THE CHARACTERISATION OF PLEOMORPHIC ACID FAST ORGANISMS ISOLATED FROM PATIENTS WITH INFLAMMATORY BOWEL DISEASE

by

Shikandhini Visuvanathan

from the Department of Medical Microbiology University College and Middlesex School of Medicine School of Pathology

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FOREWARD

The recent isolation of mycobacteria from Crohn's disease (CD) has caused worldwide interest. However, many more isolates of unidentified acid fast forms have been reported from CD tissue than mycobacterial isolates themselves. The hypothesis exists that these may be an alternate physical form of Mycobacterium. This possibility formed the basis of the investigations in this thesis.

ABSTRACT

The experimental work done consists of three components. First, the isolation of extremely slow growing pleomorphic acid fast organisms (PAFOs) from mesenteric lymph nodes of cases of Crohn's disease (17/25) and Ulcerative Colitis (11/15). This is statistically significant compared to controls (3/17).

Second, the viability of the organisms was proved by their repetitive re-isolation on subculture, and the organisms were shown to have DNA. Similar organisms previously isolated and maintained since 1976 were viable after several years in culture. This high isolation rate of apparently identical organisms from Ulcerative colitis (UC) as well as Crohn's disease (CD) has not been reported by other groups.

The third aim of this thesis was to characterise the acid-fast organisms to determine if the organisms belonged to the genus *Mycobacterium* and were indeed homogeneous. Electron microscopy showed that the isolates had a cell wall.

An extremely useful DNA extraction technique applicable to all bacteria, mycobacteria and IBD-derived acid fast organisms tested is described. 21/33 PAFO derived DNA samples hybridised with probes prepared from the DNA of *Mycobacterium kansasii* and *Mycobacterium paratuberculosis* on dot blot hybridisation. Cloned probes prepared from PAFO DNA did not show hybridisation with a specific mycobacterial species.

Investigation for the presence of tuberculostearic acid by gas chromatography and mass spectrometry suggested its presence in 13/16 CD cultures, 6/10 UC cultures and 3/8 control cultures which grew the acid-fast organisms.

The relationship of the bacteria to mycobacteria were further investigated by protein analysis, analysis for the 30 kDa Fibronectin binding antigen and mycolic acid analysis. A few CD cultures showed the presence of the 30 kDa antigen and mycobacterial mycolates.

Despite attempts to find alternative culture methods for higher yields, Robertson's cooked meat broth remains overall the best available medium for the growth of these organisms.

In conclusion, the organisms show many mycobacterial markers. The association of these bacteria with IBD is certain but their aetiological role remains unproven. The work has been brought forward to a stage where DNA has been amplified for nucleotide sequencing and a long term study in nude mice has been initiated in an attempt to establish an animal model.

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CHAPTER ONE

SECTION 1: INTRODUCTION

Inflammatory bowel disease (IBD) is a collection of diseases affecting the bowel, the most common of which are Ulcerative Colitis and Crohn's Disease. Others include amoebic and bacillary dysenteries and tuberculous enterocolitis. Although IBD is neither common nor fatal, it is important as a medical condition, because its highest incidence is early in life - in the late teens and early twenties, its treatment involves major surgery including a possible colectomy for ulcerative colitis, and having the disease increases the risk of intestinal carcinoma certainly for ulcerative colitis and less so for Crohn's Disease cases.

There is much confusion over the relationship between Crohn's disease (CD) and ulcerative colitis (UC). Some gastroenterologists believe they are two distinct entities and others believe they are two ends of the same disease spectrum. Therefore, an investigation into a possible aetiology for Crohn's disease would not be complete without mention and inclusion of ulcerative colitis.

CROHN'S DISEASE

Crohn's disease (CD) was first described as a clinical and pathological entity by Crohn, Ginzburg and Oppenheimer in 1932. However, CD may first have been described as early as 1806 or even earlier (Kirsner, 1984). Undoubtedly, a surgeon from Glasgow, Sir T Kennedy Dalziel, described a condition "Chronic Interstitial Enteritis" in 1913, which was almost certainly a description of Crohn's disease.

DEFINITION

CD is a chronic granulomatous inflammatory disorder, principally of the terminal ileum and also large intestine, but lesions may occur in any part of the alimentary tract. Recurrent disease, even years after resection of all macroscopic disease, is well recognised. The disease is characterised by chronic inflammatory infiltrates and fibrosis, affecting all layers of the bowel.

PATHOLOGY OF CROHN'S DISEASE

Macroscopically, the bowel is engorged and oedematous. The mucosa is oedematous showing a "cobble-stone" pattern with linear ulceration and fissuring. The changes are patchy with the change from normal mucosa to the affected part being abrupt. Skip lesions are characteristic. Affected lymph nodes are enlarged and the mesentery thickened.

MICROSCOPIC APPEARANCE

Inflammatory change involves all coats of the bowel wall. Focal lymphocytic infiltration is most prominent in the submucosa. There is oedema and hyperplasia of the lymphoid follicles. Non caseating epithelioid-cell granulomas occur in about 60% of cases and resemble closely those of sarcoidosis. They may occur in all layers of the bowel wall and even in the mesentery and mesenteric lymph nodes. Most often, they are found in and around lymphatic channels.

Marked oedema, especially in the submucosa occurs due to lymphatic obstruction. Fibrosis is prominant in chronic cases. Fissures arise from ulcer bases and may penetrate the whole thickness of the bowel wall.

CLINICAL FEATURES

Many diseases may be mimicked, and features vary in part depending on the site and extent of bowel affected. In an acute form, the picture may be identical to that of acute appendicitis. However, usually intermittent abdominal pain in the right lower quadrant of the abdomen and diarrhoea are the main symptoms. A mass due to inflammed loops of bowel, with or without an abscess, and subacute intestinal obstruction is common. Other features include weight loss, a low- grade fever, moderate anaemia, perianal disease (skin tags, perianal abscesses and fistulae) and malabsorption. Most cases have a chronic course with exacerbations and remissions. Extra-intestinal manifestations include arthritis, ankylosing spondylitis, iritis, aphthous stomatitis and erythema nodosum.

CANCER RISK

Colorectal carcinoma (CA) occurs more commonly in CD patients than in the general population. Like UC, colorectal CA occurs at the site of macroscopic disease in patients with extensive colitis of long standing. There is also a small risk of upper gastrointestinal (GI) tract cancer (pharynx, oesophagus, stomach). Although there is a definite statisticlly increased risk of cancer, the absolute numbers remain small.

GENERAL MANAGEMENT OF CROHN'S DISEASE

CD is a chronic condition with remissions and relapses over many years. Problems are managed as they arise. Vitamin, folate and iron defeciency are corrected. Diarrhoea is relieved with codeine phosphate or a suitable alternative. A low residue diet reduces the frequency of intestinal colic. A low fat diet is required when malabsorption occurs.

DRUG TREATMENT

Drug treatment consists of corticosteroids and sulphasalazine for acute disease. Immunosuppressive agents, such as azathioprine are used in patients unresponsive to other forms of therapy.

In contrast to colitis oral antibiotics may be of some benefit. Oral metronidazole is only of proven value for short term treatment. Also in patients with severe perianal disease, oral metronidazole is useful for relieving symptoms in the short term.

SURGICAL TREATMENT

70-80% of patients require surgical treatment. Surgery is required for obstructive symptoms due to fibrous stricture, abscess formation, enterocutaneous fistulae and failed medical treatment. In most patients, the interval between diagnosis and resection is short (approximately 5 years). Recurrence may occur especially at/or around the site of a previous anastomosis.

MORTALITY

Factors contributing to a two fold excess mortality among CD patients include diffuse small bowel disease, post operative sepsis (this can be minimised with per operative antibiotic prophylaxis), pumonary embolus and colorectal CA.

CROHN'S DISEASE IN CHILDHOOD

Diffuse small bowel disease and recurrence are more frequent than in adults. Growth and height retardation including corticosteroid-induced growth stunting are particular problems of CD in childhood.

ULCERATIVE COLITIS

Again, like CD, the cause of this condition is not known. It may be difficult to distinguish (particularly in the early stages) from Crohn's disease of the colon.

DEFINITION

UC is a recurrent inflammatory and ulcerative disease of the colon and rectum characterised clinically by rectal bleeding, diarrhoea, cramping abdominal pain and weight loss.

PATHOLOGY OF ULCERATIVE COLITIS

UC typically involves the sigmoid colon and rectum. The entire colon is affected in about half the cases. The terminal ileum may be inflamed. The colonic mucosa is deeply congested and bleeds easily. Punctate erosions herald the onset of ulceration. Between the ulcers, especially in chronic cases, the surviving mucosa becomes swollen and hyperplastic giving the appearance of pseudopolyposis. Fibrosis may finally cause the colon to behave like a rigid tube.

MICROSCOPIC APPEARANCES

Unlike CD, it is the mucosa which is primarily involved in UC. During active phases, it is congested and densely infiltrated with leucocytes and plasma cells. Crypt abscesses are characteristic. They are an accumulation of neutophils, eosinophils, red cells and mucus within crypt lumina. Ulceration is produced by coalescence of ruptured crypt abscesses and seldom extends more deeply than the submucosa. The base is formed by vascular granulation tissue. There is goblet cell depletion.

CLINICAL FEATURES

The principal symptom is diarrhoea with loose bloody stools containing mucus. Tenesmus, pain or lower abdominal discomfort on defaecation, bleeding per rectum due to proctitis, malaise, anorexia and weight loss may occur. Most cases have a chronic relapsing course with exacerbations and remissions but some cases are mild and possibly self-limiting. Exhausting diarrhoea with fever, tachycardia and dehydration, or toxic dilatation represent severe forms of the disease. Systemic complications include arthropathy, ankylosing spondylitis, aphthous stomatitis, erythema nodosum, pyoderma gangrenosum, cholangitis, hepatitis and septicaemia with gram negative bacteria.

CANCER RISK

The development of CA of the colon is a considerable risk, especially if there is entire colonic involvement, early disease onset (as a child) and presence of disease for more than ten years. There is an individual risk of 1/200 of carcinoma in ulcerative colitis of ten years duration between the tenth and twentieth year (Rhodes and Mayberry, 1986).

GENERAL MANAGEMENT

Patients with severe bowel symptoms, weight loss, anaemia, fever and tachycardia are admitted for correction of dehydration and electrolyte deficiencies, anaemia and hypoprotinaemia. A low residue diet is given. Antibiotics have no special place in the primary management of UC except if septicaemia occurs. Codeine phosphate is used for diarrhoea.

DRUG TREATMENT

Active disease is controlled by steroids and sulphasalazine. Rectal preparation of these drugs are useful. Sulphasalazine is particularly useful in maintaining clinical remission.

SURGICAL TREATMENT

Emergency surgery is indicated for colonic perforation and toxic dilatation. Acute UC which fails to respond to medical treatment or relapses is an indication for proctocolectomy. In long standing cases, the risk of carcinoma is a further indication for surgery. Operation usually implies total proctocolectomy with permanent ileostomy.

MORTALITY

The overall mortality rate is twice the expected value. Extensive colonic involvement, major surgery and carcinoma are the main contributary factors.

DIFFERENTIATION OF ULCERATIVE COLITIS FROM CROHN'S COLITIS

These two conditions can be differentiated in at least 80% of patients (Lockhart-Mummery and Morson, 1960; Kirsner, 1975), however neither disease has pathognomonic findings present in every case of the one and absent in every case of the other. Differentiation may be difficult because of a limited morphological response of the colon to disease or the occurrence of an "indeterminate" colitis with features of both UC and CD. The simultaneous presence of UC and CD probably occurs (Eyer et al, 1980), but is difficult to document (Lockhart-Mummery and Morson, 1960).

SECTION 2: CLUES TO THE AETIOLOGY OF CROHN'S DISEASE

I. EPIDEMIOLOGY OF CD

Wherever relevant, I have included mention of UC specifically, or IBD where both CD and UC are considered together.

CD occurs most often in USA, UK, Scandinavia, is less frequent in Central Europe and the Middle East and is only occasionally encountered in African, Asian and South American populations. The overall incidence of CD has been steadily increasing worldwide over the last few decades (see Table 1.1). Incidence and prevalence of CD and UC has been reviewed by Calkins and Mendeloff, 1986. Large increases in CD incidence rates are reported from Minnesota, England and Wales, Clydesdale in Scotland, Norway, Finland and Israel. North Tees, Scotland, Cardiff, Wales and Baltimore report either stabilising or declining rates in the past decade after a sharp increase in rates in the 1950's and 1960's.

Prevalence rates reported vary from 9 per 100,000 population in Oxford, England (Evans and Acheson, 1965) to 56 per 100,000 population in Cardiff, Wales (Mayberry, Rhodes and Hughes, 1979).

Incidence rates for ulcerative colitis are also shown in Table 1.1. Calkins and Mendeloff, 1986, reviewed data on incidence and prevalence. Incidence rates for Norway and North Tees in Scotland have increased, rates for England and Wales, Israel and Denmark have stablised after a steady increase over decades and rates in Baltimore and Sweden show a decline. Prevalence rates vary from 66 per 100,000 population in Oxford, England (Evans and Acheson, 1965) to 117 per 100,000 in Copenhagen, Denmark (Binder *et al.*, 1982).

TABLE 1.1

Age Adjusted Incidence Rates (per 100,000 population) of Crohn's Disease and Olcerative Colitis

Place	Time Period	Incidence	References
<u>Crohn's Disease</u>			
Baltimore, USA	1960-1963 1977-1979	1.2 3.1	Monk <i>et al</i> , 1967 Calkins <i>et al</i> , 1984
Fifteen areas of the USA	1973	2.4	Garland <i>et al</i> , 1981
Olmsted County, Minnesota, USA	1935-1954 1965-1975	8.4 13.5	Sedlack <i>et al</i> , 1980
Gothenburg, Sweden	1951-1955 1966-1970	1.2 6.3	Kewenter, Hulten & Kock, 1974
Cardiff, Wales	1936-1940 1971-1975	0.18 4.83	Mayberry, Rhodes & Hughes, 1979
Blackpool, England	1971-1975 1976-1980	3.3 6.1	Lee & Costello, 1985
Aberdeen, Scotland	1955-1957 1970-1972 1973-1975	1.2 4.3 2.6	Kyle, 1971 Kyle & Stark, 1980
Copenhagen, Denmark	1961-1959 1970-1978	1.3 2.7	Binder et al, 1982
<u>Olcerative Colitis</u>			
Baltimore, USA	1960-1963 1977-1979	3.0 2.2	Monk et al, 1967 Calkins et al, 1984
Fifteen areas of the USA	1973	3.5	Garland <i>et al</i> , 1981
Cardiff, Wales	1968-1977	7.2	Morris & Rhodes, 1984
Oxford, England	1951-1960	5.2	Evans and Acheson, 1965
Copenhagen, Denmark	1962-1978	8.1	Binder et al, 1982

ETHNICITY

The incidence of CD is higher in Western populations. The Jewish population is at higher risk of developing inflammatory bowel disease (Acheson, 1960) and there is a remarkable predominance in North American Jews.

The study of a migrant population of Ashkenazi Jews has provided an opportunity to study differences in the incidence of CD by geographical location and consider the effect of local environmental influences on this population.

Gilat and Rozen, 1980 reviewed the data (Tables 1.2 and 1.3). There is a wide variation in the incidence of CD among Ashkenazi Jews at different geographical locations (Table 1.2). This suggests an environmental factor(s), for if the basis was genetic as is primary adult lactase deficiency, incidence figures are constant regardless of the geographical location (Table 1.3).

The overall picture suggested is that the incidence of CD in Ashkenazi Jews varies because of environmental influences. It also seems that the incidence varies according to the general incidence of CD in the local population.

SOCIAL FACTORS

Α lower incidence in CD and UC has been suggested in rural residents in Baltimore, Scotland and New Zealand; however, studies in Britain and Scandinavia show no urban-rural differences Mendeloff, 1986). No definite (review by Calkins and conclusions have been drawn regarding the association of socioeconomic factors and IBD incidence, but an association with professional and managerial occupations has been suggested (Monk, Mendeloff, Siegel et al, 1969).

Table 1.2

	Incidence/10 ⁵ of CD	
	Ashkenazi Jews	General Population
Tel-Aviv (1970-76)	1.6	1.2
Basel (1960-69)	2.2	1.6
Cape Town (1970-74)	2.8	0.8
Baltimore (1960-63)	7.2	2.5
Malmo (1958-73)	24.0	4.8

Review by Gilat and Rozen, 1980

Table 1.3

Adult lactase Deficiency in Jews (Genetic trait)

% with deficiency Tel-Aviv 66 Connecticut, USA 71 Vancouver, Canada 69

Review by Gilat and Rozen, 1980

II. GENETIC FACTORS AND INFLAMMATORY BOWEL DISEASE

Several studies suggest genetic factors as a component of susceptibility. There are four main lines of evidence for this. data The first is ethnic variation. Epidemiological has already been outlined. Secondly, IBD is associated with specific genetically determined diseases including ankylosing spondylitis (Moll, 1985), Turner's syndrome (Price, 1979) and Hermansky-Pudlak syndrome (Schinella et al, 1980). Thirdly, there have been reports in some populations of associations of IBD with certain HLA antigens. Asakura, Tsuchiya, Aiso et al, 1982 reported an association between Japanese ulcerative colitis and lymphocyte-DR2 antigen. However, there is no clear consensus at present as to the firm existence of a relationship between HLA antigens and IBD. For example, even though ankylosing spondylitis is highly associated (80%) with HLA-B27, when anklyosing spondylitis or sacroiliitis appears in IBD patients about 50% of such patients do not possess the HLA-B27 haplotype (Van den Berg-Loonen, Kekken-Saeys, Meuwissen et al, 1977).

Finally, familial aggregation of IBD has been extensively documented. The data does not fit a simple Mendelian pattern of inheritance, but a positive family history in patients with IBD ranges from 1-23% depending on the study (Mayberry, Rhodes and Newcombe 1980; Farmer, Michener and Sivak 1981, Weterman and Pena 1984, Lashner *et al* 1986).

Familial association may be genetic or environmental in origin but evidence for a genetic component certainly exists. Roth *et al* 1989, calculated age - corrected empiric risk estimates for IBD in Ashkenazi Jews as 8.9% to offspring, 8.8% to siblings and 3.5% to parents and suggested these figures could be used for genetic counselling and

modeling. Apart from this increased risk in first degree relatives, there is a high monozygotic twin concordance especially for Crohn's disease (Weterman and Pena 1984). There is a higher risk of IBD in children when both parents have IBD (Bennett, Rubin and Present 1988). Also numerous instances of family members with onset of disease separated greatly in time (Kirsner, 1973) and IBD in siblings living geographically apart for many years (Farmer, Michever and Mortimer 1980) have been reported. There is a lack of increase of disease in spouses .These reports are rare (Kirsner 1982) and are likely to be due to chance (Farmer, Michever and Mortimer 1980; Whorwell, Eade, Hossenbocus *et al* 1978b, Zetsel 1978).

Occurrence of CD and UC in the same family (Kirsner 1973) suggests that CD & UC may represent variability of the same disease entity (i.e. genetic pleiotropism) or they share one or more genes in common.

111. OTHER AETIOLOGICAL THEORIES PROPOSED

Other causes postulated as aetiological factors include food allergy, autoimmune disease, defective immunological and other (eg mucus) defence mechanisms. Some investigators consider that an autoimmune mechanism is indicated by the presence in the serum of gut binding antibodies (Nagai and Das, 1981; Hibi *et al*, 1982 and Chapman *et al*, 1986). Others consider an autoimmune mechanism may occur because of the presence in the lamina propria, lymph nodes or peripheral blood of lymphocytes, which recognise surface components of gut epithelial cells (Aronson, Cook and Roche, 1983; Shorter, McGill and Bahn, 1984; Roche,Fiocchi and Youngman, 1985; Hunt and Trotters, 1973).

Evidence for a role for food allergy (Wright and Truelove, 1965) comes mainly from clinical improvement seen when patients are

given antigen depleted diets (Jones *et al*, 1985). Although insufficient as single theories, collectively they probably do play some part in the pathogenesis of CD but are unlikely aetiological agents.

IMMUNOLOGICAL ASPECTS

Immunological disturbances do occur in CD. Treatment of CD involves the use of immunosuppressive drugs, such as corticosteroids and azathioprine. However, no immunological phenomena have been found to explain aetiology or pathogenesis.

COMPLEMENT

Abnormalities of complement have been demonstrated in Crohn's disease. Release of chemotactic activity for neutrophils elicited via C5a is subnormal (Elmgreen, Berkowicz and Sorensen, 1983). Increased C3 catabolism has also been shown (Hodgson, Potter and Jewell 1977). Elmgreen, Both and Binder (1985) suggested that complement abnormalities seen in CD patients may not just reflect mucosal inflamation or hypercatabolism of complement. However the possible pathogenetic significance of the complement abnormality is not known. If tissue damage due to activation of the complement system was then circulating levels of complement might be occurring, However, there are no deficiencies in the major diminished. complement components or their regulators in inflammatory bowel disease, and the significance of any complement abnormalities described remains to be established (Tytgat and Mulder, 1986).

The occurrence of circulating immune complexes during severe attacks of CD, is probably secondary to the activity of the inflammatory process and not primarily related to aetiology (Tytgat and Mulder, 1986).

LEUCOCYTE ABNORMALITIES

A decrease of H_2O_2 production by neutrophils in active CD has been demonstrated (Verspaget *et al*, 1984). Leucocyte mobilisation to skin windows was reduced in patients with CD compared to controls. This finding was not correlated with disease activity, duration or localisation of the disease (Wandall and Binder 1982).

Allan and Hodgson (1986) suggest that the migration of neutrophils to the gut in IBD (Saverymuttu *et al.*, 1986) simply leaves behind an abnormal population for study elsewhere. This would then account for the accepted defect in granulocyte migration (Wandall and Binder, 1982) observed, which however does not have a role in the disease. The migration of the polymorphonuclear cell leucocytes through the gut wall into the lumen with colonic inflammation has been postulated to be due to chemotactic peptides from intestinal bacteria (Le Duc *et al.*, 1987; Granger *et al.*, 1987). A deficiency of colonic mucosal carboxypeptidase has been demonstrated in UC (Chadwick *et al.*, 1987) and the authors postulate that this allows a build up of bacterial peptides.

NON-SPECIFIC MEDIATORS

The role of prostaglandins (PG) is unclear. Recently they have been less favoured as inflamatory mediators in IBD. Indomethacin (a PG inhibitor) is not therapeutic. Also dimethyel PGE2 has been shown to ameliorate experimental colitis in rats (Allgayer and Stenson 1987) rather than worsen it.

There is an increased density of T cells in inflamed mucosa but T- lymphocyte subclasses in CD do not differ from controls (Selby et al, 1984). No consistent deficiencies in spontaneous or mitogen induced lymphoproliferative reponses were demonstrated in gut mucosal lymphocytes derived from CD and UC, as compared to

patient matched peripheral blood lymphocytes or normal gut mucosal lymphocytes (Fiocchi, Battisto and Farmer, 1981). Natural Killer (NK) cells have been postulated to have a pathogenic role in IBD but the NK cells of intestinal mucosa have been shown to behave similarly in IBD, carcinoma of the colon and other intestinal diseases (Gibson and Jewell 1986).

Recently, multifocal gastrointestinal infarction has been implicated in the pathogenesis of Crohn's disease (Wakefield et al, 1989). A pathogenetic sequence of events - vascular injury, focal artertis, fibrin deposition, arterial occlusion, tissue infarction or neovascularisation was seen in CD affected segments but not in normal bowel. The mechanism of the thrombogenic injury is the interaction between immune cells and the vascular endothelium - the usual anticoagulant status being displaced by cellular procoagulant activity. T-helper cell stimulated, lymphokine-mediated monocyte activation with subsequent synthesis and cell surface expression of monocyte procoagulant activity is postulated. Such procoagulant activity is favoured by a range of biological stimuli, including various micro organisms. (An infectious actiology of CD is entirely compatible with these findings - see Section V).

IV. ENVIRONMENTAL FACTORS

DIETARY FACTORS, SMOKING, ORAL CONTRACEPTIVE PILL

Among environmental factors implicated are dietary factors such as a low fibre diet, (Thornton, Emmett and Heaton, 1979), a high intake of refined sugar and a deficit of raw fruit and vegetable. Increased formula feeding in childhood has been associated with later development of both CD and UC (Whorwell, Holdstock, Whorwell *et al*, 1979). A dietary hypothesis has been suggested as an explanation

for the lower risk among Jews in Israel (who eat less fat and meat and more fibre and carbohydrate) compared with American Jews (Rozen, Zonis, Yekutiel *et al.*, 1979).

Patients with CD have been found to have the same or higher frequencies of cigarette smoking than comparison groups in contrast to patients with UC who were either non smokers or ex smokers; Somerville et al (1984) have suggested that non smokers acquire UC and smokers acquire Crohn's disease, ie that smoking may be a determinant factor in predisposed individuals. UC and CD have been reported to be more prevalent among oral contraceptive users than among non users but it may be that UC and CD patients use the oral contraceptive pill because of their disease, to avoid pregnancy (Calkins and Mendeloff, 1986). None of these factors have been accepted as being causal in IBD.

V. INFECTIOUS AGENTS

The infectious theory remains and is a natural one for inflammatory bowel disease, because of the large number of bacterial, viral and other organisms associated with the gastro intestinal tract, the lymphocyte and polymorphonuclear leucocyte infiltration and granuloma formation. Also, the pathology of the disease, the multiple lesion sites and multiple familial occurrences all suggest an infectious process.

Crohn, noting the resemblance of the disease to tuberculous ileitis, looked for tubercle bacilli in smears and cultures but did not find any acid fast bacilli. He was unsuccessful in attempts to infect guinea pigs, rabbits and chickens with diseased intestine and mesenteric lymph nodes (Crohn, Ginzburg and Oppenheimer, 1932).

The next concerted effort to isolate mycobacteria from CD tissue was by Van Patter in 1952. He found three cultures with acid fast organisms in 3/43 patients which he could not subculture or identify further. 131 animals inoculated with disease tissues remained normal (Van Patter, PhD thesis, University of Minnesota, 1952).

In 1969, Mitchell and Rees presented evidence of a transmissible agent to mice from human sarcoid tissue. Due to histological similarities of CD to sarcoidosis, especially the presence of granulomas they extended their studies to Crohn's disease.

ANIMAL TRANSMISSION STUDIES

The demonstration of a possible transmissibe agent by Mitchell and Rees in 1970 was crucial in the history of the search for an infectious agent in CD tissue. They injected homogenised gut and lymph node tissue from CD patients into mouse footpads. They modelled their experiments on the mouse using the footpad as the inoculation site because of the success of these methods in leprosy. 13/56 of the footpads showed typical focal granulomas 6-24 months after inoculation. Early histological lesions (at 26-46 days) showed characteristic granulomas clearly distinguished from а cellular inflammatory reponse seen in mice injected with non-Crohn's lymph node homogenates. Late histological lesions (169-500 days) showed persistence of epithelioid and giant cell granulomas in footpads inoculated with CD tissue homogenates but footpads injected with non CD lymph node homogenates no longer showed the cellular inflammatory response observed earlier.

Initially, other research groups confirmed their observations. Taub and Siltzbach in 1972 detected granulomas following footpad injection in 5/16 mice. Cave, Mitchell, Kane and Brooke

(1973) demonstrated epithelioid and giant cell granulomas after intra-ileal injection of CD tissue in New Zealand white rabbits, which was not observed in controls. They obtained successful passage into a second group of rabbits using mesenteric lymph node homogenates of rabbits injected with CD tissue three months earlier. However, negative reports followed (Bolton et al, 1973; Heatley et al, 1975). Bolton used 334 animals and Heatley 91 animals but failed to support previous experiments. Observations on transmissibility were extended to ulcerative colitis (Cave, Mitchell Brooke, 1975). This and does not necessarily mean that ulcerative colitis also has a transmissible agent. It reinforces the concept that other inflammatory non-Crohn's disease homogenates must be used as controls to distinguish tissue specific from more general responses. All work done for this thesis compared ulcerative colitis cultures to CD cultures, not only for this reason, but also because ulcerative colitis and CD share so many other epidemiological and clinical features.

