD therapy formed a distinct CD subgroup, the effects of SOD being non-specific. Cluster analyses of epidemiological data have indicated that CD patients form two distinct groups.

Although, CD is pathologically similar to JD, it is clinically different in many respects. The case for mycobacteria, and more specifically *M. paratuberculosis*, as the aetiological agent of CD, can neither be proved nor disproved. Further research is required to clarify whether these organisms play a causative role in the aetiology of CD.

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