Das and co-workers induced lymphomas and hyperplastic lymph nodes in 16% athymic (nu/nu) mice by CD tissue filtrates, and found that the lymphomas contained an antigen(s) recognised by Crohn's disease sera (Das *et al*, 1983). These antigens may be glycoproteins of 110-200,000 molecular weight (Das *et al*, 1988). Pena *et al*, (1988), however showed that this reaction was not specific as the CD sera reacted with some nude mice lymph node cells in general. Collins *et al*, 1988, also report that lymph node hyperplasia is non specific and find no specificity for CD sera for the lymphoma antigen.

The initial work of Mitchell and Rees in transmitting granulomas to mouse foot pads by CD ilieal tissue prompted several workers to look for this transmissible agent.

MICROBIOLOGICAL REVIEW OF CROHN'S DISEASE

A. VIRAL AGENTS IMPLICATED

A variety of viruses have been implicated. Cytomegalovirus (Farmer et al, 1973) was detected in tissue culture. Aronson, Phillips and Beeken (1974) suggested the transmissible agent was a small RNA virus as a cytopathic effect (CPE) was detected when CD intestinal tissue was cultured in WI-38 human diploid fibroblast cells, and the agent grew well in the presence of DNA inhibitors.

Continuous rabbit ileal cultures were used (Gitnick, Arthur and Shibata, 1976) and electron microscopic studies were consistent with a picornavirus (Gitnick and Rosen, 1976). Another report followed of a CPE in WI-38 tissue culture monolayers detectable with 6/10 Crohn's disease intestinal filtrates but absent in controls (Whorwell *et al.*, 1977).

Other workers were unsuccessful in attempts to find a consistent viral agent. Phillpotts, Hermon-Taylor and Brooke (1979) used a series of eight cell lines including those used previously by other workers, and concluded that a CPE similar to a viral CPE was produced using CD, UC and control gut and lymph node tissue. A search for antibodies to rotavirus (De Groote *et al.*, 1977) and the Norwalk virus (Greenberg *et al.*, 1978) were negative.

Despite another publication by Gitnick *et al* (1979), suggesting a small enveloped RNA virus as a viral agent, other negative reports followed. A cytopathic effect was found with ulcerative colitis tissue as well as CD tissue (Morain et al, 1981), and the CPE was recorded as not being due to a virus (McLaren and Gitnick, 1982). Mycoplasma contamination was noted in some specimens which produced a CPE (Kapikian *et al*, 1979). Phillpotts *et al* (1980) found no evidence of a non-cytocidal virus infection.

Yoshimura, Estes and Graham (1984) cultured tissues from 95 patients in several cell lines and concluded in agreement with Phillpotts and other workers that any cytopathic effect noted was due to a non- replicating cytotoxic factor(s) released from intestinal tissues of both IBD and non-IBD patients.

B. BACTERIA IMPLICATED IN CROHN'S DISEASE

Aluwihare (1971) reported intra mural bacteria of differing morphology in 6/16 CD patients in an electron microscopic study of non-ulcerated Crohn's colon. Such bacteria were not seen in normal colon or in ulcerative colitis specimens with intact mucosa.

Parent and Mitchell (1976) isolated cell wall defective Pseudomonas maltophilia and Pseudomonas like bacteria from filtrates of homogenised Crohn's disease bowel and lymph node tissue, but not from ulcerative colitis or controls. They suggested that the earlier animal transmission studies using CD filtrates could indicate cell wall defective bacteria rather than viruses. However, Pseudomonas species are normally present in the gut, so evaluation of their significance is difficult. Whorwell et al (1978a) however, found no evidence by indirect immunofluorescence methods for the presence of Pseudomonas maltophilia (now reclassified as Xanthomonas maltophilia) and Mycobacterium kansasii (implicated as a possible aetiological agent by Burnham et al, 1978 and discussed in detail in Section C). Belsheim et al (1983) isolated bacterial L forms, especially L forms of Eschericia coli and Streptococcus faecalis from 40-50% of patients with CD and UC but not from controls. They maintained that because of the lack of cell wall antigens, L forms present less of an

antigenic challenge to the host and this may aid their persistence in host tissue. Also L form isolations had been reported from a variety of human illnesses.

Ibbotson, Pease and Allan (1987) reported cell wall deficient forms bacteria from 40-60% of patients with active IBD, 15-25% of of patients with inactive disease and only 5-7% of controls. They concluded that a range of such bacteria (including Escherichia coli and Streptococcus faecalis) had a possible aetiological role in IBD. However, the possibility still remained that these organisms were merely secondary invaders. Apart from cell wall defective forms of bacteria, various other bacteria have been implicated in the aetiology of CD. The recent emergence of newly recognised gasto-intestinal pathogens such as Clostridium difficile and Helicobacter pylori suggest that a bacterial aetiology should not be readily dismissed. Higher numbers of Bacteroides species have been reported from the faecal flora of CD patients (Keighley et al, 1978). Wensinck et al (1981) reported that up to 10% of the faecal flora in CD patients constituted gram-positive coccoid rods identified as Eubacterium, Peptostreptococcus and Coprococcus species.

Tabaqchali *et al* (1978) and other authors describe an increased incidence of antibodies against strains of *Escherichia coli* in CD and UC patients compared to controls. Increased antibodies have also been reported to *Bacteroides fragilis* in both CD and UC (Brown and Lee, 1974), *Bacteroides vulgatus* in CD patients, (Helphingstine *et al*, 1979), Pseudomonas like cell wall defective bacteria in CD patients, but not UC patients or healthy controls (Parent and Mitchell, 1978) and *Mycobacterium kansasii* in both CD and UC patients (White *et al*, 1978).

Antibodies against Chlamydia of lymphogranuloma-venereum type was detected in 38 of 55 (69%), CD patients as compared to 2/21 control patients (Schuller *et al.*, 1979). There is a contradictory report by Taylor-Robinson *et al* (1979), in which both CD and UC patients were reported to have a low frequency of chlamydial antibodies.

Van De Merwe (1984) identified four strains of anaerobic coccoid rods, *Eubacterium contortum* (ME44 and ME47), *Peptostreptococcus* (C18) species and *Coprococcus comes* (Me46), isolated from CD patients, to which CD patients more often had serum antibodies than healthy subjects. They suggested that the occurrence of serum antibodies to these coccoid rods be used for diagnostic purposes for CD, but their relevance for aetiology and pathogenesis is not yet clear.

In conclusion, no consistent virus or bacterium has been isolated from IBD tissue, and attention has returned to mycobacteria as possible aetiological agents.
C. MYCOBACTERIAL ISOLATES FROM CROHN'S DISEASE TISSUE

Mycobacterial associations with CD have long been suspected but it is only in the last twelve years that mycobacteria have been isolated from CD tissue. The results are summarised in Table 1.4.

As stated earlier, Van Patter in 1952 made the first major effort to culture mycobacateria from Crohn's tissue since Crohn himself in 1932. Van Patter set up 1,762 cultures from 43 patients and inoculated 131 animals including guinea pigs, rabbits, cats, rats and chickens with diseased tissues. All animals remained normal. He used seven different types of media and incubated cultures for up to 15 months. He isolated acid fast organisms from three patients at 6, 7.5 and 8 months incubation. These organisms could not be subcultured and were never formally identified.

Burnham et al in 1978 described the isolation of Mycobacterium kansasii from the lymph node of a single patient with Crohn's disease. mycobacterial associations with CD having long been Despite suspected, this was the first mycobacterial isolate and hence initiation of further attempts to culture important in the mycobacteria from CD tissue. Apart from the isolate of M. kansasii, Burnham et al reported the growth of pleomorphic acid fast organisms (PAFOs) from 22/27 CD patients, 7/13 UC patients and 1/11 controls. The work in this thesis is based on the characterisation of these PAFOs.

To date, unidentified acid fast forms have been isolated by several research groups from Australia (Coloe *et al*, 1986,qouted by Chiodini,1989), Japan (Haga, 1986, Japanese Journal of Gastroenterology 23:2325-2333,quoted by Chiodini 1989), USA (Collins *et al*, 1986) and the Netherlands (Haagsma *et al*, 1988). Chiodini *et al* (1986a) and Graham *et al* (1987a) both describe unidentified spheroplasts which were ZN negative, but the exact staining method used is not known.

Table 1.4

MYCOBACTERIAL ISOLATES FROM PATIENTS WITH CROHN'S DISEASE

Van Patter	1952	AFB	3/43
Burnham et al	1978	M. kansasii Unidentified AF organisms	1/27 22/27
Chiodini et al	1984	M. paratuberculosis	2/11
	1986	M. paratuberculosis Unidentified spheroplasts	4/26 12/26
Coloe et al	1986	M. paratuberculosis Unidentified AF growth	1/30 NK/50
Haga	1986	Unidentified AF coccoid bodies	1/31*
Collins et al	1986	M. chelonei M. paratuberculosis Unidentified AF rods and/or spherules	1/12 1/12 5/12
Graham <i>et al</i>	1987 1988	M. kansassii M. fortuitum complex M. avium complex Unidentified spheroplasts Unidentified spheroplasts	1/59 2/59 2/59 12/59 17/87
Colemont <i>et al</i>	1988	M. chelonei	2/32
Haagsma <i>et al</i>	1988	M. paratuberculosis M. fortuitum Unidentified AF materia l	2/75 1/75 2/75
Thorel et al	1989	M. paratuberculosis	1/NK

NK = not known

* Number of specimens cultured (number of patients not known)

M. PARATUBERCULOSIS AND CROHN'S DISEASE

Chiodini *et al* in 1984 isolated two strains of a *Mycobacterium paratuberculosis*-like organism named strain Linda and strain Dominic, after the two CD patients from whom the organisms were isolated. Three UC and 3 control cultures (from patients with other bowel diseases) were negative (Chiodini *et al*, 1984 a).

Strain Linda was isolated from cultures from a 15 year old girl. isolate was noted after $3^{1}/_{2}$ months incubation on Herrold's The egg yolk medium (HEYM) with mycobactin. Strain Dominic was from cultures obtained from a 12 year old boy. Again, growth was noted on HEYM with mycobactin but after $5^{1}/_{2}$ months. A highly complicated culture technique was used . Tissues were first washed in sterile water, aseptically trimmed of fat and placed in sterile Butterfield's buffer at 3-5°C overnight. The next day, 20g of gut mucosa was placed in trypsin for 45 minutes. Tissues were then filtered through cheese cloth, centrifuged and the resulting sediment suspended in 0.1% benzalkonium chloride. After 24 hours at temperature, the sediment was distributed onto 8 HEYM slants room supplemented with 2µg/ml mycobactin and tubes were incubated for over The two isolates, strain Linda and strain Dominic, 30 months. were thus grown from GI mucosa, were mycobactin dependant and acid fast. They belonged to Runyon's group III, the non-pigmented slow growers.

The organisms were pathogenic for mice when injected intravenously or intraperitoneally; the mice developed hepatic and splemic granulomas which contained numerous acid fast mycobacteria. The strains were not pathogenic for rats, guinea pigs, rabbits or chickens. A seven day old goat was inoculated orally with 50mg of the organism. Humoral and cell mediated immunological responses were noted in 2-3 weeks. A non

caseating granulomatous ileocolitis occurred in 5 months. Acid fast bacilli were seen in sections of intestine, but a single organism was seen in each of two micro granulomas of the mesenteric lymph nodes. The mycobacterium species was reisolated from the lymph node but not from the intestine, Chiodini et al published a series of further papers (Chiodini et al, 1984 a-d) describing detailed characteristics of the isolates. They also described the isolation of strain Ben, cultured froma 78 year old man with Crohn's disease after 18 months incubation. Chiodini et al in 1986 (1986a) reported their fourth mycobacterial isolate from CD tissue - strain Leona isolated from an 80 year old patient. On primary isolation, this and the previous CD derived mycobacterial isolates described appeared in spheroplast form as non acid-fast coccobacilli which after several months transformed to the parent mycobacterium. Mycobacterial spheroplasts and their respective unclassified mycobacterial parent forms were isolated in total from 4/26 CD patients. No spheroplasts or mycobacteria were isolated from 13 UC or 13 control specimens.

Chiodini and co-workers used restriction polymorphism of the ribosomal DNA genes to determine the relationship between the cellwall deficient and bacillary forms (Chiodini et al, 1986 a,b). Identical restriction patterns were found between the spheroplasts and the four Mycobacterium paratuberculosis-like isolates providing definitive evidence of their origin. On biochemical testing the mycobacterial strains isolated from the CD patients were said to resemble M_{\star} paratuberculosis and these were later being identical to ruminant confirmed as strains of M. paratuberculosis by DNA probes (McFadden et al, 1987 a,b).

An additional 12 CD patients had unidentified spheroplasts isolated from their cultures. Seven out of ten tested

seroagglutinated with specific *M. paratuberculosis* antisera, suggesting these unidentified forms were also *M. paratuberculosis*.

Chiodini suggested that the presence of mycobacteria from CD tissue and not UC or control tissue supported a role for mycobacteria as aetiological agents in CD. Furthermore, the presence of cell wall defective (CWD) forms could then account for the inability to demonstrate acid fast bacilli in tissues and the failure to demonstrate a strong and consistent immunologic response because CWD mycobacteria are generally of low immunogenicity. He suggested that the previous failure to isolate these organisms was because of the caustic nature of other techniques for processing mycobacterial specimens. Also that a slow rate of reversion with subsequent local hypersensitivity-type immunologic responses could account for the chronicity of CD, assuming that only the bacillary forms are pathogenic (Chiodini, 1989).

The first confirmation of *M. paratuberculosis* from CD tissue came from Coloe *et al* in Australia (P J Coloe, C R Wilks, D Lightfoot and F A Tosolini (1986), Aust Microbiol., 7;188, quoted by Chiodini, 1989). They isolated *M. paratuberculosis* from 1/30 CD patients from colonic material but not the draining lymph nodes, after 16 weeks incubation. Approximtely 50 CD patients and 50 patients with UC or other bowel diseases have had biopsy specimens cultured. Some acid fast growth has been observed on some CD cultures (no definite figure has been published). These slow growing isolates have not yet been characterised (Coloe, personal communication, quoted by Chiodini, 1989). Using an immunoblot technique, Whitehead (Proc. 2nd Int. Colloq. Paratuberc., quoted by Chiodini, 1989) found identical antigen bands when he reacted *M. paratuberculosis* antigens separated by

polyacrylamide gel electrophoresis with serum from the CD patient who yielded the Australian paratuberculosis strain and cattle naturally infected with M. paratuberculosis.

Collins, Beaman and Arthur et al (1986) thoroughly investigated the role of M. chelonei, subsp. abscessus, isolated from the ileum of a CD 109 M. chelonei was inoculated orally into new born patient. All fifteen goats inoculated developed transient goats. diarrhoea 3-7 days past inoculation. Three goats died at 5-9 days after an 8-12 hour course of bloody diarrhoea. Intestinal lesions were limited to mild inflammation of the terminal ileum and colonic infiltration with polymorphonuclear cells. Stool cultures rectal swabs had acid fast bacilli at four days, but not and at weekly intervals thereafter. M. chelonei was isolated from colonic and ileal mucosa. An acute intestinal disorder with M. chelonei could have significant meaning as this organism generally associated with immunocompromised hosts or traumatic is wounds. However, the authors did not rule out other neonatal diseases of goats as a possible cause of the observed diarrhoea and acute bowel inflammation (Chiodini, 1989). Gitnick et al (1988) in an update of their work (initially reported by Collins et al., 1986) report culturing 24 samples (surgically resected intestinal tissues) from 12 CD patients, 29 specimens from UC patients and 26 specimens from patients with other bowel disease (22 non CD patients in total). They isolated a M. paratuberculosis-like strain from the colon of a CD patient after 12 months incubation on Herrold's egg yolk medium and an uncharacterised slow grower from a patient with adenocarcinoma of the bowel (apart from the M. chelonei strain reported in 1986). As well as these mycobacteria, they isolated acid fast spherules from 2 CD tissues, and acid fast rods from 3 CD tissues. They also isolated acid

fast spherules from 2 UC samples and 4 control samples. These have not as yet reverted to mycobacteria. Gitnick's cultures were processed according to Chiodini's methods (already described). Samples were cultured on HEYM with mycobactin and on 7H 10 medium. Mycobacterial L forms were sought in L form media in T-flasks, When the M. *paratuberculosis*-like strain was fed to neonatal goats, all remained well, in contrast to Chiodini's previous experiments (1984a).

Colemont *et al* (1988) isolated two strains of mycobactin dependant *M. chelonei* from 32 CD patients, and they saw acid fast bacilli, but did not successfully culture them in nine other biopsies. In contrast to the findings of Graham *et al*,(1987a) and Collins *et al*,(1986) no cultures or strains were positive for mycobacteria in non-Crohn's disease cultures (7 ulcerative colitis, 10 other bowel diseases).

Haagsma et al (1988) cultured 66 CD surgical specimens and 7 CD biopsy specimens and isolated 1 strain of *M. fortuitum* and 1 strain of *M. paratuberculosis* (after 11 months on Herrold's egg yolk medium and 16 months on Ogawa medium). A further *M. paratuberculosis* strain was also later isolated. They only cultured CD tissue and not control tissue which was a flaw in their study. Acid fast material was reported in 2 cultures. Chiodini (1988) showed that Haagsma's initial *M. paratuberculosis* isolate was genetically identical to Chiodini's *M. paratuberculosis* strains.

Thorel *et al* (personal communication, quoted by Chiodini, 1989) also isolated a strain of *M. paratuberculosis* from a 45 year old woman with CD and subsequently a study has been initiated in Lyon to study biopsy and faecal specimens from 50 patients with CD.

To date there have been 9 isolates of *M. paratuberculosis* from CD tissue from five centres (see Table 1.4) worldwide (Connecticut, California, Australia, Netherlands, France). *M. paratuberculosis*

belongs to the M. avium complex and cannot be distinguished biochemically or serologically from mycobactin dependent M. avium strains (McIntyre and Stanford, 1986 a,b). However, McFadden et al (1987c) have used DNA probes to distinguish M. paratuberculosis from M. avium and M. intracellulare, although it is still questionable whether this is at the species level. M_{\star} avium is an environmental opportunistic organism. M. paratuberculosis is a well recognised pathogen in a variety of animal species and its existence as an environmental organism has not been formally proven. It is the slowest growing of the cultivable mycobacteria and it generally takes years to become proficient at working with the species. Hence M. paratuberculosis with its prediliction for the GI tract would be an attractive aetiological agent of CD. Hampson et = al(1989b)failed to demonstrate M. paratuberculosis as a coloniser in AIDS patients and suggest that its isolation only from disease states and Crohn's disease, lends support to the idea that it is significant in Crohn's disease. Also the isolation of the pathogen M. paratuberculosis from a disease with a clear animal parallel is intriguing. Johne's disease is caused by M. paratuberculosis, where the organism has a strict preference for the gastrointestinal tract and does not produce caseation necrosis. The similarities between CD and Johne's disease have long been noted (Dalziel, 1913).

Johne's disease is a contagious, chronic and usually fatal enteritis in ruminants. Clinical sings of the disease are characterised by episodes of chronic diarrhoea and progressive emaciation. Histologically, there is a chronic inflammatory reaction, principally of the terminal ileum but also of the colon and other regions of the gut.

Cattle, sheep and goats aged less than six months are easily infected and become diseased whereas adult animals seem to be resistant. The incubation period is very long. For example, cattle infected as calves do not show clinical signs until three years of age. Morgan (1987) argued the case that if we assume that Johne's and CD are similar in not only their pathological lesions but also in their actiology, then a hypothesis would be formed that some infants are infected with a mycobacterium in the first few months of life and CD develops in young adult life. The same claim can be made for leprosy in young people. This would explain the familial association and the occurrence in monozygotic twins especially in those living apart since childhood. The low incidence in married couples would reflect the difficulty of transmitting the disease between adults. It is also noteworthy that Chiodini's first two M. paratuberculosis isolates were from two patients aged 15 and 12 years. Together with this, Chiodini possibly used the best conditions to detect a fastidious organism like M. paratuberculosis - use of mycobactin, meticulous decontamination and culture procedure and prolonged incubation (months or even years).

Overcrowding, stress and poor hygiene favours the spread of Johne's disease. Morgan further suggests that, if the hypothesis is true, the increased incidence of CD in Ashkenazi Jews could be related to the experience of similar poor conditions during childhood in Europe.

Considering the isolation of *M. paratuberculosis*, the chances of successfully isolating *M. paratuberculosis* from the affected gut in Johne's disease depends to some extend on the severity of the pathological lesion. In early lesions, *M. paratuberculosis* is easily identified with masses of acid fast bacilli (AFB), but in severe

lesions with encapsulation and necrosis, no AFB are visible histologically. By analogy, material obtained surgically from patients with CD is often from the most severely affected areas with long standing lesions and by Morgan's hypothesis, the chances of culturing mycobacteria would be small.

ANIMAL STUDIES

Attempts to transmit CD to laboratory animals has given equivocal results (discussed earlier under Animal Transmission Studies, Section V). Animal studies with the CD-derived *M. paratuberculosis* strain were done by Chiodini *et al* (1984a) and Gitnick *et al* (1988). It was surprising that Gitnick's *M. paratuberculosis* strain did not produce any disease in neonatal goats, whilst Chiodini's strain caused a granulomatous ileocolitis at 5 months. The only difference between the two experiments was that Gitnick fed his animals with a stomach tube. It is hard to image that this could have altered any observed effects, unless infection of tonsils, for example, predisposes to subsequent disease by a mechanism of locally induced immunosuppression – not an impossibility.

Apart from *M. paratuberculosis*, CD derived *M. chelonei* has been used in animal studies. The role of *M. chelonei* in neonatal goats was assessed by Collins *et al* (1986) and is discussed above. Briefly, a granulomatous intestinal disease was not produced, the animals developed diarrhoea, had acid fast bacilli in stool samples and 3/15 goats died. Also Colemont *et al* (1988) were unable to reproduce a Crohn's like lesion in mice with 2 CD-derived *M. chelonei* strains. Hence, disappointing results have been obtained in transmitting CD derived mycobacteria to animals. If a mycobacterium is involved in CD, this may mean that the "correct" mycobacterium has not been used in

animal studies, the correct animal host for such studies has not been found, or as presumed in humans only a small minority of those infected go on to develop the disease.

Experiments with Johne's disease have interesting parallels. There are considerable species, strain and individual differences and difficulties in demonstrating Koch's postulates(review by Morgan, 1987).

Johne's disease was documented in primates for the first time in 1987 (McClure *et al*, 1987). Infection occurred in 29/38 monkeys in a colony of stumptail macaques (*Macaca arctoides*) extending the natural host range of *M. paratuberculosis* from ruminants to sub human primates. Hence, it was possible to consider that humans too could be infected.

Against this background, there are however, several factors which do not support the idea of M. paratuberculosis causing all or many cases of CD, however attractive the hypothesis may seem. First, M. paratuberculosis in animals is a wasting disease with diarrhoea and usually results in death of the animal; this is not seen in CD. However, the immune response to mycobacterial antigens could modify the disease and mycobacterial diseases are known to be immunologically mediated. It is interesting that all those investigators who isolated M. paratuberculosis have been trained in veterinary microbiology. This could be interpreted in two ways. First, perhaps they managed to isolate M. paratuberculosis because there were experienced with the techniques required to culture this fastidious organism. However, it is conceivable that M. paratuberculosis may be a contaminant in such laboratories and this was the reason it was isolated. Also, in contrast to the work of other research groups, it is surprising that Chiodini's group has failed to isolate any other mycobacteria apart from M_{\star}

paratuberculosis. Interesting parallels exist in leprosy, in which a limited group of organisms, often of the H. avium group, or sometimes of fast growers, have been isolated from leprous tissues, sometimes initially as cell-wall defective organisms (Skinsnes et al 1975, Bapat and Modak, 1978).

Finally, despite all the effort and time put into culturing CD tissues over several years, only 9 isolates of *M. paratuberculosis* have been made. The conclusion is that if *M. paratuberculosis* is a significant isolate, it causes only a few cases of CD and the aetiology of the majority of cases remains unknown.

OTHER MYCOBACTERIAL ISOLATES

Considering other mycobacteria isolated from CD tissue, *M. kansasii* was first isolated by Burnham et al in 1978. Even though initial skin testing (with antigens prepared from the strain of *M. kansasii* isolated), supported *M. kansasii* as an aetiological agent in Crohn's disease, (Burnham *et al*, 1978; White *et al*, 1981) further data on skin testing showed that increased responsiveness was also found in the controls (Elliott *et al*, 1980). The authors concluded that *M. kansasii* was unlikely to be the aetiological agent, and was probably a chance contaminant. Also Whorwell *et al* (1978a) failed to demonstrate *M. kansasii* in tissues by immunofluoresence.

Graham et al (1987a) in the USA isolated M. kansasii from one CD specimen. The rate of isolation of mycobacteria from IBD tissue by Graham and co-workers was high. They brought to light the widespread occurrence of mycobacteria in diseased tissues. Graham et al isolated mycobacteria from 47.6% of CD, UC and control tissue but did not isolate M. paratuberculosis. They cultured intestinal resection specimens and biopsy specimens (aphthous ulcers in CD patients, biopsies at colonoscopy in all groups).

Considering resection specimens, approximately lg resected tissue was cultured; no slow growing mycobacteria were isolated from 37 CD or 2 UC patients from these resection specimens. One slow grower was isolated from a total of 16 control samples (result updated by Yoshimura *et al.*, 1988).

A rapid grower was isolated from one CD resection specimen but not from UC or control resection specimens. From biopsy specimens, 7/22 CD samples, 4/17 UC samples and 8/19 control samples grew slow growing mycobacteria and 1/22 CD samples, 5/17 UC samples and 10/19 non-IBD controls grew rapid growers. All rapid growers were identified as M. fortuitum or M. chelonei subspecies chelonei or subspecies abscessus.

Spheroplasts were identified in 10/37 CD resection specimens and 2/22 CD biopsy specimens. This is contrasted with spheroplast isolation from 1/2 UC resection specimen, 0/8 control resection specimens and 2/17 UC biopsy and 0/19 control biopsy specimens. Five spheroplast cultures reverted to slow growing mycobacteria giving a total of 24 slow growing mycobacterial isolates from CD, UC and control samples. One CD biopsy isolate was *M. kansasii* and six were identified as members of the *M. avium* complex (2 from CD biopsies, 2 from UC biopsies and 2 control specimens). None of the other 17 isolates have been identified due to their extremely slow growth rate prohibiting sufficient inocula for biochemical tests. They all belong to Runyon's group III, the non-pigmented slow growers.

Spheroplasts were first detectable by the aid of the dissection microscope after 2 months incubation, and more usually after 3.5 months incubation. The isolates did not stain by Ziehl-Neelsen or Gram stain. Five of these speroplast cultures have reverted to pleomorphic acid fast organisms, but remain unidentified.

At an initial glance, it appears that mycobacteria were isolated from all tissues and no specific mycobacterium is associated with CD tissue. However, if all this data is analysed closely, it is seen that except for a single strain of *M. fortuitum*, conventional mycobacteria have not been isolated from resected CD, UC or control tissues. However, spheroplasts have been isolated primarily from resection specimens (from 10/37 CD patients, 1/2 UC patients but 0/8 controls). Thirty-five strains of mycobacteria (mainly *M. avium* complex and *M. fortuitum* complex) were isolated from biopsy specimens, indicating that biopsy sources such as aphthous ulcers provide a suitable microenvironment for the propagation of such environmental organisms. Hence, it is important to analyse not only method of culture but the source of material cultured.

The isolation of *M. kansasii*, *M. chelonei*, *M. fortuitum* and *M. avium* from CD, UC and control specimens should not come as a surprise. These organisms are all environmental species, is they occur as freeliving organisms within the environment.

This is in contrast to *M. tuberculosis* for example, which is not known to have an environmental reservoir other than that associated with human contamination such as sewage. *M. kansasii* is present in tap water, aquaria and waste water. *M. chelonei* has frequently been isolated from rivers, lakes, waste and sea water and dust. *M. fortuitum* has been found almost anywhere in the environment including water, swimming pools, soil and dust. *M. avium* has been isolated mainly from soil but also from rivers and waste water of animal origin.

Although considered to be environmental species, these mycobacteria are capable of causing opportunistic infection. However, they seem unlikely candidates as aetiological agents of IBD. *M. kansasii* is a

well recognised opportunist cause of pulmonary disease with a similar disease pattern to tuberculosis. The M. avium complex is now a major cause of disseminated infection in AIDS patients, where the great majority of incriminated strains belong to agglutination types 4 and 8, and causes a wide range of diseases in aminals and humans, primarily as opportunistic pathogens.

At all centres, different methods of isolation, different decontamination steps and different culture media have been used, consequently varied results have been obtained.

The range of media used included HEYM, Ogawa, Middlebrooks 7H10 and 11 Media, Lowenstein Jensen and Robertson's cooked meat broth. However, the primary isolation time was very long in all cases. Acid fast forms took between 3 and 30 months to grow, Van Patter isolated AFB at 6, 7.5 and 8 months, Burnham *et al* isolated the pleomorphic acid fast forms at 2-6 months and Graham *et al*, isolated spheroplasts at a median time of 3.5 months. It is clear that primary isolation of these organisms is very much a long-term procedure.

HISTOCHEMICAL DATA

Demonstration of a consistent microbe mycobacterial or otherwise directly in CD specimens has not been possible. Colemont *et al* (1988) used simple acid fast staining techniques and identified mycobacteria in 11/32 CD tissue specimens. However, routine acid fast staining has generally not been successful in CD (Burnham, 1980). Various histochemical techniques have been used to identify mycobacteria in tissue sections. Whorwell *et al* (1978a) found no evidence of infection with *M. kansasii*, rotavirus or *Xanthomonas maltophilia* in tissues from CD and UC patients when they searched for the relevant antigens by immunofluoresence.

Haga (Jpn. J. Gastroenterol. 1986 23: 2325-2333, quoted by Chiodini) used immunohistochemistry and could not demonstrate antigens of *M. paratuberculosis* in 18 CD tissues. Van Kruiningen *et al* (1988) could only confidently demonstrate one CD tissue section as being positive using an immunoperoxidase method for detection of *M. paratuberculosis*.

Inconsistent results have been obtained by different workers. Yoshimura et al (1987) used liquid genomic DNA-DNA hybridisation and detected mycobacterium - related sequences in 53% CD patients, 33% UC patients and 17% of controls. In contrast Kobayashi, Blaser and Brown (1989) did not observe any staining in 67 CD specimens using immunohistochemical methods. These authors used monoclonal antibodies to M. paratuberculosis, M. tuberculosis and lipoarabinomannan. Although different techniques have different levels of sensivity and specificity none of them show any consistent positive result and are mostly negative. The organisms may be present in a form that is difficult to stain - for example a cell wall defective (OWD) form may not stain in the tissues with Ziehl-Neelsen. It is notable that many of the human isolates have been recovered in this form. Also it is possible that any infectious agent may be present in extremely low numbers or be scattered in small foci accounting for inability to demonstrate the organisms within the lesions.

It may be that the organism is simply not present and acted as an initial antigenic stimulus triggering various immunological mechanisms.

SEROLOGICAL DATA

Grange, Gibson and Nassau (1980) reported increased IgA and IgM antibodies to *M. tuberculosis* in CD patients. Matthews *et al* (1980) showed that 79-96% of CD sera agglutinated with antigens from *M. paratuberculosis* and *M. avium*, but no clear difference was shown between CD and control sera.

Thayer *et al* (1984) used an ELISA (Enzyme linked immuno-sorbent assay) to detect antibody to a crude preparation of *M. paratuberculosis* antigen and found increased antibody titres in 23% of CD patients. Cho *et al* (1986) found no evidence for increased antibody titres in CD sera to a species specific glycolipid antigen from *M. paratuberculosis* or to common mycobacterial antigens. McFadden and Houdayer (1988) found no evidence for antibodies to Cocito's mycobacterial A60 (a membrane glycoprotein found in most mycobacteria) antigen prepared from *M. bovis* BCG, *M. avium* and *M. paratuberculosis* in sera from CD patients. Jiwa *et al* (1988) showed elevated IgG levels in CD sera by ELISA to mycobacterial purified protein derivatives (prepared from *M. tuberculosis*, *M. kansasii*, *M.phlei*, *M. paratuberculosis* and *M. smegmatis*.)

Kobayashi *et al* (1988) could not demonstrate elevated antibody levels in CD to lipoarabinomannan (LAM) or *H. paratuberculosis*. Blaser *et al* (1984) found no difference between CD and controls in reactivity to arabinomannan, a common mycobacterial antigen. Chiodini (1989) postulated that OWD mycobacterial forms would lack common mycobacterial antigens such as LAM and thus account for lack of anti-LAM antibodies in CD.

In conclusion, no consistent serological responses against mycobacterial antigens have been demonstrated. Mycobacteria are environmental organisms and human exposure is common. Serum antibodies may be ubiquitous so that CD and control patients may have raised levels. Common antigens exist between mycobacteria and other families of the order *Actinomycetales* such as Nocardia and Corynebacteria, hence a wide range of reactivity amongst normal and diseased is likely.

Serum antibodies to antigens of several bacterial pathogens have been reported (Blaser *et al.*, 1984) indicating a non specific response, perhaps secondary to increased bowel wall permeability. These results indicate that serum reactivity is not reliable for examining the relationship between intestinal disease and mycobacteria. However, this does not necessarily contraindicate the association of mycobacteria with CD.

SIMILARITIES TO OTHER MYCOBACTERIAL DISEASES

Similarities between CD and mycobacterioses support a mycobacterial aetiology. The similarity to tuberculous ileitis is well known. In the western world, intestinal tuberculosis (TB) is generally misdiagnosed as CD and diagnosed correctly only after surgery. In developing countries where TB is common, cases of granulomatous intestinal disease are diagnosed as TB.

Every clinical, radiological, endoscopic and pathological feature of CD may occur in primary intestinal tuberculosis, and the two diseases may be virtually impossible to distinguish. The only distinguishing criteria are the presence of caseating granulomas or acid fast bacilli in the intestinal wall or regional lymph nodes in tuberculosis (Lockhart - Mummery and Morson 1960).

The similarity to Johne's disease has already been considered. *M. paratuberculosis* with its absence of caseation is a more likely candidate for CD than *M. tuberculosis*. Non caseating granulomas are also seen in Sarcoidosis, another disease where mycobacteria have been implicated.

There are many parallels between IBD and leprosy in the presence of an immunopathological spectrum, with the tuberculoid form of leprosy having many granulomata but few acid fact bacilli and the lack of

cultivability of the causative organism. The absence of detectable organisms in tissues may be analogous to the situation in tuberculoid leprosy.

Crohn's disease and the mycobacterioses also share extra intestinal manifestations including arthritis, erythema nodosum and amyloidosis amongst their sequele.

ANTIMYCOBACTERIAL CHEMOTHERAPY AND CD

The use of antimycobacterial drugs in CD has received renewed attention in the last few years after scattered case reports of CD patients in remission following co-incidental quadruple antituberculosis therapy (Warren, Rees and Cox 1986; Schultz *et al*, 1987). Ward and McManus (1975) had reported that dapsone was effective in 4 of 6 cases of CD. Prantera *et al* (1988) also reported that a CD patient improved dramatically after 2 weeks on dapsone and was in remission at one year on dapsone alone.

Picciotto *et al* (1988) found anti-mycobacterial chemotherapy effective for 9 months in a 22 year old man with ulcerative colitis and for 8 months in a 26 year old CD patient. It is interesting that their patient with Ulcerative Colitis also improved on antimycobacterial drugs.

Thayer *et al* (1988) used rifabutin and streptomycin to treat six refractory CD patients.

Thayer's group used rifabutin 300mg per day orally and streptomycin lg i.m. for 5 days per week for two months. Preliminary results suggested that therapy was effective in moderating the course of CD, with marked improvement in their Crohn's disease activity index (defined by Best *et al.*, 1976).

There has been recent interest in rifabutin (a rifampicin derivative) due to high in vitro activity against *M. paratuberculosis* (Chiodini *et al.*, 1984c) and its successful use in the treatment of *M. paratuberculosis* infection in the colony of stumptail macaque monkeys where 7/7 monkeys treated with rifabutive survived (McClure *et al.*, 1987).

Hampson *et al* (1989, personal communication) treated 17 CD patients with rifampicin, ethambutol, isoniazid and pyrazinamide or clofazimine. Twelve (71%) patients showed statistically significant improvement in their CD activity index and 9/10 patients were completely withdrawn from steroids. After 9 months treatment, 10/20 patients treated were in remission (Hampson *et al*, 1989a) and after one year of treatment objective improvement was found in patients based on indium-111 scans (Saverymuttu *et al*, 1986). Mulder (personal communication, quoted by Chiedini, 1989) also reported some improvement in 8 CD patients treated with 6 months rifabutin and ethambutol in an open trial. Clearly this data is encouraging.

However, negative results when using antimycobacterial chemotherapy for CD have also been reported. Shaffer et al (1984) published the results of a two year randomised double blinded cross over controlled trial of antimycobacterial agents in 27 CD patients. They used rifampicin, ethambutol, sulfasalazine and steroids versus sulfasalazine Thirteen patients withdrew due to poor steroids alone. and compliance, need for surgery or adverse effects. They noted no significant difference in the CD activity index or any other clinical disease indicators. Rutgeerts et al (1988) noted no improvement when rifabutin and ethambutol were used in 16 CD patients for 6 months. They reported a high occurrence of a 'flu-like syndrome (also reported by Shaffer et al, 1984).

The 'flu-like syndrome with ot without leucopenia, frequently observed in CD patients receiving rifampcin or its derivatives (Chiodini, 1989) is not understood. It is not seen in TB, leprosy or AIDS when these drugs are used, and is peculiar to Crohn's disease. Patients receiving steroids when rifabutin is initiated do not get the 'flu-like symptoms and perhaps the disorder is an immune phenomenon. Some authors withdrew the drug due to the severity of associated symptoms (Shaffer, 1984) but others report the syndrome to be transient (Hampson *et al.*, 1989 a).

If Crohn's disease is caused by an organism such as M. paratuberculosis or an atypical mycobacterium, then treatment schemes would need to follow current recommendations for mycobacterial infections other than tuberculosis. Even if only a small proportion of CD cases were due to a mycobacterial infection then it would be of great relevance to distinguish this group and use chemotherapy rather than surgery on these patients. The treatment of pulmonary mycobacterioses in nonimmunocompromised patients is difficult and includes at least quadruple drug therapy for 2-3 years (Tsukamura, 1988; Hornick et al, 1988). Current evidence suggests that treatment for Mycobacterium avium intracellulare may be effective after prolonged periods (Bass, 1986); but in AIDS, although the underlying immune defect in AIDS is largely responsible, it is impossible to clear these patients of mycobacteria with conventional antituberculosis agents (Armstrong et al, 1985).

It may be that in CD, therapy is needed for a very long time. However, multiple drug regimens for prolonged periods of time may be associated with problems. Also, even if antimycobacterial drugs were effective in CD, their broad spectrum of activity means that this is supportive but not conclusive evidence for a mycobacterial aetiology.

Spontaneous exacerbations and remissions in Crohn's disease further complicate assessment of the effect of these drugs; however, more clinical trials are certainly warrented to assess more fully the use of antimycobacterial drugs in CD. At present, larger trials are underway at St George's Hospital Medical School (Hampson J S, personal comminication) and Cardiff (Jenkins A, personal communication) to try and answer these questions.

CONCLUSIONS

A host of different mycobacteria have been isolated from CD patients as well as controls. It is possible that in CD, there is an abnormal immune response to a common antigen. Also, properties of the host must not be forgotten.

CD may have multiple causes and also may be multifactorial. The correct immunogenetic background may be necessary. It is possible that the intestine may react in a narrow way to different mycobacteria and that mycobacteria may only cause some cases of Crohn's disease.

The particular difficulties associated with studies of CD are notable. Assumption that CD is a single disease eutity, grouping of CD along with UC, and mis-classifications as CD (Calkins and Mandeloff 1986, estimate that more than 20% CD diognoses are not in fact CD). A possible "latent period", a prolonged onset-diognosis period and the possibility that the process is immunologically mediated makes interpretation of results all the more difficult.

CHAPTER TWO

The increasing number of unidentified acid fast organisms isolated from Crohn's disease tissue is notable. Many more of these organisms have been isolated than mycobacteria themselves. Whilst isolates of mycobacteria from CD tissue are no doubt of great interest, the emerging picture of consistent isolation of unidentified acid fast forms and spheroplasts may be of even greater significance. No single mycobacterium has been isolated consistently (Chapter 1, Table 1.4). However, almost every reseach group has either visualised (Colemont *et al*, 1988) or grown acid fast forms (Van Patter, 1952; Burnham *et al*, 1978; Coloe *et al*, 1986; Haga, 1986 and Haagsma *et al*, 1988) or spheroplasts (Chiodini *et al*, 1984b,1986a; Graham *et al*, 1987a). Certainly, no one has reported protoplast isolation; all isolates seem to have some cell wall.

Techniques of Ziehl-Neelsen staining are no doubt different in all research centres and some groups report Ziehl-Neelsen negative spheroplasts. However, some of these spheroplasts have reverted to acid fast bacilli (Chiodini *et al.*, 1984b; Graham *et al.*, 1987a). Table 2.1 lists the isolation of such unidentified acid fast forms and spheroplasts from CD tissue as compared to UC tissue and non-IBD tissue. Some workers have grouped isolates from UC and non-IBD together as "controls" (Coloe *et al.*, 1986; Collins *et al.*, 1986), and some authors do not report figures for UC or controls (Van Patter, 1952; Haga, 1986; Haagsma *et al.*, 1988).

Table 2.1

Isolation of unidentified acid-fast organisms or spheroplasts from patients with Crohn's disease, Ulcerative Colitis and controls.

		<u>CD</u>	<u>UC</u>	CONTROLS (non-IBD)
Van Patter, 1952		3/43	Not done	Not done
Burnham <i>et al</i> , 1978 (updated by Elliott <i>et al</i> , 1980)		42/76	14/27	3/41
Chiodini <i>et al</i> , 1986		12/26	0/13	0/13
Coloe et al, 1986		NK/501	0/50*	0/50*
Haga, 1986		1/31+	NK	NK
Collins <i>et al</i> , 1986 (updated by Gitnick <i>et al</i> , 1988)		5/12	2/22*	4/22*
Graham <i>et al</i> , 1987a (updated by Yoshimura <i>et al</i> , 1988)		17/87	3/25	5/41
Haagsma <i>et al</i> , 1988		2/75	Not done	Not done
	TOTAL	81/319	31/232	

* - Total of non-CD patients

NK - Not Known

- ¹ Some cultures stated as positive for acid fast growth, but no figure stated. Hence, omitted in total.
- Total of 31 samples from CD patients, but number of patients not known. Hence, omitted in total figure

To date, acid fast forms/spheroplasts have been isolated from 81/319 CD patients but only 31/232 patients with UC or other bowel diseases (Total figures, Table 2.1). This is a statistically significant difference (P<0.0005) by Fisher's exact test. Only Burnham *et al* (1978) have isolated a large number of acid fast forms from UC tissue. If Burnham's figures are omitted, 5 isolates have been reported from UC patients and only 9 isolates have been reported from non-IBD patients. It is of note that Burnham's group cultured only mesenteric lymph nodes from patients with CD, UC and non-IBD patients, whereas all other groups cultured mainly bowel resection and biopsy specimens, and Haga also cultured faecal specimens.

Despite different observations with ulcerative colitis, the overall picture remains clear. More unidentified acid fast forms have been isolated from CD tissue than mycobacterial isolates themselves. Also some acid forms were initially isolated as spheroplasts.

Although mycobacteria are classically bacilli, there is evidence that alternate forms may exist. Cell wall defective forms (Chiodini *et al*, 1984b; Rastogi, Levy-Frebault and David, 1983) or yet another form known as Much's granules (Much, 1907). These granules described by Much in 1907 are filtrable forms of *M. tuberculosis*, which are just visible through the light microscope. They are not classical bacilli.



Much found these granular forms in tuberculous tissues in situations where classical bacilli were difficult to demonstrate such as in tuberculous abscesses. They were not properly acid fast however, some of the bigger granules were acid fast. When injected into animals, they did not give rise to typical tuberculous disease, but if tissues from the first animal were injected into a second, the second animal developed tuberculous lesions with classical tubercle bacilli.

Stanford (1987) points out that Brieger, 1953; Csillag, 1964 and Khomenko, 1987, probably describe observation of Much's granules. Khomenko and Golyshevskaya (1984) in Moscow describe in detail filtrable forms of *M. tuberculosis* which on repeated biological passages in guinea pigs resulted in typical bacterial forms. These ultra-fine filtrable forms were detected in increasing numbers in patients who had been on triple anti-tuberculosis chemotherapy for open-cavitatory lung disease, the numbers of the ultra-fine forms increasing with the duration of therapy (Khomenko, 1987). By the third month of treatment, 82% of 130 patients had these forms, despite rapid disappearance of *M. tuberculosis* in sputum. However, the cavities

still remained open. Hence, patients who are considered to be bacteriologically negative may in fact be carriers of changed forms which are capable of reverting to classical mycobacteria. These ultrafine forms have also been suggested for *M. leprae*, *M. avium* and *M. paratuberculosis* (Yuschenko, 1970; Potter, 1927; Morin and Valtis, 1927; quoted by Khomenko, 1987), and possibly for the order *Actinomyces*.

Professor Chandrasekhar from Delhi, India also suggests that cell wall deficient forms of tubercle bacilli contribute to microbial persistence in latent infections (Chandrasekhar, 1978). Guinea pigs injected with spheroplasts (obtained using cycloserine treatment) of *M. tuberculosis* did not develop tuberculin hypersensitivity or pathological changes. However, reversion of the spheroplasts to the bacillary state resulted in initiation of infection (Ratnam and Chandrasekhar, 1976). Spheroplast reversion was more likely when the duration of stay within the host was long.

It is possible that these forms could not only explain the failure to control tuberculosis worldwide due to bacterial persisters causing relapse or re-infection, but could also be applicable to other mycobacterioses, in particular leprosy. Chatterjee (1983) claims growth of *M. leprae* in a redox system using biological reductants in the liquid medium and describes the appearance of non-acid fast coccoids and bacilli as part of the initial replication process. Stanford postulated a mycobacterial life cycle including bacilli, granules and globi from which smaller pleomorphic forms could arise (Stanford *et el*, 1987).

This could be one of the possible explanations for the presence of the pleomorphic acid fast forms isolated so far from inflammatory bowel disease tissue. It could be possible for them to revert to classical bacilli occassionally.

PURPOSE OF PRESENT RESEARCH

In 1976, Dr Burnham and Professor J E Lennard-Jones from St Mark's Hospital and Dr J L Stanford from the Middlesex Hospital, London, decided to try and isolate mycobacteria from the mesenteric lymph nodes of patients with CD.

Apart from Crohn (1932) and Van Patter (1952), this was the next concerted effort in a search to associate mycobacteria and CD. Difficulty in identifying bacteria in tuberculoid leprosy and the known difficulty of culturing the leprosy bacillus encouraged this fresh approach to Crohn's disease.

They were the first group to report the growth of pleomorphic acid fast organisms (PAFOS) in several CD cultures in 1978. Dr R G Bird performed electron microscopy on these organisms and reported probable cell-wall deficient organisms, some of which appeared to be in association with particles of cell wall. Burnham *et al* (1978) described the PAFOS in 22/27 CD cultures, 7/13 UC cultures and 1/11 control cultures. The culture medium was RCM broth (initially used in the study to detect contaminants). They also described growth on Lowenstein-Jensen medium and Sauton media made hypertonic with sodium chloride. Growth occured as a fine surface growth after 2-6 months incubation. Lowenstein-Jensen cultures with ileal extracts did not improve growth any further. Occasional acid fast bacilli were seen on Ziehl-Neelsen staining. However more numerous were small PAFOs which stained poorly with Gram stain and appeared mycoplasma-like when stained with Giemsa.

Elliott *et al* (1980) updated Burnham's figures of cultures yielding PAFOs, to 42/76 nodes from patients with Crohn's disease, 14/27 nodes from patients with UC and 3/41 control nodes. By Fishers exact test, the CD and UC isolation figures ar highly significant compared to the controls (p<0.00001 and p<0.0001 for CD and UC respectively).

Subculture of these organisms was possible in some cases.

Dr Susan White in her PhD thesis (1981) noted that a significant number of IBD patients (12/76 CD patients, 2/27 UC patients and 3/41 control cultures) grew Gram positive coryneform-like organisms from the same long term cultures which contained acid fast material. She thoroughly investigated these coryneforms and divided them into two groups by primary chromatographic cell wall analysis. One group was probably the genus Propionibacterium and did not possess mycolic acids and the other group did possess mycolic acids. The second group fell into the Corynebacterium/Mycobacterium axis. She concluded that the coryneform organisms were connected with or were another form of the acid fast material; that the organisms assume an unusual, possibly cell wall deficient form in the lymph nodes, and become cultivable generating acid fast material at the surface of the medium under culture conditions used. At a late stage in culture these would develop into gram positive coryneform organisms.

This idea was not altogether accepted as coryneforms are well known contaminants of skin and the GI tract and this may have explained her findings.White performed cell wall and mycolic acid analysis only on coryneform isolates. For the purpose of this study, these organisms were considered to be likely contaminants and cultures growing coryneforms were discarded (Chapter 3).

Many questions remained unanswered. Were the PAFOs truly cell wall deficient bacteria? Were they secondary invaders through diseased intestine or could they be reproducibly isolated only from Crohn's disease tissue? Could the slow growth rate be improved? How significant were the occassional mycobacterial isolates from CD tissue reported by other investigators compared to the acid fast forms

reported? Were the acid fast forms pathogenic and could lesions be induced to resemble CD lesions? Was there a suitable animal model that could be used?

Stanford (1980) suggested that it was quite within the theorectical capabilities of such an organism (PAFOs) to induce the basic pathological changes of CD.

Further progress with the PAFOs did not prove possible at the time (early 1980's) but all cultures were retained in the hope that technical advances would permit progress at a later date. As part of the work for this thesis, the pleomorphic forms in these cultures were passaged and maintained in culture.

Since November 1984, a fresh set of mesenteric lymph node cultures were collected from patients with CD, UC and controls (patients without IBD). Some of the lymph node preparations were used for the production of monoclonal antibodies from hybridomas generated from activated B cells in the lymph nodes of the IBD patients. Chao et al (1988) tried to determine the specificity of antibodies secreted by the hybridomas for binding to food antigens, human gut and bacteria. They concluded that even this analysis of the antibody response of CD and UC patients at the clonal level failed to point to a particular genus. A high percentage of hybridomas did secrete antibodies which bound to mycobacteria and two of the CD derived PAFOs, (hence the data is compatible with a mycobacterial cause) but binding to E. coli was more frequent. Autoantibodies and antibodies to food antigens were rare. Apart from use in this study, lymph nodes were cultured as described in Chapter 3.

The purpose of my research was in the first instance to confirm the findings of Burnham et al, 1978, that PAFOs could be cultured from a significant proportion of CD and UC mesenteric lymph nodes. Not only

was verification of these findings important, but fresh (new) isolates were needed. Only mesenteric lymph nodes were cultured as the inevitable isolation of bowel flora from bowel tissue could complicate the interpretation of results. Other research workers world wide have all used bowel resection or biopsy specimens and have used various decontamination procedures in order to culture mycobacteria. However, in this study, the decontamination procedure was to be specifically omitted in case it inhibited a fastidious organism or a cell-wall defective one. Also if an organism was isolated from a mesenteric lymph node, it means this organism was actively taken up into the lymph node and its isolation would probably be more significant than its isolation from the gut mucosa.

The PAFOs isolated in 1978 had been maintained for several years in culture. Several hundred cultures were available. In order to improve growth rate, if cultures were selected at random, then the possibility remained that I would be attempting to improve culture techniques on a variety of organisms and no clear answer would be obtained. If a group of homogeneous organisms could be found, then attempts at improving growth rate could be focussed down to this group.

First it was essential to determine if all these PAFOs were similar to each other or not. The high isolation rate of these organisms from UC tissue as compared to other research groups (Table 2.1) was intriguing and it was important to determine if the CD and UC isolates were homogeneous. Also, were these organisms different to those PAFOs isolated from non-IBD tissues? Burnham (1980) and White (1981) had not been able to characterise the organisms mainly because they were present in such small amounts, even after months in culture.

Burnham in his thesis considered any culture bottle which was Ziehl Neelsen positive to be a "positive" culture, but could not obtain

enough growth to perform mycolic acid analysis. Only electron microscopy was performed on these isolates (see Chapter 3, Section D). Hence, it was decided to try and characterise the organisms.

If acid fast isolates worldwide are to be compared, each research group must identify their organisms as far as possible to determine if a consistent organism is being isolated. Comparison of biochemical and cell wall characteristics (if a cell wall is present) would be useful, and genomic analysis would be ideal as it is so definitive.

Since the organisms were acid fast or partially acid fast it seemed likely that they belonged to the *Corynebacterium-Rhodococcus-Nocardia-Mycobacterium* axis. Attempts were made to support this theory, and especially the possibility that the organisms were mycobacteria were considered. The role of the PAFOs in Crohn's disease and indeed in UC would then have to be assessed. A suitable animal model would need to be found if Koch's postulates were to be fulfilled.

The following factors were analysed by Burnham in his MD thesis, and no significant difference was found in the PAFO positve culture group as compared to the negative culture group when considering

- age and sex distribution of patients
- any clinical features of the lymph node donors
- length of disease history
- cultures obtained from lymph nodes draining macroscopically diseased as opposed to non-diseased bowel.
- drugs of any kind taken by patients at the time of operation (Prednisolone, Salazopyrine, Azathioprine, Septrin, Metronidazole, Gentamicin, combinations of any of these)
- type of operation (hemicolectomy, small bowel resection, anastomotic resection, laparotomy, proctocolectomy). Although more colonic operations are performed in UC compared to CD, no apparent differences emerged.

Burnham attempted to relate the presence of granulomata in surgical specimens to culture results. In leprosy, there is an inverse correlation between bacillary numbers and numbers of tissue granulomata. This was not found to be the case in for CD. However, it was noted that if a lot of growth for PAFOs was obtained (2+ or more on a O-4+ growth assessment scale), less than 3 granulomas only were noted per tissue section.

No attempt was made to analyse any of the above factors in this thesis.

White (PhD thesis, 1981) cultured serum samples from CD patients and healthy controls for cell wall defective or other organisms but obtained no growth from any of these. This was not attempted by the author.

In summary, the aim of the work was to determine if PAFOs isolated from IBD tissue could be included within the genus *Mycobacterium* and also determine if the organisms belonged to a homogeneous group. It was especially relevant to know if the CD, UC and control isolates were similar. Finally, it was hoped that a small homogenous group of isolates could be chosen and then attempts made to satisfy Koch's postulates.

CHAPTER THREE

SECTION A

BACTERIOLGICAL CULTURE OF PLEOMORPHIC ACID FAST ORGANISMS FROM PATIENTS WITH INFLAMMATORY BOWEL DISEASE

Al. BACTERIOLOGICAL CULTURE OF MESENTERIC LYMPH NODES OBTAINED FROM PATIENTS WITH CROHN'S DISEASE, ULCERATIVE COLITIS AND OTHER BOWEL DISEASES

INTRODUCTION

Mesenteric lymph nodes were used in this study to try and reproduce the work of Burnham *et al.*, 1978, in attempting to grow acid fast organisms from cases of CD and UC.

Mesenteric lymph nodes often show histological abnormalities and granulomas. They would be less likely to be contaminated with bowel flora. Also abnormalities of the intestinal lymphatic system are often seen early in CD. For these reasons, only mesenteric lymph nodes were cultured. Intestinal tissue was not used. Burnham had initially used Robertson's cooked meat (RCM) broth to detect anaerobic contaminants. However, the PAFOS were demonstrated in this medium with Lowenstein-Jensen being the next most useful medium. Hence both these media were used, with others, for primary culture of lymph node specimens.

MATERIALS AND METHODS

All mesenteric lymph nodes were obtained at laparotomy. There were no ethical considerations in this study, as nodes were only taken from specimens necessarily removed at surgery. Most lymph nodes were obtained from St Mark's Hospital, London, but a few were from the Middlesex Hospital, St Bartholomew's Hospital and St George's Hospital.

Nodes were obtained from patients with CD, UC and other bowel diseases (control nodes). Control nodes were from patients with familial polyposis coli, radiation enteritis, carcinoma of the bowel, idiopathic megacolon, rectal prolapse, constipation, volvulus and diverticular disease. The ages of the control group were on average higher than in the IBD group, an inevitable consequence of the nature of diseases and operations from which control nodes could be obtained.

Samples from 6.11.84 (initiation of this study) to 4.12.85 were collected and cultured by Miss Caroline Parmiter. Samples from 4.12.85 to 1.8.86 were collected and cultured by Mr Robert Anderson. From 1.8.86, samples were collected and cultured by the author.

All intestinal resection specimens were placed in a sterile pot in theatre and were collected personally at the time of surgery by the author or Miss Parmiter or Mr. Anderson to ensure aseptic handling of the resection specimen. Lymph nodes were then dissected from the rest of the bowel specimen using a sterile scalpel and forceps. Where possible more than one lymph node was taken. The nodes were immediately transported in a sterile container to the laboratory and cultured. If immediate culture was not possible (only a small proportion of samples), the nodes were stored at 4°C.

Once in the laboratory, work was done in a Category 1 safety cabinet. The lymph node(s) was placed in a sterile petri dish using sterile forceps. Great care was taken to ensure asepsis. The node was cut in half using a sterile scalpel and scrapings taken from the middle of the lymph node under aseptic conditions. The scrapings were inoculated into a large Robertson's cooked meat (RCM) broth bottle (20ml glass universal), a small (5ml bijou bottle) RCM bottle, and a Lowenstein-Jensen (LJ) slope. Herrold's egg yolk medium (HEYM) with mycobactin J at 3µg/ml and Sauton's medium solidified with agar was

sometimes used for primary inoculation (see Table 3.A.2). If more than one lymph node was obtained, each node was inoculated separately. If the lymph nodes were very small, only RCM bottles and LJ slopes were inoculated. Each node had an average of two RCM bottles inoculated. Each node had an average of two RCM bottles (15 patients) and some patients had more than one node inoculated (6 patients). Each node was inoculated onto one LJ slope but 6 nodes were inoculated onto two LJ slopes. Herrold's egg yolk medium with mycobactin and Sauton's media were used as primary inoculation media after 1.8.86. Also rub plates on blood agar were used after 1.8.86 to detect any contamination by bowel flora. In all,74 patients had lymph nodes cultured for this study. All bottle caps were shut to fingertightness.Cultures were incubated at 32°C in a warm room.

A.2 CULTURE REVIEW FOR PAFOS

METHODS

Each inoculated bottle or slope was examined weekly for one month and monthly thereafter. Due to the large number of cultures(>200 in total), some could only be examined three monthly.

The bacteriology of the cultures was conducted in several stages. First, the aim was to select cultures that were Ziehl Neelsen(ZN) positive (ie, this indicates that the PAFOs were present). The second aim was to obtain cultures that had the PAFOs in pure culture.

The third aim of the bacteriological work entailed subculturing all positive cultures to prove they were viable and to maintain the organisms. This procedure was carried out for both the new set of lymph node cultures and the 1976 culture series (see Section B).

All cultures were examined by the author. Each culture was numbered and at the time of examintion, the author had no knowledge of whether the lymph node inoculated into a particular bottle originated from a CD, UC or control patient. ZN and Gram staining was done and
assessed by the author. For all cultures, whether RCM or LJ, each Gram stain or ZN stain was performed on a separate glass slide.

Fifteen ZN stains were also coded by number and given to Dr J L Stanford to be assessed at various times during the study.

A culture was termed "positive" if there was a visible surface growth in RCM medium (see Fig 3.A.1) or if there was slight granulation or an iridescent sheen on LJ medium, and this was ZN positive (Fig 3.A.3).

Surface growth in RCM was visible by eye, but LJ slopes were examined using the dissecting microscope unless granulation was clearly visible to the naked eye. Colonies causing the granulation appearance were colourless and dome shaped. Hence, if visible growth was observed on RCM, or on the LJ slope, or HEYM (colonies on HEYM gave a similar appearance to those on LJ and cultures on Sauton's medium were never positive), then Gram and ZN stains were performed.

A.3 SELECTION OF CULTURES FOR FURTHER WORK

Selection of cultures for further work had to be done meticulously as it was vital to choose cultures with a pure growth of PAFOs. If contaminants were not screened out, any DNA detected might be due to them. Probes may hybridise to contaminant DNA and any protein patterns detected may be mixtures of patterns of PAFOs and contaminants, a situation I wanted to avoid.

Any sample which yielded gut flora on the rub plate or appeared contaminated in <u>all</u> bottles inoculated within one week was not used for any further investigation as the original lymph node must have been contaminated. Obvious heavy growth, due to contaminants was easily noticed. However contamination of RCM broth may not be readily visible, or may be difficult to assess by the naked eye; thus a contaminant

screen was necessary for every culture bottle which was not grossly contaminated.

A contamination screen consisted of subculture of each bottle using a 10µl loopful onto

1. Blood agar incubated aerobically for one week

2. Blood agar incubated anaerobically for one week

3. Chocolate agar incubated in CO₂ for 5 days

4. Mycoplasma medium (2-5 days)

5. Sabouraud's slopes kept up for 3 weeks.

The Gram stain was used to assess the presence of contaminants and/or PAFOs.

All those RCM bottles screened by this method which gave no growth on the above and whose surface growth was ZN positive were suitable for further investigation (eg by electrom microscopy, DNA analysis etc).

LOWENSTEIN-JENSEN (LJ) SLOPES

LJ slopes, if grossly contaminated, were usually obvious since the slopes turned yellow, disintegrated, or produced large amounts of bacterial growth of non acid-fast organisms. The PAFOs never gave this appearance and were just visible to the naked eye as granulation or were only visible under the dissecting microscope. Hence, the amount of subculturing onto blood agar plates etc from LJ slopes was minimal.

All contaminants were identified, in case a pattern emerged where for example, the same organism was repeatedly cultured.Great care was taken to isolate anaerobic contaminants (such as *Propionibacterium* sp.) and the Coryneform group organisms. This group of organisms in particular could have a Gram stain appearance that could be confused with that of the PAFOs but would be readily differentiated by growth on blood agar (PAFOs would not grow on blood agar). Some of the cultures were incubating for between two and three years and contaminants, if

present, may not have grown in 48 hours. Hence, extended incubation periods for up to 7 days were used.

Contaminants were identified by Gram stain and tests suitable to the organism, such as catalase, coagulase, carbohydrate fermentation, API strips, urease or other suitable biochemical tests.

Cultures that appeared "negative" (ie no surface growth in RCM, no granulation on Lowenstein-Jensen) were left undisturbed even for several (6-8) months. The cultures were opened only when used for DNA analysis, electron microscopy, TSA analysis or whichever investigation was required. If a "negative" (negative for PAFOs), culture was used for further investigation, the culture was screened for contaminants as for a "positive" culture. Also, a Gram stain and ZN stain was done.

SUBCULTURE

Some of the surface growth and some broth (usually a 10µl loopful) was subcultured to fresh RCM broth (one large and one small bottle), a Sauton's slope and an LJ slope to make sure the organisms could be passaged and to maintain fresh cultures. The subcultures were incubated at 32°C for long periods and examined at weekly intervals for the first month and then monthly thereafter.

In order to avoid opening the RCM culture bottles on several occasions which would increase the contamination rate (a well known problem especially with broth cultures), all the above were done on the same occasion. The organisms were not easy to pick out with the plastic bacteriological loop and a nichrome loop was better for this purpose.

If no growth was seen on an LJ slope, then no subculture was performed, but the surface was gently scraped and emulsified in a small amount of saline on glass slides, and a Gram stain and ZN stain was performed.

MYCOBACTIN

8 RCM cultures positive for PAFO growth were subcultured to 16 RCM bottles with added mycobactin $(3\mu g/ml)$ to observe if mycobactin improved PAFO growth.

By the end of the study, at least one culture (usually several cultures) per patient have been examined. Certainly every large RCM bottle was examined unless obviously contaminated when an alternate bottle was used. Each and every node from all patients was subjected to the above procedures. The following terminology has been used throughout this chapter and thesis.

Positive culture = RCM or other culture positive for growth of PAFOs Negative culture = RCM or other culture negative for growth of PAFOs Control node = Node obtained from a patient with neither CD or UC (control culture) (non IBD cultures) Positive control = Standard mycobacteria, eg M. kansasii, M. smegmatis grown in RCM medium at 32°C Negative control = Uninoculated RCM bottle at 32°C for the same length (uninoculated control) of time as the CD, UC and non IBD cultures.

RESULTS

PRESENT CULTURE SERIES

SECTION A.1 AND A.3

In all, 74 patients had mesenteric lymph nodes cultured in RCM medium and LJ medium and several were cultured on HEYM and Sauton's medium as well.

Of these, 17 cultures were considered unsuitable for further investigation due to contamination of all media with intestinal or skin bacteria.

Table 3.A.1

	CD	NO OF UC	PATIENTS Non-IBD Controls	Total
Number of lymph node donors	35	20	19	74
Contamination detected in all media/rub plate	10	5	2	17
No of patients from whom lymph node cultures could be investigated further	25	15	17	57

No one organism was cultured. The range of organisms cultured included Escherichia coli, Proteus mirabilis, Clostridium species, (mainly C.perfringens), Bacteroides fragilis, Klebsiella sp., Micrococcus, Enterococcus sp., Lactobacillus sp.,Staphylococcus epidermidis, Diptheroids, Propionibacterium sp., Candida sp. and Torulopsis glabrata.

Even if no growth was obtained, but the Gram stain of the culture showed Gram positive cocci, Gram negative rods etc, or was in any way inconsistent with the presence of PAFOs in pure culture, then that culture was not used for further work.

A few of the subcultures were inadvertently contaminated - an inevitable problem associated with handling broth cultures. These were not used for further study.

DISCUSSION

Contamination by intestinal bacteria was a predicted problem despite the use of mesenteric lymph nodes. Ambrose *et al* (1984) isolated intestinal bacteria from 33% of 46 CD mesenteric lymph nodes and 5% of 43 non Crohn's nodes. In the present study, 29% of mesenteric lymph nodes (10/35) from CD patients were found to be contaminated, as were 25% of UC patient nodes (5/20) and 11% of non-

IBD nodes (2/19). This suggests that bacteria do leak from the small bowel lumen in IBD.

A few of the cultures were not contaminated with intestinal flora, but with diptheroid skin flora and this could have occurred at the time of surgery, or at the time of resection and culture of the lymph node specimen. *Corynebacterium* species could have originated from the intestine or from hands but were not considered significant in either case (contrary to Sue White's PhD thesis, 1981). Of 74 patients who were lymph node donors, cultures from 57 were chosen for further investigation. At least one of the cultures of the set inoculated per patient in this group of 57 were not contaminated by either intestinal bacteria or ordinary skin flora and could therefore be used for further analyses.

SECTION A2

PRIMARY ISOLATION OF PAFOS FROM ROM CULTURES

Culture review: A total of 175 cultures from 57 patients were reviewed. Growth was observed 3-8 months after inoculation of the lymph node scrapings. If the bottles were left untouched, growth continued to increase slowly. Hence, cultures were not always stained by ZN, subcultured and checked for contamination as soon as growth was observed. They were left for a further 3-6 months enabling growth to continue, so that enough bacterial cells would be present for them to be characterised. The lymph node samples were collected over a period of more than two years, hence cultures were staggered in time and work was not held up whilst waiting for more growth to occur.

The time noted for evolution of positive growth is not necessarily accurate as some cultures were reviewed only after three months of incubation. However, none of the cultures reviewed monthly showed any growth before three months and this was considered the minimum time for positive growth to be noted in RCM medium.

Successful isolation of the PAFOs, using RCM as the primary isolation medium was achieved in 17/25 CD patients, 11/15 UC patients and 3/17 controls (Table 3.A.2 and 3.A.3). The isolation rate from CD and UC are highly significant compared to the controls. The actual culture numbers rather than numbers of patients from whom PAFOs were isolated is shown in Table 3.A.3. It is seen that duplicate positive cultures from each patient were avaiable in most cases.

Of the 15 Ziehl-Neelsen stains examined by Dr J L Stanford, those scored as positive were also scored as positive by the author, indicating consistency in slide assessment.

GRAM STAIN APPEARANCE

On Gram staining, the appearance of the organisms was variable. Usually they stained very poorly, others were faintly Gram positive.

PAFO isolation in the present series is compared with the old series (updated figures, Elliot et al, 1980 in Table 3.A.4). The results from control nodes in the present series appears high (18%, Table 3.A.4) due to the small number of nodes cultured. If this figure is assessed in comparison with the total number of cultures set up from control nodes (Table 3.A.3), only 6/47 control cultures are positive for PAFO growth. Results are seen to be comparable between the present and the old series, although the isolation rate in the present series from CD, UC and non-IBD cultures was higher than the old series.

PRIMARY ISOLATION OF PAFOS FROM LJ MEDIUM

If slight granulation was observed on the surface of LJ medium, ZN staining was performed. However, most slopes needed to be checked under the dissecting microscope before growth could be seen. 7/25 CD cultures, 4/15 UC cultures and 1/17 non-IBD controls grew PAFOs (Table 3.A.2). All cultures that were positive on LJ were also positive in

RCM. Cultures were positive on LJ media only at approximately 10 months incubation (range was 9-12 months), and only very little growth was observed on the surface on the LJ slope.

OTHER MEDIA FOR PRIMARY ISOLATION

Two CD lymph nodes did yield PAFOs on Herrold's egg yolk medium with mycobactin at 7 and 11 months-also noticed in RCM at 7&5months. Sauton's slopes were of no use for primary isolation of the organisms (Table 3.A.2).

RESULTS OF SUBCULTURE

All cultures with PAFOs were subcultured. By this stage, all those with contaminant bacteria had been discarded. The results of subculture are shown in Table 3.A.5. Subculture from RCM was always more successful than subculture from LJ medium partly because the amount of growth on LJ was so small. Also subculture from RCM to LJ was not very successful - subculture was successful in only 3/17 cases to LJ(CD) and took more than 4 months to become positive. With RCM however, 15/17 CD cultures with the PAFOs could be passaged without any difficulty. Subcultures were positive by three months. In UC cases, 9/11 cultures were passaged and 3/3 control (non IBD) cultures were passaged. The figures represent PAFOs cultured per numbers of patients. In actual fact as each subculture was passaged to two Robertson's cooked meat broth bottles, it meant that at least four cultures were available after each subculture.

Passage from either RCM or LJ to Sauton's medium was unsuccessful.

SECOND PASSAGE

Due to successful subculture being obtained mainly in RCM medium, second passage was attempted only from RCM to RCM cultures. 24/27 cultures subcultured (14 CD, 8 UC and 2 non IBD controls) grew PAFOs within 2-3 months of subculture.

No differences were noticed either in growth rate or amount of growth in large or small RCM bottles. However, due to the larger volume of material, the large RCM bottle was expected to have more organisms in total.

MYCOBACTIN DEPENDENCY

Two cultures yielded PAFOs on HEYM with mycobactin. However they were not mycobactin dependant as they grew also in RCM (without added mycobactin). Further they were subcultured onto RCM & HEYM without mycobactin and took 2 months and 4.5 months (1 culture) and 2.5 months and 6 months (2nd culture) to grow on RCM & HEYM respectively.

8 RCM cultures were subcultured to 16 RCM bottles with added mycobactin. Growth was observed at the usual time. No enhanced growth was observed.

DISCUSSION

No whole mycobacterium was isolated from any of the lymph nodes cultured. However, a large percentage of RCM cultures showed a surface growth at 3-8 months which was ZN positive. Subcultures took 2 or more months to grow. A much smaller percentage of LJ slopes were positive at 9-12 months. Subcultures took 4 months to grow. RCM medium at 32°C proved to be the best medium overall.

The isolation of PAFOs from these mesenteric lymph node cultures confirmed previous observations by Burnham et al in 1978. In the present series, 17/25 CD nodes and 11/15 UC nodes grew PAFOs compared to 3/17 control nodes. These isolates could be passaged in vitro. All

organisms could be grown on mycobactin free media. The 2 cultures that grew in HEYM with mycobactin grew later in mycobactin free media. Hence these organisms were unlikely to be M. paratuberculosis. This was later confirmed by genomic analysis (Chapter 5).

In conclusion, PAFOs were isolated from a high proportion of IBD tissue compared to controls. These organisms were shown to be viable as re-isolation was possible by subculture. Pure cultures free of bacterial contaminants were chosen for DNA analysis (Chapters 4 and 5) and other studies (Chapters 6 and 7).

ISOLATION OF PAFOS FROM ULCERATIVE COLITIS

The finding of a high proportion of positive cultures in this group raises several points. The observation is consistent with previous evidence for a transmissible agent in UC as well as CD (Cave, Mitchell and Brooke, 1975). It could be postulated that the difference in the pathology of UC and CD could be due to a different host reaction to the same agent. Assuming that the UC isolates are the same as the CD isolates may not be correct. For this reason alone, comparing the characteristics of the CD and UC isolates was important.



Bottle 1

2

1957

4

3

Fig 3.A.1

Appearance in RCM culture of PAFOs (Bottle 2). Note surface growth. This is Ziehl-Neelsen positive when stained. All cultures incubated at 32°C.

- Bottle 1 Four week old culture of M. kansasii.
- Bottle 2 Growth of the PAFOs from a CD lymph node after 18 months in culture.
- Bottle 3 Uninoculated RCM bottle incubated at 32°C for 18 months (No surface growth)

Bottle 4 Four week old culture of M. smegmatis

<u>Note:</u> There is less growth after 18 months in culture of CD-tissue derived organism, compared with 1 month old cultures of M. *kansasii* or M. *smegmatis*.

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Fig 3.A.2

Ziehl-Neelsen stain of some broth from bottle 3 in Fig 3.A.1. (Uninoculated RCM culture at 32°C) . ZN stain is negative.

This is to be contrasted with Fig 3.A.3 a-d.

Fig 3.A.3 a, b, c, d

Representative examples of PAFOs (Ziehl-Neelsen stain). Note pleomorphic and variable appearances. Sometimes occassional acid fast bacilli were present.



Fig. 3.A.3.a Appearance after 9 months in RCM broth culture: CD culture



Fig 3.A.3 b UC culture showing pleomorphic forms

<u>Fig 3.A.3.c</u> ZN stain showing PAFOs - appearance after several months in RCM : CD culture



Fig 3.A.3.d ZN stain of organisms from LJ slope (CD patient D.H.) Organisms failed to grow for further identification.

TABLE 3.A.2

Group	Total	RCM		LJ		HEYM with	nycobactin	Sauton's	
	No of LNs in study	No cultured	No positive	No cultured	No positive	No cultured	No positive	No cultured	No positive
CD	25	25	17	25	7	5	2	4	0
0C	15	15	11	15	4	2	0	2	0
Controls	17	17	3	17	1	2	0	2	0

Cultures Positive for PAFOs on Primary Isolation on Various Media

Table 3.A.3

PAFO isolation in RCM cultures (present series)

	Positive Cultures*/ No of Patients	Positive Cultures*/ No of Cultures Studied
CD	17/25	44/61
UC	11/15	47/67
Control (non IBD)	3/17	6/47

*A positive culture is one where visible growth was obtained in RCM medium and this was Ziehl-Neelsen positive.

Table 3.A.4

PAFO isolation rate in RCM: Comparison of old and new series

 Positive Cultures*/No of Patients

 1976 Series
 New Series

 CD
 42/76 (55%) p<0.00001</td>
 17/25 (68%) p<0.002</td>

 UC
 14/27 (52%) p<0.0001</td>
 11/15 (73%) p=0.002

 Control
 3/41 (7%)
 3/17 (18%)

P values (Fisher's exact test) are in comparison to the respective control node isolation rates.

*A positive culture is one where visible growth was obtained in RCM medium and this was Ziehl-Neelsen positive.

Table 3.A.5

Results of subculture of positive cultures from RCM and LJ

	No Positive RCM cultures	Positive cultures on Subculture from RCM to		No Positive	Positive cultures on Subculture from LJ to			
		RCM	LJ	Sautons	LJ culures	RCM	LJ	Sautons
Crohn's Disease	17	15	3	0	7	3	2	0
Olcerative Colitis	11	9	3	0	4	1	0	0
Controls	3	3	1	0	1	0	0	0

SECTION B : 1976 SERIES OF CULTURES

INTRODUCTION

The purpose of analysing this set of cultures was not to determine numbers of positive cultures (for PAFOS) for CD, UC and controls. This had already been published by Burnham $et \ al$ (1978).

These long term cultures needed to be screened as for the new sets of cultures and bottles with a pure growth of PAFO identified. These PAFOs needed to be subcultured as before to obtain more growth for investigation. The primary aim of identifying the RCM bottles that contained the PAFO from this series was to compare the characteristics of these organisms to those of the new series. Also as these cultures were over ten years old, viability studies needed to be done.

MATERIALS AND METHODS

The mesenteric lymph node cultures originally collected by Burnham et al. and White were stored as long term cultures in the warm room at 32°C. Even though 362 cultures from 73 patients were available for investigation, it was impossible for all to be analysed. Already, 175 newly inoculated cultures from the 1984 series were being investigated. 136 of the 362 cultures (at least one culture/patient) were studied. 11 addition, extra cultures were chosen, based on the appearance of good surface growth in RCM. Most LJ cultures were either dry or extremely old and were not studied. Also, previous work by Burnham and White with this culture series had shown that their RCM cultures gave better growth than LJ cultures. However, 10 LJ slopes were examined. Each culture was subcultured as described in Section A so fresh cultures were available. A full contaminant screen, Gram stain and ZN stain were performed (on cultures and subcultures) as described.

RESULTS

RCM CULTURES

Results are shown in Table 3.B.1 for RCM cultures. 16 cultures from 10 CD patients, 5 cultures from 4 UC patients and 6 cultures from 6 control (non IBD) patients were found to have contaminants and excluded from the study. ZN stains were done on the rest of the cultures and those that were positive were used for further investigation (cultures from 22 CD nodes, 6 UC nodes and 2 control nodes).

	No Available for Study		Number	used	No of nodes investigated	Number	ZN Positive
	No. of Nodes	No. of Cultures	No. of Nodes	No. of Cultures	(Other cultures excluded due to bacterial contamination)	Nodes	
CD	43	247	43	77	33	22	
0C	13	72	13	29	9	6	
Controls (non-IBD)	17	43	17	30	11	2	

Table 3.B.1 - 1976 SERIES

LJ Slopes

ZN staining was negative in 7 of 10 (4 CD, 4 UC and 2 non-IBD cultures) LJ slopes examined. One LJ slope from a CD patient showed a good growth of acid fast organisms which was identified as *Nocardia asteroides*. These organisms grew on subculture within one week and were not confused with the PAFOs which have quite a different ZN appearance and growth rate.

DISCUSSION

The organisms from the 1976 culture series were bacteriologically identical to those isolated from the present series. Growth was observed best in RCM cultures incubated at 32°C. Mycobactin had not been added to any of the cultures. Lowenstein-Jensen proved not to be very useful in the culture of these acid fast organisms, although poor growth was obtained on some slopes.

The viability of the organisms was proved by ability to re-isolate them on subculture to fresh media. Surprisingly cultures which had been incubated for 7-10 years were viable. The time taken for regrowth was shortened to 2-4 months (compared to 3-8 months or more for primary isolation), however, growth still occurred in extremely small quantities. Subcultures were then available not only as a test of viability of the organisms, but to serve as a reserve bank of organisms to be used for characterisation.

SECTION C

MYCOBACTERIA GROWN IN ROM BROTH ("MYCOBACTERIAL CONTROLS")

In addition to inoculation of RCM cultures with lymph nodes from patients with and without IBD, several species of known mycobacteria were inoculated into RCM broth cultures. This should help establish whether normal mycobacteria would grow in RCM. If they did grow in RCM then these cultures could be used as positive controls.

If PAFOs cultured on LJ medium were used in any characterisation experiment, then normal mycobacteria grown on LJ would be used for comparison.

METHODS

When the PAFOs were subcultured, a $10 \,\mu$ l loop was used to transfer growth into fresh RCM cultures. Hence a $10 \,\mu$ l loopful of growth of the following mycobacterial species grown on LJ slopes was transferred into two large and two small RCM broth cultures.

M. smegmatis (NCTC 333), M. chelonei (ATCC 19977), M. avium (NCTC 8559) M. fortuitum (NCTC 8573), M. kansasii (Clinical Isolate K1386), M. tuberculosis (Clinical Isolate).

These cultures were examined every fortnight for one month and monthly thereafter. At one, three and six months post inoculation, ZN stains were performed to check the morphology of the organisms and subcultures were made on LJ and ROM medium. Subcultures were performed at 3-6 month intervals, so that if for example a 6 month old culture from a CD lymph node was investigated, then a 6 month old culture of a normal *Mycobacterium* sp. in a ROM culture was available for comparison to act as a positive control. As in all experiments performed with the PAFOs, the bottle caps were shut to finger tightness.

RESULTS and DISCUSSION

A typical surface growth (Fig 3.A.1) was noticed in all RCM bottles by 3 weeks and a large amount of surface growth was obvious at 4 weeks with all the mycobacterial species tested. ZN staining at one month, three and six months post inoculation, showed well stained acid fast organisms. The morphology of the organisms was normal.

Subcultures also grew the mycobacteria well and a profuse growth was noted on subculturing from RCM to Lowenstein-Jensen, indicating that mycobacteria did grow well in RCM cultures.

These RCM cultures would be better controls than mycobacteria grown on LJ medium since they would be growing in the same medium (RCM) as the PAFOS. If there were artefacts or problems associated with the presence of RCM itself in any further analyses, then this should be revealed.

Section C.1

Uninoculated RCM bottles also were incubated at 32°C for variable lengths of time. If an 18 month old CD lymph node culture was used in any investigation, then an 18 month old uninoculated RCM culture was used as the negative control for that investigation. This would enable any problems due to the presence of RCM alone to be identified.

SECTION D: ELECTRON MICROSCOPY

INTRODUCTION

Electron Microscopy (EM) on thin sections of Crohn's disease lymph nodes did not reveal any definite organisms (White 1981).Electron Microscopy using negative staining carried out on some of the PAFO positive RCM cultures by Dr. R.G.Bird (Burnham *et al* 1978) showed isolates to be cell wall defective. Some organisms appeared in association with fragments of cell wall. Hence it was unclear whether the organisms did or did not have a cell wall.

Electron Microscopy (EM) was carried out on the present culture series (and some of the old series) by Dr. R. Dourmashkin at the Middlesex Hospital Medical School. Samples for EM were made available by the author.

MATERIALS AND METHODS

Sixty mesenteric lymph node cultures in RCM were analysed. A sterile sharpened orange stick was used to pick up the surface growth in RCM cultures (the PAFOS). The colony material was fixed at room temperature by gently inserting the orange stick into an Eppendorf containing fixative. 3% glutaraldehyde in Hank's buffered saline solution with added calcium and magnesium ions was used as fixative. After 2-6 hours, the material was washed with several changes of buffer, then fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer pH 7.4 at 0°C for one hour and washed in distilled water.

The material was put in 2% agar (Noble) in water, than dehydrated in acidified 2,2 - methoxypropane and embedded in TAAB EM resin.

Sections were made with a diamond knife and stained briefly with uranyly acetate in 50% alcohol and Reynold's lead citrate and examined in a Phillips EM 200. Magnification was up to x 45,500.

RESULTS

All cultures examined had a cell wall. Unfortunately none of the elctron micrographs were made available by Dr. R. Dourmashkin for inclusion in this thesis.

Some samples did not show any organisms and were probably unsuccessful for technical reasons or because an inadequate sample was taken (in some cases very little material was available for analysis). Several different types of organisms were demonstrated and these were assigned arbitrary numbers. Type 1 were bacillary forms with a thick cell wall and capsule. Type 2 were mycobacteria like bacilli with thin walls (by EM) and vacuoles. Type 3 were unique organisms (bacilli) with extracellular material. Type 4 were bacilli with a cell wall and capsule growing in strings. Type 5 appeared cell wall deficient but turned out to be staphylococci. Types 6 & 7 were with a cell wall and extracellular materix. Dividing forms were also seen.

DISCUSSION

The organisms from the CD, UC & non IBD cultures seem to have cell walls and are certainly pleomorphic. Although EM evidence was presented that some forms were cell wall defective in 1978 (Burnham et al, 1978), this was not found to be the case in the present study.

Mr. G. McIntyre at the Middlesex Hospital prepared cell wall defective forms of mycobacteria (M.Kansasii, M. Smegmatis, M. tuberculosis) and found their EM appearance to be quite different from the disease isolates.

There did not seem to be any relationship between the type of organisms (arbitrary designation) found and a particular ZN appearance or time in culture before sample analysis. CD,UC and non IBD cultures did not associate with a particular morphological type.

In conclusion, the most important information obtained from the EM work was that thin section electron micrographs showed the PAFOs to have a cell wall. This is consistent with the organisms being acid fast, as acid fastness is at least in part a property of the cell wall. (Beaman 1976, Murohashi *et al.*, 1969).

CHAPTER FOUR:

SECTION A: PREPARATION OF DNA BANK I

As the pleomorphic organisms isolated from the long term cultures were acid fast, it seemed likely that the organisms belonged to the *Corynebacterium-Nocardia-Mycobacterium-Rhodococcus* axis. However they grew poorly or did not grow on the usual media used for this group of organisms (blood agar, Lowenstein-Jensen, Sauton's). Characterisation would be helpful in three ways.

- (i) Could the organisms be segregated into clusters of similar strains and perhaps into species? If this could be done, would there be associations between these clusters and disease? Were the organisms derived from patients with CD the same as those from patients with UC? Were the organisms from non-IBD patients the same as those from patients with disease, or only superficially similar? Representatives of defined clusters could then be selected for detailed study.
- (ii) If a better idea was obtained on the taxonomy of these organisms, improved cultural conditions and media could be tried appropriate to the identified genus.
- (iii) The pleomorphic forms could possibly be a generally unrecognised form perhaps of *Mycobacterium* and perhaps analogous to Much's granules (Much, 1907), which could revert to classical bacilli occassionally. The presence of occassional acid-fast bacilli was noted in some of the smears (Chapter 3). Whatever the form of the organism, the best way forward was to analyse them at genomic level.

DNA analysis was to be attempted in two ways.



 A very large DNA bank (labelled DNA bank I) which included the groups of organisms listed below would be probed with cloned probes obtained from the DNA isolated from the pleomorphic forms.

Groups of organisms.

a. Representative strains from the genera

Mycobacterium Nocardia Corynebacterium Rhodococcus and related genera

- b. common gastrointestinal flora, as these could have been present when the culture was originally inoculated
 gastrointestinal pathogens, since newly discovered GI pathogens (Clostridium difficile, Campylobacter jejuni, Helicobacter pylori) have been associated with disease.
- c. Skin contaminants which could have been introduced into the broth culture during initial inoculation or subculture.

The aim was to find out which of the above organisms had DNA most homologous to the DNA isolated from the pleomorphic forms. 2. DNA from the cultured acid fast forms (comprising DNA bank II) would be probed with mycobacterial probes to determine if they had homologous DNA that would hybridise with the probes. The probes would first be characterised against the large DNA bank I prepared. It was hoped that mycobacterial probes would be able to hybridise to as little as long of PAFO DNA despite presence of DNA from either gut or skin flora (hence all these organisms were included in the DNA bank). If the probes were specific for mycobacteria, they could then be used directly on DNA isolated from RCM cultures, whether or not contaminating bacteria were present.

First it was necessary to establish a DNA isolation procedure that would successfully isolate high molecular weight (MW) DNA from diverse bacterial species including mycobacteria and the IBD tissue derived organisms. Different DNA isolation procedures for different bacterial genera would be inconvenient with so many strains. DNA was isolated from :

1. <u>77 diverse bacterial species</u> from 35 genera

2. Mycobacteria:

The isolation of high MW DNA from mycobacteria is difficult because of their impermeable cell walls. Patel, Kvach and Mounts 1986, described a method for the isolation of mycobacterial DNA which yielded high MW DNA suitable for restriction endonuclease analysis and which did not shear DNA as in previously used cell disruption procedures. McFadden *et al* (1987b), used a modification of this procedure when examining Crohn's disease - isolated *M. paratuberculosis* strains.

Further modifications to the procedure described by Patel, Kvach and Mounts were made and applied to all bacterial species tested including mycobacteria and the IBD tissue derived acid-fast forms. The procedure is described below.

3. The isolation of DNA from IBD-tissue derived pleomorphic forms. The nature of the cell wall of these organisms was not known, but a procedure which could extract mycobacterial DNA would be expected to be successful for these organisms.However, the main problem anticipated was the extraction of DNA from very small quantities of bacterial cells. At most the IBD tissue derived organisms were present in microgramme quantities. Hence, apart from extracting DNA from the surface growth in Robertson's cooked meat medium (RCM), it was decided to extract DNA from the RCM broth, as the organisms would also be present in the broth.

It was also necessary to establish that meat-derived DNA in RCM would not interfere with subsequent DNA analaysis of the organisms.

The rest of this chapter is divided into two sections:-

- a. Extraction of DNA from conventional bacterial species including mycobacteria.
- b. DNA extraction from the IBD tissue derived organisms.

MATERIALS AND METHODS

a. Genomic DNA extraction was done on the following groups of organisms. Groups of Organisms and Original Sources

Representative mycobacterial strains

Apart from those mycobacterial strains listed in Table 4.A.1. the following were also used.

Mycobacterium species	Original Source: -
M. chelonei	ATCC 19977
M. haemophilum	Clinical isolate(C.I.)

DNA from M. bovis, M. tuberculosis, M. vaccae and M. phlei (NCTC 8157) were gifts from Dr M Moss, DNA from M. simiae was a gift from Mr S Hampson, and DNA from *H. leprae* (100ng) was a gift from Dr J McFadden, all from St George's Hospital Medical School.

Bacterial strains from genera related to mycobacteria

See Table 4.A.1 for Mocardia, Rhodococcus, Propionibacterium

Cellulomonas, and Rothia sp.

Corynebacterium species

1. Corynebacterium JH	NCTC 11913
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- 2. Corynebacterium JK NCTC 11915
- 3. Corynebacterium minutissimum NCTC 10284
- 4. Corynebacterium xerosis NCTC 7883

Arthrobacter species

1.	Arthrobacter	simplex	NCIB	8929
2.	Arthrobacter	globiformis	NCIB	8907

Brevibacterium species

1.	Brevibacterium		
	lipolyticum	IAM	1413

2. Brevibacterium linens NCIB 8546

Representative strains of organisms which are gastrointestinal (G1) pathogens or are implicated in GI disease:-

(listed in alphabetical order)

Aeromonas hydrophila, Porton Down 13109 (Ref lab); Campylobacter jejuni, Clinical Isolate (C.I.); Helicobacter pylori, C.I.; Clostridium difficile, C.I.; Clostridium perfringens, C.I.; Salmonella enteritidis, C.I.; Yersinia enterocolitica , C.I.

Representative strains of organisms which are associated with the GI tract:-

Alcaligenes odorans, NCTC 10388; Bacillus subtilis, C.I.; Bacteroides asaccharolyticus, NCTC 9337; Bacteroides fragilis, NCTC 8343; Clostridium septicum, C.I.; Enterobacter cloacae, C.I.; Escherichia coli, NCTC 10418; Streptococcus faecalis, C.I.; Fusobacterium varium, NCTC 10560; Proteus mirabilis, C.I.; Pseudomonas aeruginosa, NCTC 10662.

Yeast

Candida albicans (Clinical isolate)

Skin Flora

Staphylococcus epidermidis, C.I.; Staphylococcus aureus, NCTC 6571.

Representative strains of bacterial genera which have been implicated as having some association with IBD, in particular CD:-

Bacteroides vulgatus, NCTC 10583; Eubacterium aerofaciens, NCTC 11838; Eubacterium lentum, NCTC 11813; Peptostreptococcus magnus, C.I.; Wolinella recta, NCTC 11489

Studies on faecal flora of CD patients were reviewed by Van De Merwe,(1984). Eubacterium and Peptostreptococcus species and Bacteroides vulgatus were present more often than in healthy controls.

A Molinella species was isolated as being predominant in the

periodontal lesions of IBD-affected patients by Van Dyke *et al*, (1986). They suggested that this unusual micro-organism may have a role in the pathogenesis of IBD.

Other

Uninoculated Robertson's cooked meat broth (Southern Group Labs); Listeria monocytogenes, NCTC 7973; Kurthia zopfii, NCTC 405; Lactobacillus casei var rhamnosus, NCIB 8010; Gardinerella vaginalis, C.I.; Klebsiella pneumoniae, C.I.; McCoy cells infected with Lymphogranuloma venerium (LGV₂), SA2F strain; McCoy cells, Chlamydia lab, University College Hospital; Mycoplasma species, C.I.; Peptococcus asaccharolyticus, NCTC 11461; Streptococcus pneumoniae, C.I; Streptococcus milleri, C.I.

Total - DNA from 77 bacterial species + 1 yeast + 1 RCM DNA + 6 gifts of mycobacterial DNA ----85 DNA samples

Negative Controls

Uninoculated RCM broth acted as a negative control in this study.

DNA was extracted from 500µl aliquots of

- 1. RCM broth at room temperature.
- RCM broth at 32°C (temperature of incubation of all IBD cultures) for 12 months.
- 3. RCM broth at 32°C for 18 months.

Each sample was done in triplicate, hence in all, DNA extractions from nine ROM broth cultures were done. There was no surface growth (see Fig 3.A.1 in Chapter 3), as the cultures were uninoculated.

Organisms included in any one of the categories above does not exclude itself from inclusion in another category. They have been listed as such to indicate the primary reason for being chosen for inclusion in the DNA bank.

Culture Conditions

Mycobacterial species:

All strains were those present in our laboratory and originally collected by Dr J L Stanford. They were all subcultured onto 3 Lowenstein-Jensen (LJ) slopes which were incubated at 35°C for appropriate lengths of time suitable to obtain a medium to heavy growth on each LJ slope. 1-2 slopes were used for DNA extraction.

Other bacterial species:

All NCTC, NCIB, IAM, IMRV, ATCC and VPI strains were obtained freeze dried. The organisms were solubilised in nutrient broth and grown on either Columbia blood agar (BA) or Corynebacterium agar (CA) at 37°C. *Corynebacterium, Propionibacterium and Rhodococcus* species were first cultured on CA medium (Appendix). All clinical isolates were subcultured on Columbia blood agar at 37°C. The organisms were incubated aerobically or anaerobically (in anaerobic jars) as appropriate to the organism concerned. *M. haemophilum* was grown on chocolate agar. *Campylobacter* and *Helicobacter* species were grown on Campylobacter medium.

DNA Extraction Procedures

Bacterial cells were scraped off solid media (Lowenstein-Jensen (LJ), Corynebacterium (CA) agar, Blood agar (BA)) into 10mls TEN buffer in a universal tube. The contents of either 1 or 2 LJ slopes/BA or CA plates were used depending on whether the culture was of medium (2 slopes/plates used) or heavy growth (1 slope/plate used). Work was carried out in a category I safety cabinet when mycobacterial samples were handled. The universals were spun at 1500rpm for 10 minutes to centrifuge the pellet. The wet weight of the cells was determined after the supernatant was decanted off.

The pellet was resuspended in $300-500\mu$ l of TEN buffer. The universals were placed in a water bath at 70° C for 1 hour. *M. tuberculosis* is inactivated at 60° C in 15-20 minutes, hence a temperature of 70° C for 1 hour was used to minimise biological hazard and enable further work with all the organisms to be carried out on the open bench.

Subtilisin (Sigma, type VIII) was added to a final concentration of 10mg/ml to each universal and incubated overnight at 37° C (18 hours). Lysozyme (Sigma) was added to 50mg/ml and samples were incubated at 50° C for 5-6 hours. Lysis was completed by adding sodium dodecyl sulphate (20% W/V) and Pronase (Cal biochem) to a final concentration of 1% (W/V) and 3mg/ml respectively. This was incubated at 37° C overnight (15 hours). A further addition of Pronase (Cal biochem) (final concentration 3mg/ml) was made.

All enzymes used were made up fresh in TEN buffer just prior to use. Subtilisin was usually made up as a 100mg/ml stock solution and 33µl of this was added per tube. Lysozyme was made up as a 250mg/ml stock solution (this dissolves with difficulty unless the solution is warmed to 37°C) and 83µl was added to each tube. Pronase was made up as a stock solution of 45 mg/ml and 30µl of pronase was added per tube. Four hours later the universals were centrifuged at 7000rpm for 5 minutes to pellet the cell debris. The supernatant was carefully recovered using a fine-tipped pipette and transferred into an autoclaved eppendorf tube. Phenol-chloroform extraction was then carried out.

An equal volume of phenol:chloroform:isoamyl alcohol (50:48:2 v/v) was added to the supernatant. Gentle mixing by hand was done for 10 minutes. The phases were then separated by centrifugation for 5-10 minutes at high speed using a bench top micro-centrifuge.

The upper aqueous phase containing nucleic acids was carefully removed using a fine-tipped pipette, taking care not to disturb the interface (contains protein). DNA was precipitated by adding 0.2 volumes of 10M ammonium acetate and 2 volumes of 100% ethanol (stored at -20°C). The eppendorf tubes were mixed by inversion, by hand and often DNA could be spooled. The spooled DNA was washed in 70% ethanol (stored at -20°C) and then air dried. Alternatively, if DNA could not be spooled after mixing with 100% ethanol, the tubes were stored at -20°C overnight and DNA recovered by centrifugation using a benchtop microcentrifuge (high speed for 15 minutes). The DNA was washed in 70% ethanol and then air dried.

All samples were then resuspended in 100µl TE buffer containing 10µgml⁻¹ of boiled ribonuclease A (Sigma). The DNA was stored at 4°C overnight to ensure it had fully dissolved. The DNA samples could then be used or frozen as appropriate.

DNA MANIPULATIONS

DNA yield was determined spectrophotometrically. Optical density measurements were made using a Unicam SP1800 UV spectrophotometer (details in appendix to Chapter 4) and DNA concentrations were estimated for each sample. 300ng aliquots of all DNA samples were electrophoresed in 1% agarose gels in TBE-running buffer containing ethidium bromide (0.5 µgml⁻¹), to determine the approximate molecular weight of intact DNA. This gel also provided an alternative means of quantification, namely estimating the DNA concentration of each sample against a standardised amount of DNA used. This was useful especially in instances where DNA quantities were so small that dilutions used for OD values caused inaccuracies in calculated DNA concentrations.

The gel was electrophoresed at a maximum voltage of 120 volts (65 amps) for $l^1/_2$ hours. The gel tank contained 1.5L of 1 x TBE buffer (see Appendix) with 0.5µg ml⁻¹ ethidium bromide. Each 20µl volume

loaded contained 300ng DNA as estimated by OD260 values in its appropriate TE buffer volume, and 2µl DNA loading buffer containing 50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol, 15% pharmacia ficoll 400 dissolved in water. The volume to 20µl was made up with distilled water.

Restriction Endonuclease Analysis

Restriction endonuclease (RE) digests of DNA (0.2-5µg), depending on the amount of DNA available after extraction, were performed in 20µl volumes in small Eppendorf tubes. These were done in accordance with the manufacturer's recommendations but using buffers which contained spermidine hydrochloride at 4 µg ml⁻¹. The relevant volumes containing the required amount of DNA were removed from the stock solutions. The DNA solutions were heated to 65° C for 5 minutes, then 2µl of 10 x spermidine/BSA buffer supplied with the endonuclease by the manufacturer was added. Pvu II was added, then distilled water was used to make the volume up to 20µl. The mixtures were incubated at 37° C for a minimum of 2 hours, then run through an agarose gel containing ethidium bromide as described.

RESULTS

300ng aliquots(unless stated otherwise) of undigested DNA as determined spectrophotometrically, from the following 45 organisms were run on 1% agarose gels (see Fig 4.A.1).

Row 1	DNA from:
Lane l	Listeria monocytogenes
2	Candida albicans
3	Bacillus subtilis
4	Pseudomonas aeruginosa
5	Staphylococcus epidermidis
6	Klebsiella pneumoniae
7	Proteus mirabilis
8	Uninoculated RCM at 32°C for 12 months
9	Uninoculated RCM at 32°C for 18 months
10	l Kb DNA ladder

- 11 Mycobacterium phlei (300 ng) 12 Mycobacterium fortuitum 13 Mycobacterium thermoresistible 14 Mycobacterium diernhoferi 15 Mycobacterium duvali 16 Mycobacterium flavescens 17 Mycobacaterium smegmatis 18 Mycobacterium marinum
- 19 Mycobacterium rhodesiae
- 20

Row 2 has only 15 wells

Lane 1

- 2 Mycobacterium neoaurum
- 3 Mycobacterium gilvum
- 4 Mycobacterium nonchromogenicum
- 5 Mycobacterium chitae
- 6 Mycobacterium bovis BCG
- 7 Mycobacterium malmoense
- 8 1 Kb DNA ladder
- 9 Mycobacterium phlei (300 ng)
- 10 Corynebacterium minutissimum
- 11 Arthrobacter globiformis
- 12 Nocardia asteroides
- 13 Lactobacillus casei var rhamnosus
- 14 Mycobacterium xenopi
- 15

Row 3

- Lane l Cellulomonas biazotea
 - 2 Rhodococcus species
 - 3 Rhodococcus corallinus
 - 4 Bacteroides fragilis
 - 5 Corynebacterium JK
 - 6 Escherichia coli
 - 7 Propionibacterium acnes
 - 8 Staphylococcus aureus
 - 9 Gardinerella vaginalis
 - 10 1 Kb DNA ladder
 - 11 Mycobacterium phlei (300 ng)
 - 12 Campylobacter jejuni
 - 13 Rothia dentocariosa
 - 14 Streptococcus faecalis
 - 15 Peptostreptococcus magnus
 - 16 Bacteroides melaninogenicus var asaccharolyticus
 - 17 Fusobacterium varium
 - 18 Clostridium perfringens
 - 19 Clostridium difficile
 - 20

These 45 undigested DNA samples from diverse bacterial species are seen in Fig.4.A.1. All samples <u>except</u> those in Row 1 lanes 8 and 9 (RCM DNA discussed below) show high MW DNA. The DNA was of good quality and produced by the single extraction method described.
All mycobacterial samples (Row 1 lane 11-19, Row 2 lanes 2-7 and 14) produced neat high MW bands. The DNA in each lane was estimated by comparison with the amount of M_{\star} phiei DNA ,1 µg/ml stocks made and these were stored at -20°C.

<u>RCM DNA</u>, Row 1 lanes 8 and 9. The undigested DNA from meat is seen to be degraded and of very low molecular weight, and no high MW band is present. Nine RCM samples were run on agarose gels and at no time was any high MW DNA band seen - all RCM samples were similar to the two RCM samples shown in Fig 4.A.1. Hence meat derived DNA did not interfere with subsequent analysis of high MW DNA bands for organisms which were grown in RCM broth (also see Fig 4.B.1).

Every single DNA sample tested from the DNA bank (84 samples) produced similar high MW DNA bands, with the exception of ROM DNA. Typical results, showing the yield of DNA from 68 of the bacterial species tested are shown in Table 4.A.1. All DNA was isolated as described in the methods section.



Figure 4.A.1

45 undigested DNA samples electrophoresed through a 1% agarose gel and stained with ethidium bromide. (Gel photographed under ultra-violet light). Rows 1 and 3 have 20 wells, row 2 has only 15 wells. A 1Kb DNA ladder was used as a size marker (row 1 lane 10, row 2 lane 8 and row 3 lane 10) and 300ng Mycobacterium phlei DNA (row 1 lane 11, row 2 lane 9, row 3 lane 11) was used as a DNA concentration marker. All lanes show intact high molecular weight DNA except Row 1 lanes 8 and 9. These two lanes show low molecular weight degraded RCM DNA.

TABLE 4.A.1: DNA YIELDS FROM BACTERIAL STRAINS

ORGANISM	SOURCE	YIELD* [µg DNA (g wet wt cells) ⁻¹]
Aeromonas hydrophila	Porton Down 13109	625
Alcaligenes odorans	NCTC 10388	294
Arthrobacter globiformis	NCIB 8907	333
Bacillus subtilis	C.I.	115
Bacteroides asaccharolyticus	NCTC 9337	192
Bacteroides fragilis	NCTC 8343	225
Bacteroides vulgatus	NCTC 10583	582
Brevibacterium lipolyticum	IAM 1413	149
Cellulomonas biazotea	NCIB 8077	275
Cellulomonas fimi	NCTC 7547	150
Campylobacter jejuni	C.I.	267
Helicobacter pylori	C.I.	156
Clostridium difficile	C.I.	108
Clostridium perfringens	C.I.	134
Clostridium septicum	C.I.	110
Corynebacterium JK	NCTC 11915	167
Corynebacterium minutissimum	NCTC 10284	370
Enterobacter cloacae	C.I.	200
Escherichia coli	NCTC 10418	215
Eubacterium aerofaciens	NCTC 11838	145
Eubacterium lentum	NCTC 11813	250
Fusobacterium varium	NCTC 10560	111
Gardinerella vaginalis	C.I.	93
Klebsiella pneumoniae	C.I.	66

Table 4.A.1 contd.

Lactobacillus casei var rhamnosus	NCIB 8010	182
Listeria monocytogenes	C.I.	128
Mycobacterium asiaticum	Institute Pasteur	53
Mycobacterium avium	NCTC 8559	76
Mycobacterium bovis 800	Glaxo strain	125
Mycobacterium chitae	ATCC 19630	54
Mycobacterium diernhoferi	E.I. (Vienna)	64
Mycobacterium duvalii	NCTC 358	59
Mycobacterium flavescens	NCTC 10270	84
Mycobacterium fortuitum	NCTC 8573	97
Mycobacterium gastri	N.S.C.I.	63
Mycobacterium gilvum	C.I.	179
Mycobacterium gordonae	NCTC 9822	54
Mycobacterium intracellulare	ATCC 13950	73
Mycobacterium kansasii	C.I.	104
Mycobacterium malmoense	NCTC 11299	208
Mycobacterium marinum	C.I.	97
Mycobacterium neoaurum	NCTC 10439	130
Mycobacterium nonchromogenicum	E.I. (Uganda)	106
Mycobacaterium paratuberculosis	V.I.	78
Mycobacterium rhodesiae	ATCC 27024	128
Mycobacterium smegmatis	NCTC 333	78
Mycobacterium thermoresistibile	ATCC 19528	116
Mycobacterium xenopi	N.S.C.I.	99
Mycoplasma species	C.I.	76
Nocardia asteroides	ATCC 3318	124
Nocardia brasiliensis	IMRV 800	111

Table 4.A.1 contd

Peptococcus asaccharolyticus	NCTC 11461	185
Peptostreptococcus magnus	C.I.	159
Propionibacterium acnes	VPI 0162	58
Propionibacterium avidum	VPI 0589	50
Proteus mirabilis	C.I.	189
Pseudomonas aeruginosa	NCTC 10662	182
Rhodococcus corallinus	NCTC 10668	44
Rhodococcus species	NCIB 9784	29
Rothia dentocariosa	NCTC 10207	233
Salmonella enteritidis	C.I.	263
Staphylococcus aureus	NCTC 6571	84
Staphylococcus epidermidis	C.I.	63
Streptococcos faecalis	C.I.	84
Streptococcus milleri	C.I.	35
Streptococcus pneumoniae	C.I.	55
Wolinella recta	NCTC 11489	149
Yersinia enterocolitica	C.I.	383

 * corrected to the nearest μg

C.I. = Clinical Isolate N.S.C.I. = Non-Significant Clinical Isolate E.I. = Environmental Isolate V.I. = Veterinary Isolate

Restriction Endonuclease (RE) Analysis

RE digestions is shown in Fig 4.A.2 for five representative diverse bacterial strains including a *Mycobacterium* species. If digestion failed to occur then the DNA would remain as a heavy band of high molecular weight DNA as in Lanes 1-5 of Fig 4.A.2. If a sample failed to digest, overnight incubation with Pvu II was usually successful. Digestion was achieved with all DNA samples tested, indicating that all DNA samples were suitable for molecular biological manipulations such as Southern blotting. The smeared appearance of the digested DNA is in reality due to a very large number of discrete bands. These bands are noticeable on close inspection of the photograph. Fig 4.A.3. shows that complete digestion was achieved with all mycobacterial DNA samples (19 of these are shown in the photograph).



Figure 4.A.2

DNA samples electrophoresed through a 1% agarose gel and stained with ethidium bromide.

- Lanes 1-5 Undigested DNA samples (200ng aliquots)
- Lane 6 Bacteriophage λ digested with Hind III as size marker
- Lanes 7-11 5µg aliquots of DNA samples from Lanes 1-5 digested with Pvu II showing complete digestion
- Lanes 1,7 Staphylococcus epidermidis
- Lanes 2,8 Fusobacterium varium
- Lanes 3,9 Clostridium perfringens
- Lanes 4,10 Mycobacterium kansasii
- Lanes 5,11 Nocardia asteroides



Fig 4.A.3

Digested mycobacterial DNA samples electrophoresed through a 1% agarose gel and stained with ethidium bromide.

Lane 11: Bacteriophage λ digested with Hind III as size marker. Lane 1-10:) Aliquots of mycobacterial DNA samples digested with PvuII and) Lanes 12-20) restriction endonuclease showing complete digestion.

These 19 DNA samples are DNA extracts from the first nineteen mycobacterial species listed in Table 4.A.1

DISCUSSION

DNA was obtained reproducibly in good quantities by this method from all species analysed without exception, <u>ie</u> all aerobic and anaerobic gram negative and positive bacteria and mycobacteria. The yield was variable (Table 4.A.1) but in all cases substantial. The minimum amount obtained was 22µg DNA (g wet weight cells)⁻¹ for a *Rhodococcus* species and the maximum yield was from *Aeromonas hydrophila* (625µg DNA (g wet weight cells)⁻¹).

A total of 235µg of M. smegmatis DNA was extracted by this method the first time the extraction was done. This DNA was sufficient not only for myself for the whole duration of this thesis, but it was used by most members of the laboratory for research, and gifts of this DNA have also been made to other laboratories. Other such examples include 220µg from Corynebacterium minutissimum, 283µg from Streptococcus faecalis, 450µg from Fusobacterium varium, 150µg from Campylobacter jejuni and many more. The yeast, Candida albicans also gave a good yield of DNA. The more the amount of bacterial cells scraped off the solid media, the larger the DNA yield. Except for Streptococcus milleri and Streptococcus pneumoniae, enough DNA was obtained after the one extraction to last the whole duration of this thesis. However, all samples had second extractions done purely to assess reproducability. In the case of the streptococci, I had great difficulty scraping off enough organisms off the B.A. plates for DNA extraction, and this was the reason that the extraction had to be repeated.

The DNA obtained was not only of good quantity, it was of good quality suitable for molecular biological manipulations. High molecular weight DNA was obtained which digested completely with restriction endonucleases. Despite envisaged problems (associated with the thick cell wall of mycobacteria) all mycobacterial DNA samples gave good DNA yields and digested completely.

The method of DNA isolation was simple and relatively rapid. Enzyme incubation steps were considerably shortened and similar results were obtained (data not shown); incubation time was decreased from 18 hours to 2 hours for subtilisin, from 6 hours to 4 hours for lysozyme and from 20 hours to 12 hours for pronase, without any loss of yield. The longer incubation periods (as described in Methods) were chosen for convenience so that other work could be carried out between enzyme additions.

Quantified DNA was now available from 83 bacterial species including 30 mycobacterial species and from one yeast.

b. The DNA isolation procedure was then applied to extracting DNA from the IBD tissue derived organisms.

Section B

Isolation of DNA from pleomorphic acid fast organisms cultured from cases of CD, UC and controls.

Preparation of a PAFO DNA bank: DNA Bank II

Genomic DNA extraction was carried out on ROM cultures which were positive for the PAFOs. The growth on Lowenstein-Jensen was insufficient in most cases for DNA extraction . Hence, DNA extraction was attempted on 5 LJ samples only. Cultures and subcultures from the new and old (1976) series were analysed.

MATERIALS AND METHODS

RCM Cultures

DNA was isolated from the surface growth of the RCM cultures. A 1 µl loopful was transferred into 500µl TEN buffer and the pellet was centrifuged for five minutes using a bench top microcentrifuge. In some cases it was not possible to obtain a whole 1µl loopful of growth; then only 300µl TEN buffer was used to suspend the organisms. It was not possible to use all of the surface growth for DNA analysis alone as several other investigations were planned. Hence, it was decided to use 400-500µl of RCM broth (400µl from small RCM bottles, 500µl from large RCM bottles) as a separate sample to the surface growth sample, or as the only sample where no/very little surface growth could be obtained.

The broth samples were centrifuged for 10 minutes using a benchtop microcentrifuge. They were resuspended in 300-500µl TEN buffer depending on the size of the pellet. From this point on, the identical DNA isolation procedure listed in Section A, Materials and Methods section was used for all the samples (RCM surface growth and broth samples), except at the final stage, where samples were resuspended in either 20µl or 50µl volumes of TE/RNAse (rather than 100µl TE/RNAse) buffer.

Lowenstein-Jensen Cultures

All visible growth from one slope was transferred using a bacteriological loop to 300µl TEN buffer. The DNA isolation procedure was then identical to the procedure for the RCM surface growth samples.

Preparation of a PAFO DNA bank: DNA bank II

In order to use probes to analyse PAFO DNA, DNA was extracted from 80 PAFO positive cultures from 17 CD patients (47 DNA extractions), 11 UC patients (20 DNA extractions) and 3 control patients (13 DNA extractions).

<u>New culture series</u>

At least one RCM culture from each patient yielding PAFOs on primary lymph node culture was used for DNA extraction. Several subcultures were used to obtain more DNA, but this DNA would also be used to show that the DNA in subcultures was identical to the DNA in the primary culture media. This would support the conclusions drawn from Chapter 3 that the PAFOs were viable, and can be passaged.

DNA was also extracted from each of the surface growth and broth cultures (and subcultures) of the standard mycobacteria grown in RCM.

RESULTS

By the method used, DNA was extracted from the pleomorphic organisms isolated from the IBD cultures even though the organisms were present in very small quantities. DNA was isolated from the organisms grown in RCM cultures, but not from the five Lowenstein-Jensen samples, presumably because there were too few bacterial cells for DNA extraction to be successful.

Table 4.B.1 shows representative results from the CD, UC and control cultures. Due to variable cell density at the time of harvest,

variable DNA yields were obtained. DNA extracted from the broth below the surface growth, is indicated by (b), next to the DNA yield in Table 4.B.1.

Aliquots of DNA samples containing 100-200ng DNA were electrophoresed in 1% agarose gels in TBE-running buffer containing 0.5 µgml⁻¹ ethidium bromide to determine the approximate molecular weight(MW) of undigested DNA using the same conditions described in Section A. Figure 4.B.1 shows DNA obtained from two representative Crohn's and two representative UC cultures. The DNA obtained is seen to be of high molecular weight except in the case of lane 5 which shows a high MW band (DNA from PAFOS) together with low molecular weight degraded DNA (DNA from RCM broth) at the bottom of the track.

From several IBD cultures, DNA yields obtained were very low. In these cultures, there was very little surface growth. In several cases, the DNA obtained could not be visualised on a gel, but were seen to hybridise with DNA probes (Chapter 5).

Mycobacteria grown in RCM

Regardless of the *Mycobacterium* sp. grown in RCM, on average 500-750ng DNA was obtained from the broth and 100-200ng DNA obtained from the surface growth. Only very little surface growth was used for these DNA extractions, as this material was needed for other investigations as well. The subcultures gave similar yields.

I attempted to extract DNA from 18 cultures in RCM (6 CD, 6 UC, 6 controls) in which visible growth was not obtained. From none of these could high molecular weight DNA be obtained. This provided additional evidence linking the microscopic appearance of the PAFOs with the presence of high molecular weight DNA extractable from positive cultures. DNA extraction from RCM cultures (for normal mycobacteria and PAFOs) made possible preparation of a PAFO DNA bank (DNA bank II) which would be used for hybridisation studies with DNA probes.

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Fig 4.B.1

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Undigested DNA obtained from two representative CD and two UC cultures (grown in RCM broth), showing high MW DNA.

- Lane 1 CD derived PAFOs (from surface growth in RCM)
- Lane 2 UC derived PAFOs (from surface growth) Lane 3 UC derived PAFOs (from surface growth)

- Lane 4 CD derived PAFOs (from surface growth) Lane 5 Same culture as lane 4, but DNA extraction from RCM broth. High MW PAFO DNA is seen (around 23 kb); RCM DNA is degraded (bottom of track) and of low molecular weight. (arrowed)
- Lane 6 Bacteriophage λ digested with Hind III

Patien	or subculture	Time in Culture	Amount of DNA
CD			
H P	subculture	9 months	125ng (s)
M A*	subculture	10 months	100ng (s)
A A*	subculture	8 months	100ng (s)
L B*	original	8 years	125ng (s)
D C 5*	original	7 years	250ng (s)
E B*	original subculture	8 years 14 months	100ng (s) (300ng (s) (200ng (b)
	succitute	20 montens	(repeat sample)
A C	<u>lymph node l</u> original subculture 2nd passage 3rd passage	23 months 12 months 12 months 6 months	200ng (s + b) 100ng (s + b) 200ng (s) 150ng (s)
	<u>lymph_node_2</u> original	23 months 25 months 32 months	100ng (s) 200ng (b) 50ng (b) 100ng (b)
			(repeat sample)
	large RCM) subculture)	12 months	<50ng (s)
	subculture)	10 months	(100ng (b) (100ng (s)
JG	original	22 months	No DNA visualised on gel
BB	subculture	12.5 months 22 months	100ng (s + b) 125ng (b) (repeat sample)
RH	original	22 months	No DNA visualised $(s + b)$
L G	original (large RCM)	16 months	No DNA visualised on gel (s + b)
	original (small RCM)	16 months	500ng (s + b)
D H	original	3 months	<50ng (s + b)
			contd.

<u>trol</u> (non- G R P C [*] E D [*]	IBD) subculture original original subculture	9 months 6 years 10 years 12 months	100ng (b) 600ng (s + b) 100ng (s) 100ng (b)
<u>trol</u> (non- G R P C*	IBD) subculture original	9 months 6 years	100ng (b) 600ng (s + b)
<u>trol</u> (non- G R	IBD) subculture	9 months	100ng (b)
RI	original	20 months	<50ng (s) 50ng (b)
	original	23 months	50ng (b)
AB	<u>lymph node 1</u> original repeat	17 months 23 months	200ng (s + b) 100ng (b)
JS	original repeat	18 months 24 months	100ng (s + b) 125ng (s + b)
V J*	original	3 years	250ng (s + b)
AA	original	8 years	500ng (s)
J G*	original	26 months	433ng (s)
мв	subculture	9 months	125ng (s)
	M B J G* A A V J* J S A B	M BsubcultureJ G*originalA AoriginalV J*originalJ Soriginal repeatA Blymph node 1 original repeat lymph node 2 originalR Ioriginal	M Bsubculture9 monthsJ G*original26 monthsA Aoriginal8 yearsV J*original3 yearsJ Soriginal repeat18 months 24 monthsA Blymph node 1 original repeat lymph node 2 original17 months 23 monthsR Ioriginal20 months

s = surface growth in RCM culture

b = DNA extraction from RCM broth. Figure stated does not include amount of RCM DNA.

s + b = surface growth and broth samples combined

* = 1976 culture series

No DNA visualised on gel - samples were run on the gel but no DNA was seen by eye.

DISCUSSION

It proved possible to isolate genomic DNA from the organisms. The organisms have high molecular weight DNA. Even organisms from cultures several years old yielded good quality DNA.

There is no physical separation between different sections of the RCM culture bottle. Although the organism was most obvious macroscopically as a surface growth, DNA was also isolated from the broth below. As a total weight, potentially more DNA from the organisms would be present in the broth as more broth could be used for DNA extraction. In contrast to this, the amount of surface growth was limited and usually not more than a loopful could be obtained per bottle for DNA studies.

As expected, variable amounts of DNA were extracted, depending on how much of the organisms were present in the cultures.

DNA AMPLICATION USING THE POLYMERASE CHAIN REACTION

INTRODUCTION

Forty-five DNA samples which had been derived from IBD and non-IBD cultures were given to Dr R A Cox at the National Institute for Medical Research (Ridgeway, Mill Hill, London NW7) for analysis. We were unable to visualise DNA in these samples using gel electrophoresis by the methods described previously. This was probably because less than 30-50ng DNA was present in these samples. However, Dr Cox using the polymerase chain reaction (PCR) to amplify any DNA present, could progress further with these samples.

METHODS

Six primers were chosen from the prokaryotic (mycobacterial) 16S ribosomal (r) RNA gene.Primers (previously tested) from regions highly conserved for most (or all) bacterial 16s rRNA (called "universal"

primers) and other more specific primers were both used simultaneously in a PCR reaction.

Briefly, only 10-40ng DNA was required as starting material. 0.8 units/tube of Taq Polymerase was used. The melting temperature was 95°C (for 3-4 minutes) and annealing temperture was 58°C. Thirty-five PCR cycles were done. 1.6% agarose gel electrophoresis of PCR products with ethidium bromide staining was used to visualise DNA.

RESULTS

36 of the 45 samples showed amplified DNA - these samples all showed a 760 base pair PCR product (easily visualised) corresponding to the "universal" primers used. This amplified DNA is now available for sequencing.

DISCUSSION

Inability to detect bacterial DNA by standard methods was due to these methods being too insensitive for the extremely small amounts of DNA available. Eukaryotic DNA did not give a PCR product in this system (personal communication, Dr R Cox).

No intermediate cloning step is necessary, and the technique is highly sensitive. However, contamination of samples by "exogenous" sequences is easy, making the technique delicate and labour intensive. However, now that DNA amplification has been achieved, nucleotide sequencing of this DNA is possible. This has been started by Mr G McIntyre from the Middlesex Hospital Medical School at the Mill Hill Laboratory. This will reveal to us whether the organisms have an identical genome or not. Nucleotide sequence analysis of the genome of the organism is the ultimate detail we could hope to achieve. It is fortunate that PCR amplication has made this possible.

CHAPTER 5

THE USE OF DNA PROBES TO EXAMINE THE PLEOMORPHIC ACID FAST ORGANISMS ISOLATED

SECTION 5A

INTRODUCTION

The use of specific mycobacterial probes such as Gen-Probe (Gen Probe, Inc., California) rapid diagnostic probes for the *Mycobacterium tuberculosis* complex or *Mycobacterium avium* complex were not indicated, as it could not be assumed that the PAFOs to be characterised belonged to a specific mycobacterial genus.

If probes could be found which would hybridise only to mycobacterial DNA and not DNA from intestinal bacteria and skin flora, then this would mean that lymph node specimens could be cultured and probes tested directly against DNA from the cultures for the presence of mycobacteria.

Initially dot blotting was chosen as 96 DNA samples could be tested at once. Also much smaller amounts of DNA can be used in dot blots (The PAFOs were known to have very little DNA).

PART 1. SELECTION and CHARACTERISATION OF MYCOBACTERIAL PROBES MATERIALS AND METHODS

<u>DNA Samples</u> were those derived from mycobacteria, nocardiae, and other bacteria in DNA bank I (see Chapter 4). DNA samples were boiled prior to loading onto the membrane.

<u>Probes</u>: Four probes whose complete range of hybridisation to bacteria was as yet undefined were analysed. Three of these probes designated $pMBr_{16}$, pMB_{20} and pMB_{22} are mycobacterial DNA probes from a genomic library prepared in the vector pGEM-1 (promega Biotec, Madison, USA) from CD derived strain Ben DNA (Chiodini *et al.*, 1984b).Strain Ben

has been shown to be identical to the type strain of *M.* paratuberculosis (ATCC 19698) by McFadden *et al* (1987b). The preparation of the gene library and selection of probes was by McFadden *et al* (1987b). All probes did not react with eukaryotic DNA.

Probe pMBr16

pMBr16 is a DNA probe complementary to mycobacterial ribosomal 16s RNA and was selected as it was predicted to hybridise with a wide range of mycobacteria, as 16s ribosomal RNA genes are highly conserved between species.

Probe pMB20

Clone pMB20 consisted of a 6.6Kb DNA fragment. This probe showed different restriction-fragment-length polymorphism (RFLP) patterns with different strains of *M. avium*, and this was different to *M. paratuberculosis* (McFadden *et al.*, 1987c).

Probe pMB22

Although the PAFOs were not mycobactin dependant, (M. paratuberculosis is mycobactin dependent) the definitive investigation would be to show that the PAFOs did not have the M. paratuberculosis Green et al (1989 a,b) have identified an insertion genome. sequence (IS) present as approximately 10-20 copies in the M. paratuberculosis genome. An IS is a type of transposon (mobile genetic element). The closely related M. avium lacks this IS (named IS900 by Green et al). The probe pmB22 contains this IS but also other flanking Sub clones of pmB22 were prepared for me by Dr McFadden and Dr M DNA. Moss and used as probes. The specificity of one probe was tested as below.

Probe pp107

Clone ppl07 consists of a 1.9 Kb DNA fragment from the genome of *M. kansasii* which had been cloned into the EcoRl site of the plasmid vector PAT 153. The gene library was prepared by Mr G McIntyre at the Middlesex Hospital Medical School. The probe did not react with eukaryotic DNA.

DOT BLOTTING METHOD

This method is geneally applicable to all samples tested by dot blotting. A Hybond-N membrane (Amersham, Little Chalfont, United Kingdom) was wet for 10 minutes in distilled water. Forceps were used to avoid contamination of the membrane. The top left hand corner beside where sample Al was to be applied was cut for purposes of orientation.

Assembly of dot blot apparatus

A Bio-dot microfiltration apparatus was used. It is composed of six parts that are easily assembled (Fig 5.A.1).

The clean apparatus was assembled. The sheet of hybond-N membrane was blotted on filter paper to remove excess water and placed over the 96 well rubber sealing gasket. Any air bubbles between membrane and gasket were removed. The top 96 well sample template was placed over the membrane and the screws were tightened to finger tightness. The vacuum source was attached to the vacuum tubing and flow value and vacuum applied. The screws were retightened and the flow value was closed.

Loading of Samples

96 "wells" were available to load DNA, see Fig 5.A.1 for the template. Up to 48 DNA samples were chosen and DNA aliquots were placed in a randomised manner in duplicate. Usually two spaces were

utilised for distilled water blanks. As there were more than 80 samples of DNA in DNA bank I (Chaptar 4), two dot blot membranes, were prepared (labelled membranes 1 and 2). DNA samples spotted on membrane one are shown in Table 5.A.1.

Also another membrane was prepared to include varying DNA concentrations as shown in Figs 5.A.2 and 5.A.3 (labelled membrane 3). Unless limited amounts of DNA were available 100 or 200 ng DNA was applied per well. If there was less than 100ng DNA, (several PAFO DNA samples, M.leprae DNA) the available amount was applied and duplicate samples could not be tested. The samples were then drawn through the membrane with vacuum thus immobilising the DNA on the membrane.

The membrane was removed from the apparatus and soaked DNA side up in denaturing buffer (1.5M NaCl, 0.5M NaOH) for five minutes. The membrane was then transferred again DNA side up to neutralising buffer (1.5M Nacl, 0.5M Tris HCl pH 7.5) for five minutes.

The membrane was then dried with a blow dryer, wrapped in cling film for protection and ease of handling, and the filter was exposed DNA side down on an UV transilluminator for 3 minutes. This binds the DNA to the Hybond-N membrane by covalent linkage. The membrane was then re-moistened by agitation in a 25ml solution of 3 x SSC, 0.1% SDS at 65°C for 30 minutes.

Pre-Hybridisation

The pre-hybridisation solution is essentially the same as the hybridisation solution except it contains no radioactive probe. The pre-hybridisation mixture was made up in 100ml volumes. It contained 15mls 20 x SSC, 5mls 100 x Denhardt's solution (2% Ficoll (Pharmacia), 2% Polyvinylpyrrolidone (Average Molecular Weight 360,000, Sigma) and

2% bovine serum albumin (Sigma) in distilled water), lOgm dextran sulphate (Pharmacia), lOOµls of lOmg/ml denatured (by boiling for 5 mnts) Herring sperm DNA, 2.5mls 20% SDS (Sigma) and lml of lM phosphate buffer pH 7.2 made up to a total of lOOmls in distilled water. Dextran sulphate increases the rate at which DNA-hybridisation reactions occur by reducing the volume of solution available to the nucleotides. The herring sperm DNA was used to block non-specific DNA binding sites on the membrane.

The membrane was transferred from the 3 x SSC, 0.1% SDS solution to a sandwich box containing approximately 20mls of preheated (to 65° C) prehybridisation solution. The membrane was left overnight (for convenience, even though 4-6 hours was enough) in an agitating water bath at 65°C. Sometimes the use of dextran sulphate was associated with high background signals in some of the experiments and if it was omitted, prehybridisation was always carried out overnight.

Hybridisation

First radiolabelled DNA probes were prepared. Apart from the four mycobacteria derived DNA probes already listed, other DNA probes were used and are discussed in the appropriate sections. These DNA probes were labelled with ³²P by random hexanucleotide priming using the <u>E</u>. <u>coli</u> DNA polymerase large fragment (Klenow fragment NBL) as described by Feinberg and Vogelstein (1984). A 50µl reaction mixture was prepared on ice in an 0.5ml volume small Eppendorf tube. As a rule, about 30ng of DNA probe was used per membrane. The reaction mixture contained 10µl ABC solution (see Appendix), 2µl of bovine serum albumin 10mg/ml, 20µl probe DNA in TE buffer, previously heated to 100°C for 10 minutes to separate the DNA strands, 2.5µl ³²P-dCTP (cytidine triphosphate) equivalent to 25 µCi (Amersham), 2.5µl Klenow fragment (NEL) and 13 µl distilled H₂O. The radiolabel ³²P is a high energy

beta emitter and appropriate safety measures were taken. This was always used within 2 weeks of manufacture. The reaction mixture was left overnight at room temperature behind a radio-opaque screen before % incorporation of radiolabel into DNA was calculated.

Percentage incorporation of radiolabel into probe was calculated by TCA (Trichloroacetic acid) precipitation of the DNA.

Total count (x):

lµl of the reaction mixture described above, was added to 99µl 20MM EDTA and mixed. 10µl was removed with a pipette and spotted on to a 2.5cm circular Whatman glass microfibre filter. The disc was added to a scintillation vial, and Betafluor (complete liquid scintillation counting solution) was added up to the top of the vial. The sample was counted in the beta scintillation counter.

Bound label (y):

To the 90µl dilute probe mixture (ie, after 10µl was removed - see total count above) was added 10µl of salmon sperm DNA (1mg/ml). 80µls of 50% TCA was then added and the sample was left on ice for 15 minutes. Using a filter apparatus and vacuum pump, the whole mixture was emptied onto a second glass microfibre filter disc. Unbound radioactivity was washed through first with 5mls 5% TCA and then with 10mls 100% ethanol. The filter was dried. The radioactivity incorporated into the DNA was precipitated onto the filter paper disc. The disc was added to the scintillation vial, Betafluor added and the sample counted in the beta scintillation counter

 $\frac{1}{X}$ incorporation = $\frac{Y}{X} \times 100$ %

The probes were used within 24 hours of being made up.

Hybridisation solution

49µl of the labelled probe (1µl of the 50ul of probe reaction mixture prepared was used for TCA precipitation) was boiled at 100°C for 5 minutes to separate the double stranded DNA and was then added to 20mls pre-hybridisation solution, which had been prewarmed to 65°C. This is now the hybridisation solution. The pre-hybridisation solution in the sandwich box was poured out and the hybridisation mixture added. The membrane in the sandwich box was left overnight for hybridisation to occur in an agitating water bath thermostatically controlled at 65°C. After hybridisation, the membranes were removed for washing.

Washing of membranes

Sequential washes of the membrane of increasing stringencies will leave only the desired signal and also wash away unincorporated radioactivity. The membranes were washed in 3 x SSC, 0.1% SDS for 5 minutes, 15 minutes and 30 minutes, followed by 2 consecutive rinses in 1 x SSC, 0.1% SDS for 30 minutes. All washing was in a sandwich box in an agitating water bath at 65° C. Washing the membrane at increasing stringency (0.1SSC, 0.1% SDS) will leave signals from only very strongly bound DNA.

Autoradiography

The filter was wrapped in cling film and placed in a standard x-ray cassette. The filter was overlayed with X-OMAT S Kodak film. The cassettes were loaded under dark room conditions and incubated at -70° C. Most films were developed after 24-48 hours to get an idea of the total time that would be required before the films could be finally developed, as the time of exposure varied depending on the strength of the radioactive signal given off by a particular membrane. After removal from the cassettes, the films were immersed for 3 minutes in

Kodak x-ray developer LX24 and then transferred to a solution of fixer (Unifix, Kodak) for 10 minutes. The films were washed in cold running water for ten minutes, rinsed in distilled water and hung up to dry.

Removal of probe and re-use of DNA blots

As very little DNA was obtained from the PAFOsit was important to maximise use of the DNA spotted onto each membrane by re-use of the nylon membranes. Frequently, all DNA obtained had to be placed on the membrane on one application.

Once satisfactory images had been obtained, the hybridised probe DNA was removed by incubating the membrane in 0.4 M NaOH for 30 minutes at 45° C, and then transferring the membrane to 0.1 x SSC, 0.1% (w/v) SDS, 0.2M tris-HCl pH 7.5 for 30 minutes at 45° C (Membrane transfer and Detection methods booklet by Amersham, 1985). The membrane (with DNA samples) was then stored at 4° C wrapped in cling film until required for further hybridisation reactions with radiolabelled probes.

Assessment of Results

A graded scale of 0 to 5+ was chosen, where 0 indicates no hybridisation and 5+ indicates very strong hybridisation. The dot blots were scored blind (ie without knowledge of the DNA samples on the blot) by the author and Dr M Moss separately and results compared. If any discrepancy was noted, the result was reassessed. If results on each dot blot did not give clear positives, they were considered as negative (zero score) or repeated.

Where possible, all blots were repeated so confirmation of results could be obtained.

Probe pMB22

A dot blot containing genomic DNA from 27 mycobacterial strains and a further dot blot with 46 bacterial strains was used.

All DNA samples, except for 6 samples, were prepared by the author. DNA from Streptomyces coelicolor, M. bovis, M. phlei, M. simiae, M. vaccae and M paratubeculosis were provided by Dr Moss. The probe used was a ³²P-labelled portion of the IS from the 5'end of the IS.

Briefly, 200ng amounts of heat denatured DNA samples were spotted onto Hybond-N membranes by dot blotting. 200ng, 100ng(in triplicate), 50ng and 5ng aliquots of *M. paratuberculosis* DNA was also used. All conditions were as described for probes, pMBr16, pp107 and pMB20. The hybridisation was carried out to high stringency (ie consecutive washes in 3 x SSC 0.1% SDS, 1 x SSC 0.1% SDS and finally 0.1 x SSC 0.1% SDS) as described.



Fig 5.A.1. Bio-dot microfiltration apparatus used for dot blotting (from Bio-dot manual) consists of six parts which are assembled. The sample template (top) shows rows 1-12 across and A-H down (96 "wells" in all).

SOUTHERN BLOTTING: METHOD

The method described below is generally applicable to all samples tested by Southern blotting. Restriction Endonuclease digestion is performed as described in Chapter 4. Agarose gel electrophoresis is carried out using a 10 x 15cm, 1% agarose gel as described under Agarose Gel Electrophoresis with TAE buffer in the Appendix to Chapter For larger gel sizes, quantities of agarose and TAE buffer are 4. adjusted accordingly. Samples were loaded into the wells as described in Chapter 4, Samples contained 2µl 50% glycerol loading buffer, up to 300ng DNA (depending on amount of DNA available) and distilled water to a volume of 20 μ l. Gel electrophoresis was carried out initially at 50 volts, increasing to 100 volts within 5 minutes. When the dye front reached to about 2/3 rds down the gel, electrophoresis was stopped. Ethidium bromide staining of the gel enabled visualisation under ultra violet light and a photograph was taken. Digested DNA is seen in the lanes in contrast to discrete bands of marker DNA.After photography, the gel was washed in a 2 x SSC solution (by agitation) for ten minutes, and then in a 0.5M NacH for 30 minutes. This denatures the DNA to single stranded DNA. The gel was soaked in neutralising solution (1.5 M Nacl, 0.5M tris HCl pH 7.2, 0.001M EDTA) for 30 minutes and then blotted. Transfer to a nylon membrane (Hybond-N was used) was performed by Southern blotting (Southern, 1975). See Fig 5.A.4.

A buffer solution (0.025M sodium phosphate buffer pH 7.2, see Appendix) was placed in a large container (see Fig 5.A.4). A glass plate was supported above the buffer solution and a piece of chromatography paper draped over this such that it trailed into the buffer on either side ("wick" sheets). The gel was placed on top of this paper, bottom side up (ie, surface from bottom of mould is uppermost). A piece of nylon membrane (Hybond-N, Amersham) soaked in

phosphate buffer, corresponding exactly to the gel dimension was placed smoothly on top of the gel. The chromatography paper around the nylon membrane was covered with strips of cling film. This therefore allows buffer transfer only through the membrane. Six to seven pieces of chromatography paper of similar size were placed on top of the membrane, followed by two packs of absorbent paper towels. A one pound weight was placed above the absorbent towels.

Sodium phosphate buffer is drawn through the gel by capillary action and carries the DNA from the gel to the nylon membrane. The DNA is trapped on the membrane which acts as a filter, allowing the buffer to pass on. The gel is blotted as above for 16-24 hours (overnight blotting was convenient).

Once the paper towels and cling film were removed, the membrane was marked so the well sites and the "DNA side" (the side next to the gel) of the membrane could be identified.

The membrane was washed for 3 minutes in 2 x SSC to remove any excess agarose. The membrane was blow dried, wrapped in cling film for protection and exposed DNA side down on a UV transilluminator for 3 minutes as described previously. The membrane was washed in 3 x SSC, 0.1% SDS in an agitating water bath at 65°C for 30 minutes. The membrane was ready for probing with radiolabelled probes. The procedure from this point on is as described under the dot blotting section (Prehybridisation, Hybridisation etc).

RESULTS

DNA radiolabelling

The incorporation of DNA varied from one reaction to another. Probes were used if specific activity was more than 1×10^8 counts/µg and radioactivity incorporation above 30%. Usually radioactivity incorporation was approximately 50%.

Probing of Dot Blots

<u>Results with probe pMBrl6(mycobacterial l6s ribosomal probe)</u>

Results obtained at low stringency $(3 \times SSC, 0.1\% SDS)$ and high stringency $(0.1 \times SSC, 0.1\% SDS)$ washes with a wide range of bacteria on dot blotting are seen in Table 5.A.1. A selection of 47 bacteria and RCM DNA were tested, most in duplicate.

Results at low stringency showed a very wide range of cross reaction with a variety of bacterial species. Genera close to mycobacteria such as *Corynebacterium*, *Arthrobacter* and *Nocardia* showed cross reaction, but so did distant species such as *Clostridium perfringens*, *Fusobacterium varium* and *Gardinerella vaginalis*. Also several mycobacterial species such as *M. vaccae*, *M. fortuitum* and *M. diernhoferi* did not hybridise with the probe. This could have been due to an incorrect amount of DNA being inadvertently spotted onto the membrane, so that less than 100ng DNA was actually available for hybridisation with the probe. Because of this, DNA concentrations in the original stock were assessed once again as in Chapter 4, Secion A by visualisation on a gel. A new membrane was re-loaded and reprobed but similar results were obtained.

In an attempt to reduce cross reactions with other bacteria a high stringency wash was carried out. This was still not helpful in reducing the wide cross reaction. Southern blotting with these 48 DNA smaples showed hybridisation in the same samples as with dot-blotting but varying RFLP patterns. As this probe was so cross reactive, no further bacteria from the DNA bank were tested.

Results with probes ppl07 (from *M.kansasii*)and pMB20 (from *M.paratuberculosis*)

Hybridisation of the probe with complementary DNA sequences on the membrane results in a dark circular dot on the membrane. Representative examples of results obtained with probes pp107 (Fig 5.A.2) and pMB20 (Fig 5.A.3) are shown. Apart from DNA from mycobacteria, DNA from *Nocardia asteroides*, *Corynebacterium JK* and *Cellulomonas biazotea* (not shown in Fig 5.A.2 and Fig 5.A.3) hybridised with probe pp107, and *Nocardia asteroides* and *Corynebacterium JK* DNA hybridised with probe pMB20 (Fig 5.A.3).

Table 5.A.1

Results of Dot Blot Hybridisation of genomic DNA from several organisms probed with pMBR16 and exposed to an X-ray film for 72 hours.

Spot Intensity

Sample	Low Stringency Wash (3 x SSC, 0.1% SDS)	High Stringency Wash (0.1 x SSC, 0.1% SDS)
M. paratuberculosis	5+/5+	5+/5+
M. intracellulare	3+/3+	3+/3+
M. bovis BCG	5+/5+	4+/4+
M. smegmatis	1+/1+	1+/1+
M. scrofulaceum	4+/2+	4+/2+
M. Vaccae	0	0
M. leprae	5+	3+
M. fortuitum	0/0	0/0
M. diernhoferi	0/0	0/0
M. flavescens	1+/1+	1+/1+
M. marinum	3+/2+	3+/2+
M. rhodesiae	2+/2+	2+/2+
M. neoaurum	3+/3+	2+/3+
M. gilvum	3+/3+	3+/3+
M. nonchromogenicum	0/0	0/0
M. chitae	0/0	0/0
M. malmoense	1+/0	1+/0
M. xenopi	1+/1+	0/0
M. simiae	2+/2+	2+/2+

Sample	Low Stringency Wash (3 x SSC, 0.1% SDS)	High Stringency Wash (0.1 x SSC, 0.1% SDS)
Staphylococcus epidermidi	s 1+/2+	0/0
Klebsiella preumoniae	1+/1+	0/0
Lactobacillus casei	2+/2+	1+/1+
Gardineralla vaginalis	3+/2+	2+/2+
Staphylococcus aureus	3+/3+	2+/2+
Streptococcus faecalis	3+/3+	2+/2+
Bacteroides asacharolytic	us 1+/1+	0/0
Fusobacterium varium	3+/3+	2+/2+
Clostridium perfringens	4+/5+	3+/3+
Clostridium difficile	3+/3+	2+/2+
Bacillus subtilis	1+/0	0/0
Corynebacterium minutissi	mum 3+/3+	2+/2+
Corynebacterium JK	3+/3+	1+/0
Arthrobacter globiformis	2+/2+	1+/2+
Nocardia asteroides	3+/2+	2+/2+
Rothia dentocariosa	1+/1+	0/0
Uninoculated RCM	0/0	0/0

Spot Intensity

Cellulomonas biazotea, Rhodococcus **species**, Gordona rubra, Propionibacterium acnes, Escherichia coli, Listeria monocytogenes, Candida albicans, Peptostreptococcus magnus, Campylobacter jejuni, Bacteroides fragilis, Proteus mirabilis, Pseudomonas aeruginosa **a**ll **showed 0/0 hybridisation**.

M. paratuberculosis M. tuberculosis

Fig 5.A.2

N. asteroides

M. avium

RCM broth

M. kansasii

M. gilvum

M. flavescens

C. albicans

M. marinum

S. epidermidis

C. JK

Dot blot hybridisation with 32P-labelled probe pp107. 10-500ng aliquots of genomic DNA from representative mycobacterial and other bacterial strains, and Candida albicans. Following hybridisation at 65°C for 16 hours, the membrane was washed with 1 x SSC, 0.1% SDS, then exposed to an x-ray film for 72 hours at -70°C. Hybridisation is seen with mycobacteria, Nocardia and Corynebacterium species but not with RCM broth, other bacterial strains or Candida albicans.

Arrows indicate non-specific radioactivity.





Fig 5.A.3

Dot blot hybridisation with ³²P-labelled probe pmB20. 10-500ng aliquots of genomic DNA from representative mycobacterial and other bacterial strains and *Candida albicans*. Results are similar to those obtained with probe pp107. The faded appearance is due to this membrane having been washed several times.

Arrow: non specific area of radioactivity - not in line with areas of sample applcation.


Fig 5.A.4 Apparatus for Southern blotting (diagramatic representation from Molecular Cloning, A Laboratory Manual, Maniatis, Fritsch and Sambrook, 1982).

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DNA from the rest of the organisms in DNA bank I (Chapter 4) (apart from mycobacteria, Nocardia asteroides and Nocardia brasiliensis, Corynebacterium JK and Cellulomonas biazotea species) did not bind to probes pp107 and pME20. These were all tested by dot blotting. Of all the mycobacteria tested (listed in Chapter 4) only M. duvalii, M. fortuitum and M. nonchromogenicum did not hybridise with pp107. M. diernhoferi, M. malmoense and M. nonchromogenicum did not hybridise with pME20. Hence, if probes pME20 and pp107 are both used consecutively on the same dot blot membrane, the only mycobacterium species that would not hybridise to one or other of these probes would be M. nonchromogenicum, even at washes of low stringency. The reason for this is unclear. Consistent results were obtained on repeating the dot blots. Notably, RCM DNA does not hybridise with the probes.

The sensitivity of the method was good. As little as 10ng of mycobacterial (and other) DNA (Fig 5.A.2 and 5.A.3) hybridised with the probes.

Results with probe pmB22

Following hybridisation of the dot blot containing 27 mycobacterial strains with the paratuberculosis probe, positive signals were only obtained with *M. paratuberculosis* (Fig. 5.A.5). Good signals were obtained with concentrations as low as long of *M. paratuberculosis*. The 46 bacterial strains tested showed <u>no</u> positive signals, hence data is not shown.

Discussion

Probe pMBr16

Probe pMBr16, being a DNA probe complementary to mycobacterial 16S ribosomal RNA, was expected to cross react with a wide range of mycobacteria. However, the cross reactivity extended to so many other

bacterial species, that the probe would not be useful for the intended purpose of narrowing down the identity of the PAFOs just to the genus Mycobacterium.

This result however, was not a total surprise. Ribosomal RNA (rRNA) genes are being used in DNA fingerprinting more and more and has its basis in evolutionary history, because such sequences are highly conserved between a wide range of bacteria. For example, homologies between 16S rRNA sequences of different *Campylobacter* species are 85-90%, and is 80% with *Escherichia coli*, even though Campylobacter are phylogenetically distant from *E. coli* (review by Owen, 1989). There are now several other examples in the literature, where rRNA based probes have been used.

Owen *et al*, 1988, used a cDNA probe of 16S and 23S rRNA from *Providencia stuartii* and found distinct fingerprints when EcoRl digested chromosomal DNA of *Providencia stuartii*, other *Providencia* species and *Proteus* species were examined on Southern blotting. Even within *P. stuartii* species, 11 different patterns were detected using 26 strains.

Hence, the value of probe pmBr16 would be with its use in Southern blotting to detect similar or different chromosomal DNA patterns from the PAFOS. Dot blotting either gives a positive signal or does not (as in Figs 5.A.2 and 5.A.3) and hence would not be useful due to extensive cross reactivity; bacteria with several 16S rRNA gene copies would show the greatest hybridisation.

Probes pp107 and pmB20

Apart from mycobacteria, only DNA from N. asteroides, N. brasiliensis and Corynebacterium JK hybridised with probes pp107 and pMB20. The other species of Corynebacterium tested-C. minutissimum did

not hybridise with the probes. Similarly, *Cellulomonas fimi* did not hybridise but *C. biazotea* did hybridise to pp107. Hence one would have to assume that there was a possibility that other organisms of the genera *Nocardia* and *Corynebacterium* could hybridise to these probes and *Cellulomonas* sp. to probe pp107. Related genera such as *Rhodococcus* and *Arthrobacter* did not hybridise, even when low stringency hybridisation conditions were used, so as many mycobacteria as possible would hybridise. Results were similar at a higher stringency (x1 SSC, 0.1% SDS).

Of 30 mycobacterial species tested, if both probes are used then only *M. nonchromogenicum* is missed. Hence these 2 probes would be useful in dot blotting - with the DNA from the PAFOS (DNA bank II) placed on the membrane and probed with pp107 and then pmB20. If there was hybridisation of the probe with the DNA, and if hybridisation occurred with the PAFO DNA under the same test conditions as those in which the hybridisation range of the probes were tested, then we could infer that the organisms belonged to the genus *Mycobacterium* or possibly *Nocardia* species or *Corynebacterium* species (and *Cellulomonas* sp. if hybridisation occurred with pp107).

Once hybridisation was known to occur, then further dot blotting could be carried out at high stringency, enabling strongly complementary PAFO DNA samples to be identified.

Altogether, these results were considered the best we were likely to achieve. It was unlilkely that probes with hybridisation only to mycobacteria would be found due to close similarities between mycobacteria and other genera such as *Nocardia* and *Corynebacterium*. All mycobacteria and nocardiae contain at least four group i (common antigens) antigens and at least two of these antigens are also present

in Rhodococci and Corynebacteria (Stanford J L, 1983). Some group iii antigens (consists of four antigens shared by fast growers) are also shared by Nocardiae. Hence it was unlikely that a probe could be found which hybridised only with the 40-50 species of mycobacteria known but not to the closely related *Nocardia* or *Corynebacterium* species.

Probe pmB22

The value of this probe is in its ability to specifically detect *M.* paratuberculosis and no other organism. McFadden (1987c) *et al* have shown that *M. paratuberculosis* and *M. avium* have 98% homology elsewhere in their genomes excluding the IS element of *M. paratuberculosis*. Another part of the IS (the 3' end) would cross react with *M. avium* (personal communication, J. McFadden); but the probe tested above does not (see Fig 5.A.5). Hence, in conjunction with PCR (Polymerase chain reaction) amplification, it will be capable of rapid, sensitive (10fg) and specific detection of *M. paratuberculosis*. This could be adapted to detect very small quantities of *M. paratuberculosis* in veterinary, clinical and environmental samples (Moss *et al*, in preparation).

Conclusions

Of the four mycobacterial probes characterised:

- Probe pmB22 would hybridise only with DNA from M. paratuberculosis and could be used specifically for this purpose.
- Probes pmB20 and pp107 would hybridise with DNA from mycobacteria, but also closely related genera such as *Nocardia* and *Corynebacterium*. These would be useful as screening probes.
- 3. Probe pmBr16 is too cross-reactive to be used to screen PAFO DNA on dot blotting. It can be used on Southern blots (different RFLP patterns would be seen with different organisms).



Fig 5.A.5. Dot blot hybridisation with ³²P-labelled *M.paratuberculosis* probe pMB22. 200ng aliquots (unless otherwise stated) of genomic DNA from 27 mycobacterial strains were dot blotted onto a Hybon-N membrane:

(1A) M. thermoresistibile, (1B) M. diernhoferi, (1C) M. bovis BCG, (1D) M. duvalii, (2A) M. flavescens, (2B) M. bovis, (2C)M. paratuberculosis; 100ng, (2D) M. smegmatis, (3A) M. intracellulare, (3B) M. avium, (3C) M. marinum, (3D) M. rhodesiae, (4A) M. neoaurum, (4B) M. paratuberculosis; 100ng, (4C) M. gilvum, (4D)M. nonchromogenicum, (5A) M. chitae, (5B) M. tuberculosis, (5C) M. malmoense, (5D) M. kansasii, (6A) M. gastri, (6B) M. intracellulare, (6C) M. phlei, (6D) M. simiae, (7A) M. vaccae, (7B) M. fortuitum, (7C) M. gordonae, (7D) M. asiaticum, (8A) M. paratuberculosis; 200ng, (8B) M. paratuberculosis; 100ng, (8C) M. paratuberculosis; 50ng, (8D) M. paratuberculosis; 5ng.

Hybridisation is seen only with M. paratuberculosis and no other Mycobacterium species. The dots seen in 1C and 3B are artefacts. PART 2: INVESTIGATION TO SEE IF ROBERTSON'S COOKED MEAT DNA REDUCED THE PROBE SIGNAL WHEN MIXED WITH BACTERIAL DNA.

INTRODUCTION

Several samples of bacterial DNA were mixed with RCM DNA and tested by dot blotting, to assess whether signals from PAFO DNA would be masked by RCM DNA. If signals were not masked by RCM DNA then it would not be necessary to remove RCM DNA when testing hybridisation to PAFO DNA.

MATERIALS AND METHODS

200ng RCM DNA was mixed with 10ng DNA aliquots of samples in Fig. 5.A.6 and also with *S. aureus*, *E. cloacae*, *C. minutissimum*, *M. gastri*, *M. fortuitum*, *E. aerofaciens* and *W. recta*. 10ng of each of these \pm 200ng RCM DNA was loaded onto dot blots and probed with probe pp107. Methods are as described previously.

RESULTS

Results for 6 DNA samples and ROM DNA are shown in Fig 5.A.6. The majority of samples hybridised well on probing with pp107, even in the presence of ROM DNA. *M. fortuitum* showed a similar result to *M. paratuberculosis*, ie the signal obtained on probing was greater in the absence of ROM DNA. However, all other samples hybridised well to the probe even in the presence of 20 times as much ROM DNA as test DNA. ROM DNA on its own does not hybridise with the probe.

DISCUSSION

In most cases, the presence of Robertson's cooked meat DNA would not interfere with the signal obtained with the probes. Hence once DNA extraction was carried out from broth culture, the DNA could directly be placed on the membrane and probed, with no attempt being made to separate RCM DNA.

Lane 1 Lane 2 + 200ng RCM DNA

RCM DNA

- M. kansasii
- C. perfringens
- N. brasiliensis
- C. JK
- M. paratuberculosis
- P. avidum



Fig 5.A.6

Lanes 1 and 2 have 10ng of the DNA sample listed on the left, however, lane 1 has in addition 200ng of RCM DNA. Dot blot probed with probe pp107. Hybridisation was carried out at 65° C for 16 hours the membrane washed with 3 x SSC, 0.1% SDS and then exposed to an x-ray film overnight at -70° C. Except for *M. paratuberculosis*, the signal obtained with the addition of 200ng RCM DNA is no worse than the signal in the absence RCM DNA. Hence in most cases, 20 times as much RCM DNA as DNA tested will not affect the signal on hybridisation with probe pp107.

PART 3

Use of PAFO DNA: dot blotting with mycobacterial probes.

MATERIALS AND METHODS

DNA from *M. smegmatis* and *M. kansasii* grown in ROM broth, DNA from *M. kansasii* grown on Lowenstein Jensen medium and ROM DNA were used as controls, at concentrations down to long. Aliquots of DNA obtained from the PAFOs (from CD, UC and non IBD cases) were also placed on nylon membranes and dot blotting was carried out using probes pp107(from *M.kansasii*) pmB20 (from *M.paratuberculois*) and PME₂₂(from insertion sequence of *M.paratuberculosis*).

PAFO DERIVED DNA SAMPLES

The DNA in bank II (PAFO DNA bank) was used. In several cases, no DNA was visualised on a gel: ie less than 30ng DNA was present. All these samples were reprecipitated in ethanol but the same result was obtained. lµl aliquots of samples were spotted onto a 1% agarose gel with ethidium bromide and examined under UV light, but again no DNA was visualised.

However, it was known that long of DNA on the dot blot was sufficient for hybridisation to occur with the mycobacterial probes (Part 1, Fig 5.A.2 and 5.A.3). In these samples, RCM DNA could not be removed; this did not matter as probes pp107 and pmB20 do not react with RCM DNA (Section 5A, Part 1) and mixtures of bacterial DNA with RCM DNA still react with the probes (Section 5A, Part 2). The amount of DNA spotted onto the membrane varied according to the amount of DNA available, the minimum estimated to be 10-20ng of DNA.

SOUTHERN BLOTTING

8 Pvu II digested DNA samples were tested on Southern blotting with probes pp107, pmB20 and pMBr16. Methodology was as described

above. 3 samples were from CD derived PAFOs, 3 from UC derived organisms and 2 from non-IBD organisms.

RESULTS of dot blotting

Probes pp107 and pMB20

Hybridisation results using DNA from representative cultures from 6 CD patients, 3 UC patients and 1 non-IBD patient (details of each culture are listed in Table 5.A.2) are shown in Fig 5.A.7.

Probe pMB22 (Insertion sequence probe)

No hybridisation was detected with any of the PAFO samples tested.

10ng of mycobacterial DNA was shown to hybridise with probe pp107 (Part 1, Fig 5.A.2, Part 3, Fig 5.A.7). Considering the results from Fig 5.A.7, when DNA extracted from *M. kansasii* grown on LJ and DNA from *M. kansasii* grown in RCM are probed with probe pp107, results obtained are identical (Fig 5.A.7.a).

DNA derived from 10/12 CD samples (from 5/6 CD patients), 2/4 UC samples (from 2/4 UC patients) and 0/1 non-IBD samples (from 0/1 non-IBD patient) hybridised by dot blotting to probe pp107. Similar results were obtained with probe pmB20 (data not shown). A further 8/12 CD cultures from 12 CD patients, 4/6 UC cultures from 6 UC patients and 2/8 non IBD cultures from 24 non-IBD patients reacted with the pp107 probe. With all probes, results were consistent when repeated. Results that were inconsistent were given a negative hybridisation result.

Bottle No	Patient and Diagnosis	Details of RCM Culture from which DNA obtained*	Position or Dot Blot	n Signal with probe pp107

1	E.B. (CD)	Culture after 8 years incubation	Al	+ (positive)
2	E.B.	Subculture of bottle one after 8/12 incubation	B4	+
3	A.C. (CD)	Surface growth from culture after 14 months incubation	A3	+
4	A.C.	DNA extracted from broth from culture 3 (after 14 months incubation)	A4	+
5	A.C.	Subculture of bottle 3 after 9 months incubation (surface growth)	C2	+
6	A.C.	Subculture of bottle 3 after 9 months incubation (broth sample	C3)	+
7	A.C.	Another subculture of bottle 3 9 months incubation (surface growth)	D3	+
8	A.C.	DNA extracted from broth from bottle 7	D4 (s g1	- signal ktremely faint, raded negative)
9	B.B. (CD)	Culture after 22 months incubation	A2	+
10	D.H. (CD)	Culture after 3 months incubation	Cl	+
11	D.O. (CD)	Culture after 9 months incubation	C4	+
12	L.G. (CD)	Culture after 20 months incubation	В3	-
13	J.S. (UC)	Culture after 25 months incubation	Bl	+
			cont	d.

Table 5.A.2 Details of IBD samples used for dot blotting in Fig 5.A.7

Table 5.A.2 contd.

Bottle No	Patient and Diagnosis	Details of RCM Culture from which DNA obtained*	Position on Dot Blot	Signal with probe pp107
14	A.B. (UC)	Culture after 23 months incubation	B2	+
15	F.C. (UC)	Surface growth from culture after 20 months incubation	Dl	-
16	F.C.	DNA extraction from broth from bottle 15	D2	-
17	M.F. (non-IBD)	Culture after 14 months incubation	E1	-

+ = Positive result (hybridisation)

- = Negative result (no hybridisation)
- * = Unless stated, DNA was obtained from surface growth and broth



Fig 5.A.7

Sections a and b are part of one Hybond membrane, probed with the DNA probe pp107. Section a shows varying concentrations of DNA as stated. ROM DNA does not hybridise but 10ng of mycobacterial DNA does. DNA from 12/17 samples (from 5/6 CD patients, 2/3 UC patients and 0/1 non IBD patients) are seen to hybridise with the probe (Section b). Non specific signals are those not in line with the DNA samples applied. Row E has only one sample at position E1.

RESULTS on Southern blotting

Probes pp107 (from M.kansasii) and pmB20 (from M.paratuberculosis)

Apart from the λ Hind III marker and the positive control (DNA of *M. kansasii*), no hybridisation was detected with the PAFO samples.

Probe pmB22 (IS probe)

No hybridisation was detected with the test samples. Apart from those listed in the Methods section, a further10 CD samples, 5 UC samples and 2 non-IBD samples were tested by dot and Southern blotting with this specific *M. paratuberculosis* probe but no hybridisation was seen even at low stringency. The positive control *M. paratuberculosis* DNA did hybridise indicating the negatives were true negative results.

Probe pMBr16

DNA from one CD derived organism hybridised with probe pMBr16 on Southern blotting(Fig 5.A.8), giving a different RFLP pattern to other mycobacteria (photograph by kind permission of Dr.J.McFadden). No other samples tested showed a hybridisation pattern.

DISCUSSION

In total mycobacterial probes pp107 and pmB20 (which hybridised to most mycobacteria tested) hybridised to PAFO-derived DNA from 13/18 CD patients, 6/10 UC patients and 2/5 non-IBD patients. None of the samples hybridised with the specific *M.paratuberculosis* probe.

Hence genomic analysis suggests that the organisms are either mycobacteria or very closely related but are not *M. paratuberculosis*. DNA from one CD sample showed a unique RFLP pattern. Perhaps a more specific answer could be obtained by using PAFO derived cloned DNA probes.



Fig 5.A.8

Hybridisation of ³²P labelled cloned probe pMBrl6 to PvuII restriction endonuclease digests of mycobacteria and a CD derived PAFO (labelled PVAFO/1). The RFLP pattern of the CD derived organism is not similar to any of the mycobacteria tested.

SECTION 5B

Preparation of Probes from DNA obtained from PAFOs

INTRODUCTION

Apart from testing mycobacterial probes on dot blots with PAFO DNA, an attempt was made to use cloned PAFO DNA to probe DNA bank I. Random clones were to be used. If sufficient DNA was present, total genomic DNA probes were also prepared from some of the IBD cultures. The probe would then be a very general probe and not as specific as the segment of the DNA cloned.

MATERIALS AND METHODS

DNA from 3 CD patients (A.C., E.B., D.H.), one UC culture (patient A.B.) and from a control patient (PAFO 13) without IBD, were chosen for cloning and preparation of probes. All these cultures grew acid fast organisms in RCM broth and it would be interesting to know whether CD culture-derived DNA and non-IBD culture-derived DNA hybridised to DNA from different organisms.

Part I: Use of Total Genomic Probes

Three examples are discussed.

Dot blotting

Hybridisation was carried out using dot blotting with 47 DNA samples (in duplicate) from DNA bank I (including DNA from 22 mycobacterial samples) or other PAFO derived DNA samples. Two spaces on the template had distilled water blanks. The ³²P labelled probes were prepared from PAFO derived DNA.

Hybridisation was done at 3 x SSC, 0.1% SDS (low stringency) and 1 x SSC, 0.1% SDS (high stringency).Methods are as described previously.

RESULTS

Dot blotting

Total genomic probe prepared from:

a. <u>DNA extracted from a CD culture (from patient A.C.) - probe</u> designated PAF07

RCM DNA did not hybridise, neither did DNA from intestinal and skin bacteria which are not listed below. Signals (1+) from *B. asacharolyticus*, *B. fragilis*, *C. perfringens*, *A. odorans* and *Y. enterocolitica* were eliminated at a higher stringency wash.

All signals showing an intensity of > 3+ at low stringency were with the mycobacterial species *M. neoaurum*, *M. marinum*, *M. paratuberculosis*, *M. gilvum*, *M. avium-intracellulare*, *M. chelonei* and *M. bovis* BCG or with related organisms (*P. acnes*, *C. JK*, *N. asteroides*). At high stringency, strong hybridisation (5+) was only seen with *M. gilvum* and *M. bovis* BCG.

b. <u>PAFO 13 total genomic DNA probe</u> on dot blotting showed hybridisation with *Streptococcus faecalis*, *Clostridium perfringens*, *Staph epidermidis* and *Staph aureus* all at weak intensity (1+).

This same probe on Southern blotting showed weak bands with M. simiae, Lactobacillus casei, Faecal streptococcus, Clostridium perfringens, Clostridium difficile, and Rothia dentocariosa.

c. Total genomic probe prepared from DNA extracted from the UC culture (from patient A.B.). Hybridisation was seen with:

PAFO DNA from patient A.B.¹(4+) and A.B²(2+). Also DNA from *M.* smegmatis ,*M* kansasii and *N.* brasiliensis (3+),and *M.* paratuberculosis (1+). DNA from RCM, Clostridium perfringens and S.aureus (used as negative controls) showed no hybridisation.

- A.B.¹ = hybridisation to self
- A.B.² = hybridisation to DNA from another lymph node culture from the same patient.

When probed against DNA bank I, 18/27 mycobacteria hybridised with the probe. Also 19/50 of the remaining bacterial samples, especially *Nocardia asteroides*, *Corynebacterium JK* and *Cellulomonas biazotea* gave strong signals.

DISCUSSION

The results obtained when using PAFO 7 (CD derived) as a total genomic probe is suggestive of the DNA of a mycobacterial species. Results at low stringency are similar to hybridisation results obtained with mycobacterial probes ppl07 and pmB20 (Section 5A); the strong reactions being with mycobacteria and cross reactions seen with related genera. *M. bovis* BOG and *M. gilvum* retained strong hybridisation at high stringency indicating that these two mycobacterial species showed strong homology with PAFO7 DNA. DNA from the cultures of this CD patient (A.C.) has already shown hybridisation when tested with mycobacterial probes (Table 5.A.2,Fig 5.A.7). Some of this DNA was cloned (see Part II).

Probe PAFO13 (prepared from DNA derived from a non-IBD culture) did not show hybridisation with a mycobacterial species, apart from H. similae where weak bands were seen on Southern blotting. This was probably not a significant result. It may be concluded that PAFO 13 DNA does not orignate from a mycobacterium species.

The probe prepared from the DNA extracted from the UC culture supported the DNA being mycobacterial in origin with cross reaction extending to *Nocardia*, *Corynebacterium* and *Cellulomonas* species. However this is not conclusive.

In conclusion, probes derived from DNA from the one CD and one UC culture supported the DNA being mycobacterial in origin, but cross reactivity was present. DNA from one non-IBD culture however did not show any hybridisation to mycobacteria. Cloned probes would perhaps give more specific answers.

PART II: USE OF CLONED PROBES

a. PREPARATION OF PROBES FROM DNA (CALLED EB DNA) OBTAINED FROM PAFOS ISOLATED FROM LYMPH NODE CULTURE OF CD PATIENT E.B.

MATERIALS AND METHODS

EB DNA was digested with Sau 3A and cloned into the plasmid vector pGEM-1 at Bam-Hl sites by Dr.M.Moss. Approximately 20 clones with inserts (EB DNA) were obtained, after plasmid transformation of \mathcal{E} . coli.

METHOD

Double digests were carried out using 2 clones designated Clone 1 and Clone 2:

10µl of Clone 1 (or 2) DNA

10µl core buffer (10x) according to the manufacturer's recommendations.

78µl distilled water

lµl Hind III enzyme containing 12 units enzyme/µl

lµl Sst I enzyme (5 units enzyme/µl)

1 unit of enzyme digests lug of DNA in one hour.

The 100µl digest was left at 37°C for 3 hours.

The samples then sat on ice. 20μ l 10M ammonium acetate was added, followed by 2 volumes of 100% ethanol and the samples were placed on ice for one hour. To pellet the DNA, the tubes were spun (at 4°C) for 10 minutes. Ethanol was removed and the DNA sample was air dried. 10µl TE buffer was added to resuspend the DNA.

A 1% agarose gel was prepared (1g agarose in 100ml TBE buffer 1x). A section from the middle of this gel was cut out using a scalpel. A 1% low melting temperature agarose gel (0.4g agarose in 40mls 1 x TBE buffer using Bio-rad electrophoresis purity reagent agarose low temperature gel) was poured into the space cut out above and a spacer

was used to prepare new wells. A gel tank with 1 x TBE buffer was used. Lµl of glycerol loading buffer was added to each 10µl DNA sample and electrophoresis was carried out through the 1% low melting temperature agarose gel at 50 volts (56 milliamps) for $1^{1}/_{2}$ hours. The gel was placed in distilled water for a few minutes. 0.5µg/ml ethidium bromide was added to the water and the gel examined under UV illumination.

<u>Clone 1</u>

4 inserts were obtained of which the largest (900 base pairs) was called psp-1.

<u>Clone 2</u> yielded a 500bp insert (designated psp-3).

Small agarose slices with the EB DNA inserts were cut out, and each placed in Eppendorf tubes. The tubes were incubated at 65° C for 10 minutes until the agarose melted. Three volumes of TE buffer (300µl) was added to each tube. Each insert fragment was estimated to have a DNA concentration of approximately 400ng DNA/400µl (20ng/20µl). The samples were stored at -20°C.

Preparation of probes from psp-1 and psp-3

The two samples were defrosted and boiled for 10 minutes to obtain single stranded DNA. Probes were labelled as outlined in Section 5A using 20µl psp-1 (or psp-3). Dot blotting (DNA bank I ,100 ng samples) and Southern blotting (with Pvu II digested DNA: 200 ng DNA used) was carried out (as in Section 5A) using DNA samples listed. All blots were done in duplicate or triplicate.

If repeat probing was done, this meant that each time the probe needed to be prepared with fresh radioactivity. Also, the membrane on which the DNA samples were spotted had to be re-used and probe removed as outlined in Section 5.A. Each membrane could not be used more than

six times (maximum) as the signals appeared to be weaker and weaker with each reprobing.

If any blots were full of background radioactivity then fresh Denhardt's solution and hybridisation solutions were used to see if clear blots could be obtained.

RESULTS

DOT BLOTTING:

The % label incorporation was 58% for psp-1 and 32% for psp-3. Probing of membranes by dot blotting:

Cloned probe psp-1: Hybridisation occurred at an intensity of 1+ with M. intracellulare only.

Clone probe psp-3: No signals obtained.

DISCUSSION

Out of the many bacteria tested, only a weak reaction with *M.intracellulare* was noted when psp-1 was used. The clones psp-1 and psp-3 were randomly chosen. Any part of the PAFO DNA from partient EB may have been present in the clones. However, surprisingly the DNA in these clones did not react with a large range of organisms. Radioactive incorporation was good, however failure may have been due to other technical reasons. The experiments were repeated but identical results were obtained.

The alternative conclusion is that the DNA inserts were unique and did not have any sequences homologous to the samples tested.

SOUTHERN BLOTTING

A single band was detected with *M. simiae* and *C. minutissimum* DNA (Fig 5.B.1) and *C. JK* (data not shown, band of similar molecular weight and intensity). No signals were detected with clone psp-3.

DISCUSSION

Probe psp-1 obtained from DNA from PAFOs cultured from a CD patient E.B. showed hybridisation by Southern blotting to \mathcal{H} . similae and two species of Corynebacteria (C. minutissimum and C. JK). This could indicate cross reaction with corynebacteria. However, \mathcal{H} . similae was the only one of 10 mycobacterial species tested which hybridised with this clone .

Conversely, it could mean that the probe hybridised with Corynebacterium DNA and cross reacted with *M. similae* DNA. This would mean that Corynebacterium DNA was present but the species had not been detected by bacteriological culture.

Finally, the third possibility is that all three samples (M. similar, C. minutissimum and C. JK) were all picked up as cross reactions and the homologous mycobacterium (or other bacterial species) had not yet been identified.



Fig 5.B.1

DNA from mycobacteria, related species, and other bacteria digested with Pvu II and probed with 32 P labeled psp-1, a cloned PAFO DNA derived probe from culture E.B.

Hybridisation as a single band is seen only with DNA from M. similar and C. minutissimum (labelled tracks).

b. PREPARATION OF CLONED PROBES FROM A NON-IBD CULTURE YIELDING PAFOS (DESIGNATED PAFO 13)

MATERIALS AND METHODS

loong of this DNA labelled PAFO13 was digested with the RE Sau 3A for 2 hours at 37°C. The loµl reaction mixture contained loong PAFO13 DNA in 2.5µls TE buffer, lµl 10 x Sau 3A buffer, lµl Sau 3A enzyme (at 4u/µl) and 5.5µl distilled water.

As described in Section 5.B. Part II(a) for EB DNA, PAFO13 DNA was cloned using a ligation reaction - $l\mu$ l Sau 3A digested PAFO13 DNA (10ng) was ligated with BamH₁ digested and phosphatase (70ng) treated P-GEM1 plasmid vector ($l\mu$ l), $l\mu$ l T₄ DNA ligase (1 unit), $l\mu$ l ligation buffer (10x) together with 6µl distilled water. The reaction mixture was incubated at 15°C overnight. Competent *E. coli* cells were provided by Miss Jo Thompson. Twelve colonies (of approximately 500-1000 colonies obtained) were chosen and DNA extraction carried out. The DNA was then digested with Pst I and Sst I and gel electrophoresis performed. In 11 cases inserts of less than 320 base pairs were seen. The twelfth clone had a 1.3Kb insert which was cut out according to the method in Part II (a) and a probe was prepared from this insert as described previously.

RESULTS

The PAFO 13 Derived Clone Probe showed hybridisation with:-

Cl. perfringens, Cl. difficile, S. aureus, E.coli, Faecal strep, M. gilvum, C. JK, M. paratuberculosis, S. enteritidis, E. cloacae A. odorans, P. assacharolyticus, G. vaginalis and A. hydrophila: all showed weak hybridisation (1+).

DISCUSSION

Similar conclusions can be drawn as when PAFO 13 DNA was used as a total genomic probe - ie, that PAFO 13 DNA is unlikely to be mycobacterial in origin.

c. <u>Further cloned probes</u> were prepared by Dr. M. Moss by cloning Sau 3A digested PAFO DNA into the vector PUC 13. Clones were obtained after plasmid transformation of *E. coli*. Although several samples were tested only results from patients D.H. and A.C. are outlined below. Patient D. H.

The results from this patient were unusual and are specifically discussed. Primarily, the appearance of organisms isolated were quite different on Ziehl-Neelsen (ZN) staining to the usual appearance of PAFOs isolated.

Background

This patient had a subtotal colectomy for Crohn's disease and two small lymph nodes from the ileo caecal region were cultured on two LJ slopes, two slopes of Herrold's egg yolk medium with mycobactin and 4 RCM bottles.

Three weeks post inoculation, one of the LJ slopes was noted to have a buff coloured micro-colony and ZN staining showed acid fast bacilli with cording (see Fig. 3.4. 3 d, pg 85).

The colony was left for one month and a further ZN stain also showed acid fast bacilli. Subculture onto another LJ slope also showed very feeble growth which was positive on ZN staining. However, the organism failed to grow in any significant amount to allow further identification.

RCM (15ml) This culture was also ZN positive (see Fig. 3.A.3.d)

DNA was extracted and a positive result was obtained on probing with probes pp107 and pMB20 (Figs 5.A.7; Table 5.A.2 - C_1)

DNA obtained was cloned into a pUCl3 vector as described above and the insert from clone 3 (approx 200 base pairs) was used as a probe. On dot blotting using mycobacterial DNA and other PAFO derived DNA on membranes, positive signals were obtained. Conditions used were 3 x SSC, 0.1% SDS, six days exposure at -70° C. Hybridisation results are shown below in Table 5.B.1.

RESULTS

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Table 5.B.1	Use of Clone	ed probe from PAFO DNA (pt. D.H.) :
DNA from	Diagnosis	Signal Intensity (dot blot hybridisation)
Patient		
D.H. (self)	(CD)	4+
J.S.	(UC)	1+
A.B.	(UC)	1+
E.B.	(CD)	3+
A.C. ¹	(CD)	2+
D.O.	(CD)	1+
R.I.	(UC)	1+
A.C. ²	(CD)	1+
A.C. ³	(CD)	1+
M. smegmatis	(in RCM)	3+
M. kansasii (in RCM)		3+
M. kansasii (grown on LJ)		3+
M. paratuberculosis (in LJ)		1+

A.C. 1, 2, 3: cultures 1 and 2 are from two lymph nodes from patientA. C. Culture 3 is a subculture from culture 2.

DISCUSSION

The ZN appearance of the organisms from the microcolony (grown on primary inoculation of an LJ slope) suggested the presence of a *Mycobacterium* species - on ZN staining alone highly suggestive of *M. tuberculosis*. However, neither the original culture or subcultures showed further growth for complete identification, although significantly subcultures also showed acid fast bacilli.

Other parameters however were also suggestive of a *Mycobacterium* species. Mycobacterial probes pp107 and pmB20 hybridised to DNA from the RCM culture. Also a clones probe prepared from this DNA hybridised to DNA from other PAFOs and mycobacteria. The appearance of the organisms on ZN staining from the RCM culture was that far more acid fast bacilli were seen than usual (rather than pleomorphic acid fast material alone). However DNA hybridisation with PAFO derived genomic DNA occurred. It is also notable that traces highly suggestive of the presence of tuberculostearic acid was obtained when the RCM culture was analysed by gas chromatography and mass spectrometry (Chapter 6), supporting the presence of mycobacteria in the culture.

Patient A. C.

The dot blot (with samples from DNA bank I) was probed with total genomic PAFO DNA (PAFO 7) obtained from a CD patient A. C. - see Section 5B Part I. DNA was again cloned and the insert from clone 1 used as a probe.

RESULTS

Dot blot hybridisation with ³²P labelled cloned probe prepared from DNA derived from PAFOs (from patient A.C.):

<u>Table 5.B.2.</u>

DNA on membrane	Diagnosis	Signal Intensity			
From patient					
A.C. ¹	CD	3+			
A.C. ²	CD	2+			
D.H.	CD	2+			
R.I.	UC	2+			
A.C. ³	CD	3+			
D.O.	CD	1+			
A.C.4	CD	2+			
A.C. ⁵	CD	3+			
J. S.		-			
A.B.		-			
B.B.		-			

Other DNA Samples

M. avium-intracellulare

1+

RCM DNA, S. aureus, C. difficile) C. perfringens)

A.C.^{1,2} : alternative cultures to culture from which probe DNA was prepared (same patient). A.C.³: subculture of culture A.C.¹.

A.C.⁴: DNA from surface growth and A.C.⁵ was DNA from the broth of the same culture bottle as A.C.⁴. A.C.⁴ and A.C.⁵ were from the subculture of $A.C.^2$.

DISCUSSION

With clones from DNA from patients D.H. and A.C. hybridisation is seen with other PAFOs, suggesting homology between these different strains. However, exact identification of the PAFOs did not prove possible. It was unlikely that pursuing dot blotting with PAFO DNA would yield any more significant results and not enough DNA was available for Southern blotting. Nevertheless, a relationship between PAFOs from different CD cultures and UC cultures were shown although in most cases the hybridisation was not very intense.

Also homology has been shown between PAFOs and mycobacteria although certainly not in all cases a notable exception being the PAFOs from the control (Non - IBD) culture (PAFO 13) tested.

CHAPTER SIX

INVESTIGATION FOR THE PRESENCE OF TUBERCULOSTEARIC ACID IN THE IBD DERIVED CULTURES

Tuberculostearic acid (10-methyl octadeconoic acid) is a liquid saturated fatty acid, first isolated by Anderson and Chargaff from M. tuberculosis in 1929. In M. tuberculosis, the acid amounts to approximately 10% (wt/wt) of the total fatty acid content. Tuberculostearic acid (TSA) is a structural component of mycobacteria it is a characteristic consituent of the extractable lipids of mycobacteria (Hunter, Gaylord and Brennan, 1986; Minnikin, 1982).

TSA has been demonstrated in many mycobacterial species and in a limited number of other organisms of the order Actinomycetales. If the pleomorphic acid fast organisms isolated contain TSA, this would indicate that they belonged to a genus of this order.

Larsson *et al* (1979) used gas chromatography (GC) and mass spectrometry to estimate the amount of tuberculostearic acid in various mycobacteria. Demonstration of TSA in sputum (Odham, Larsson and Mardh, 1979; Larsson *et al*, 1980; Larsson *et al*, 1987; French *et al*, 1987a) and cerebrospinal fluid (Mardh *et al*, 1983; French *et al*, 1987b) by gas chromatography and mass spectometry (GC-MS) has been used for rapid and/or early diagnosis of mycobacterial infections. GC-MS used in the selected-ion-monitoring mode (SIM) offers a very high degree of sensitivity and selectivity enabling specific microbial constituents to be detected even at picogram levels in complex mixtures such as clinical specimens. It was decided to apply the technique of GC, and GC combined with MS to analyse the IBD cultures.

MATERIALS AND METHODS

Samples

The following 48 samples were analysed:-

- M. kansasii in RCM broth culture, incubated at 32°C for 18 months.
 Iml broth was used (positive control).
- Uninoculated RCM broth, incubated at 32°C for 18 months (negative control). 1ml broth was used.
- 3. M. tuberculosis (H37RV strain) standard grown on Lowenstein-Jensen medium. Cells were harvested and autoclaved.
- 4. 16 samples from 12 patients with CD, 10 samples from 7 patients with UC and 8 samples from 4 non-IBD patients. All these samples were from culture which grew the acid fast organisms. 1ml of broth culture, including some surface growth was used per sample. 0.5mls broth was used in some cases when 1ml was not available.
- 5. Three CD, three UC and three non-IBD cultures which did not grow the acid fast organisms were also chosen. 1ml of broth culture was used per sample (no surface growth was visible).
- 6. Straight chain C₁, methyl ester (nonadecanoic acid, C₁, 0= CH₃ (CH₂)₁₇COOCH₃). The purified ester was obtained from Dr D Minnikin, University of Newcastle-upon-Tyne.
- 7. Reagent blank fatty acid extraction and derivatisation steps were carried out without any RCM sample. All RCM samples were coded (so chromatography was done without knowledge of the origin of the cultures) and autoclaved.

SAMPLE PREPARATION - FATTY ACID EXTRACTION

Lipid extraction was carried out. The standard Bligh-Dyer method was used.

To lml of sample, 1.25ml chloroform and 2.5ml methanol (1:2) was added in glass tubes. Volumes were adjusted for 0.5ml samples.

Samples were vortexed to mix, 1.25ml chloroform was added and mixed. 1.25mls distilled water was added and gently shaken, resulting in two phases. Overnight separation was allowed in stoppered tubes. The upper (methanol/water phase) layer was removed and the lower (chloroform) phase was removed with a Pasteur pipette and used as the lipid extract. The lower phase was transferred to 25ml glass tubes and evaporated to dryness under nitrogen. When dry, 2.5ml of alcoholic KOH was added and heated for 1 hour at 80°C in a water bath. After 1 hour, stoppers were removed and 2.5ml distilled water was added. The stoppers were replaced and the tubes left for a further 10 minutes.

The tubes were removed from the water bath and allowed to cool. Two drops of 0.1% thymol blue (as indicator) were added to each tube. Next, two 5ml hexane washes were done. The hexane washes were discarded. 0.75ml of 1N HCl was added to each tube. Then 2.5ml hexane was added and left at 4° C overnight. The wash was transferred to a 10ml tube. A further 2.5ml hexane was added to the original tubes and vortexed. The upper layer (hexane wash) was added to the previous wash layer and evaporated to dryness. The samples were redissolved in heptane and transferred into small tubes. Again the samples were evaporated to approximately 0.2ml volume under nitrogen and then methylated with 1-2 drops of diazomethane using a pipette. Next they were evaporated under nitrogen for a few seconds. Methylated samples were frozen at -20° C until required for injection into the GC machine.

A. <u>GC-FID (GAS CHROMATOGRAPHY WITH FLAME IONISATION DETECTION)</u>

The samples as methyl esters were first analysed by conventional gas chromatography using FID. A Perkin Elmer Sigma 2000 GC equipped with a WCOT Chrompak Sil 5 capillary column (25m long, internal diameter 0.32mm) and a guard column (length lm) was used. Injections were through a split-splitless injector used in the splitless mode. The flow rate of the helium carrier gas was 44 cm³/sec (at 100 KPa).

The injector port temperature was 350° C and the temperature programme was set as follows - 130° C for one minute, followed by 25° C/min rise up to 205° C, then 5° C/min increase up to 275° C. The flame ionisation detector temperature was 350° C.

0.5µl of each sample was injected using a Hamilton syringe. The *M. tuberculosis* standard, *M. kansasii* sample, uninoculated RCM sample and eleven other IBD derived samples were analysed. Results showed (Fig 6.1) that this method was not sensitive enough. Hence analysis was then done by GC-MS.

B. <u>GC-MS (GAS CHROMATOGRAPHY WITH MASS SPECTROMETRY) USING POSITIVE</u> <u>ION MONITORING</u>

A quadrupole mass spectrometer (Hewlett Packard 5970, benchtop Mass Selective Detector) of ionisation energy 70ev coupled to a GC (Hewlett Packard 5890 GC) was used.

The gas chromatograph was equipped with a 25m WCOT fused silica capillary column (Chrompack CP-sil-5) of internal diameter 0.32mm. Samples were introduced using splitless injection. The injector port temperature was 250°C and the oven temperature 130-275°C.

The flow rate of the carrier gas helium (BOC) was lml/min. The mass selective detector was operated under a vacuum of 10^{-5} torr. The voltage of the electron multiplier was 2800 volts. Digital signals were processed by a Hewlett Packard (HP) 300 data system and stored on a 20 megabyte high capacity flexible disc cartridge (IOMEGA). The chromatograms and spectra were printed with a graphics printer (ThinkJet Printer HP) or a laser printer (LaserJet printer HP). lul of each methylated sample was injected and analysed for a time span of twenty minutes per sample.

SECTION B.1

First full scan data for all samples was obtained. Total ion current profiles (TICP=TIC) were studied and individual ion signals were extracted (extracted ion current profiles = EICP) at m/e(mass/charge) 312, 199 and 167 (see Table 6.1). Thus data for these few ions characteristic of TSA is extracted from the larger set used to generate a TIC. Also samples were coinjected with the *M. kansasii* sample to check for TSA peak superimposition and summation. The amount of the test extract coinjected with the *M. kansasii* extract was adjusted each time according to the proportions of TSA present in each sample. 1-2µl of the mixture was injected.

SECTION B.2

GC-MS was used in a Selection-Ion-Monitoring (SIM) mode. The mass spectrometer was focussed on fragments characteristic of methylated tuberculostearic acid (m/e 312, 199 and 167). In the SIM mode, the mass spectrometer is a very specific detector as preselected ions are scanned exclusively. The mass spectrum of TSA shows characteristic ions. The C18 branched (C_{18} CH₃) methyl ester of TSA is shown in Fig 6.1 as the molecular ion (M) at m/e 312. M/e 199 and m/e 171 are abundant fragment characteristic of the methyl side chain at position 10. The fragment m/e 199 readily loses a molecule of methanol giving m/e 167 virtually absent in mass spectra of other C_{19} methyl ester positional isomers including the straight chain ester.

Hence EICP data and SIM data represent the fragments m/e 312, m/e 199 and m/e 167.

C. <u>NEGATIVE-ION MASS SPECTROMETRY</u> (Negative Ion Chemical Ionisation -NICI) using Selected Ion Monitoring (SIM)

Samples

Pentafluorobenzyl ester derivatives were prepared from the methylated samples (already prepared for work for Sections A and B).

SAMPLE PREPARATION

5% aqueous tetrabutylammonium hydroxide was added and the mixture was heated at 100°C overnight. Once cooled to room temperature, the samples were centrifuged. To the supernatant, CH₂Cl₂ (dichloromethane) and Pentafluorobenzyl bromide (25µl) were added. After mixing for one hour and centrifuging the sample, the upper aqeuous layer was removed. Two washes using 10% HCl and two distilled water washes were carried out. PFB esters were obtained after evaporating the samples to dryness under nitrogen.

<u>GC-MS</u>

A Hewlett Packard (HP) 5890 gas chromatograph with a VG Analytical Ltd., Manchester, VSEQ Hybrid mass spectrometer and a Micro VAX2000 based data system was used. The printer was a DEC LNO3R post-script printer.

Samples were introduced and separated on a 20m (0.33mm internal diameter) quartz capillary column coated with SE-30 (stationary phase) using splitless injection. The flow rate of Helium carrier gas was approximately lml/min. The injector was a HP splitless injector, at 275°C. The oven temperature was programmed from 80°C to 275°C at a 25°C/min rise after a one minute initial delay. Negative Ion Chemical Ionisation (NICI) was done using methane as the reagent gas. SIM analyses were performed by monitoring the carboxylate ions of m/z (m/e) 297.3.

RESULTS

A. <u>GC-FID</u>

Typical results are shown in Fig 6.1. Trace (a) shows the large TSA peak seen (at a retention time of 5.91 minutes) with the *M.* tuberculosis standard (extract from culture incubated at 37°C on Lowenstein Jensen medium). Trace (b) shows hardly any peak at all at the expected retention time for TSA. This was with the *M. kansasii* sample (extract from *M. kansasii* grown in RCM). This method of detection was therefore not sensitive enough to detect TSA from the small amounts of *M. kansasii* used for the lipid extraction and subsequent derivatisation.

Comparable amounts of a CD derived sample (trace (c)) also failed to show a peak at the expected retention time for TSA.

B. GC-MS with positive ion monitoring: TIC, EICP and SIM Data <u>SECTION B.1</u>

TIC AND EICP

TIC data obtained with the extract from *M. tuberculosis* shows a large TSA peak (Fig 6.2b), but the TSA peak height is very small when the culture extract of *M. kansasii* (grown in RCM) was used (Fig 6.2a). The TSA peak height (if TSA were present) with a CD-derived culture would therefore be expected to be even smaller as there were very few organisms compared with *M. kansasii* per ml of culture (also the proportion of TSA present in relation to the total fatty acid content was unknown). This was found to be the case and a typical example is shown in Fig 6.8(a); the TSA peak is not visible in the TIC profile amongst other peaks.

Peaks other than TSA are due to other fatty acids such as myristic, palmitoleic, palmitic and oleic acids.
A highly complicated TIC profile is seen with the extract of *H. kansasii* in RCM. This was seen with all the RCM cultures, as all extractions were done directly from RCM broth. This is in contrast to the *M. tuberculosis* standard where extraction was performed from bacterial cells scraped off Lowenstein-Jensen medium.

The extracted ion current profile (EICP) for *M. tuberculosis* is shown in Fig 6.2.(c). This EICP data was obtained by extracting the data for m/e 312 from the TIC data shown in Fig 6.2(b). It is seen that the EICP simplifies the data by vastly reducing the number of peaks. This was seen with all the test cultures also. The EICP removes ion abundance data from background unresolved components and other irrelevant ions. Hence from now on, although TIC data was obtained for all samples, only EICP profiles are shown in the results. The second (small) peak seen in Fig 6.2.(c) is due to the C19 straight chain (CH₂)(CH₂)₁₇COOCH₃-nonadecanoic acid). The C₁₉ straight chain peak was seen in all extractions as expected (see discussion). This peak occurred at a retention time of approximately 7.6 minutes (Fig 6.3). This is compared to the TSA peak which occurred at approximately 7.1-7.2 mintues. Both the TSA and C19 peaks were slightly proximal to the usual retention times in Fig 6.3.

Mass chromatograms obtained when monitoring the methyl esters of an extract of *M. tuberculosis* at m/e 312, 199 and 167 (extracted from TIC data) are shown in Fig 6.4(a). The TSA peak is seen between 7.1 and 7.2 minutes (retention time). It is known that the peak area is a direct reflection of quantity of compound injected (Odham, Larsson and Mardh, 1979). When employing ion selection at m/e 167, the sensitivity is reduced compared with ion selection at m/e 312. This is in total agreement with the results seen in Fig 6.4.(a) where a much smaller peak

area is seen at m/e 167 compared with m/e 312. M/e 199 shows a peak height (and area) in between the m/e 312 and m/e 167 traces. This is in agreement with the mass spectrum of TSA (Fig 6.6c)

The peaks obtained with the extract from the culture of *M. kansasii* in ROM shows similar results to the *M. tuberculosis* standard with peaks at m/e 312, 199 and 167 at the retention time expected for TSA (between 7.1 and 7.2 minutes). The fact that the culture extract was done directly from ROM broth did not interfere in any way with the result. Results were similar to an *M. tuberculosis* extract from a Lowenstein-Jensen (LJ) slope.

The extract from uninoculated ROM broth shows no peaks corresponding to TSA. This experiment indicates that the material from ROM broth separating on the column did not interfere with determination of TSA. If an extremely sensitive method such as SIM is used, the nonadecanoic acid peak is seen (Fig 6.7c) when uninoculated ROM is run but this does not interfere in any way with TSA determination.

REPRESENTATIVE RESULTS OBTAINED WITH CD, UC AND NON-IBD CULTURES

A typical EICP result for a CD derived culture extract (again showing m/e 312 extracted from TIC data) is shown in Fig 6.5. The TSA peak (arrow) in Fig 6.5a is seen to be small compared to the peak height with the *M. tuberculosis* standard (Fig 6.2c).

This is because there is comparatively very little TSA in the CD sample. When this CD culture extract was coinjected with the extract of *M. kansasii* superimposition and summation of the TSA peaks was seen. The large C_{19} peak is seen at 7.6-7.7 minutes and the 2 'RCM culture derived' peaks are seen between the TSA and C_{19} peaks.

MASS SPECTRA

The mass spectrum of the presumed TSA peak detected in the IBD derived cultures was analysed. The mass spectrum looks at all the expected ion fragments for TSA at the expected retention time for TSA and is a very definitive test of the presence of TSA. An example is shown in Fig 6.6. Fig 6.6(a) shows a large TSA peak. This peak occurred at a retention time of 7.373 minutes, rather later than the usual TSA peak (7.1-7.2 minutes). Variation in retention time such as this was known to occur with this GC-MS machine (J Honour, personal communication).

The Cl9 straight chain peak also occurred late, ie the whole trace appeared shifted. When coinjected with the *M.kansasii* extract there was superimposition of the 7.37 peak with the TSA peak of *M. kansasii*. The mass spectrum at 7.37 minutes was analysed from the TIC data (Fig 6.6b).

Fig 6.6c shows the mass spectrum of *M. tuberculosis* derived TSA. It indicates the expected molecular weight of 312 (Molecular ion m) for the C_{18} branched methyl ester (TSA). In addition the abundant fragments at m/e 199, m/e 171 and m/e 167 are found (see Table 6.1).

In comparison, the mass spectrum in Fig 6.6(b) is very similar and highly suggestive of TSA. All ion peaks seen in the *M. tuberculosis* TSA mass spectrum are almost identically reproduced. Hence even though the TSA peak appeared shifted it appeared to be a genuine TSA peak.

SECTION B2

SIM DATA

Selected ion monitoring of the culture extracts and reference esters were performed in order to improve sensitivity. Mass chromatograms were obtained when monitoring the methyl esters of the extracts at m/e 312, 199 and 167. Several samples thought negative for

TSA from EICP data were shown to be positive by SIM. Hence collective results for SIM data rather than EICP data are shown (Table 6.2).

Representative results obtained by SIM are shown below. The scales shown reflect the extremely high sensitivity it is possible to achieve with this technique. Fig 6.7 shows the TSA peak from the *M. kansasii* extract (a) and the TSA peak from a RCM culture extract (b), from a patient with ulcerative colitis. The RCM is seen to cause no interference with the appearance of a TSA peak. Also there is no peak before 7.6 minutes in the extract from uninoculated RCM broth (c). The only peak visible with uninoculated RCM is the $C_{19}:0$ straight chain peak.

The C_{19} peak and the 2 peaks peculiar to RCM cultures are seen in the trace from the UC culture extract (b).

With some samples two peaks very close to each other occurred at the retention time expected for TSA (Fig 6.9a). However, there was no difficulty in ascertaining which peak was the TSA peak - coinjection with the *M. kansasii* extract showed superimposition of the relevant peak (Fig 6.9b) - in this case the second peak was the TSA peak (hatched) as there was summation with the TSA peak from *M. kansasii* (off the scale Fig 6.9b).

A typical example of a CD derived RCM culture extract starting from the TIC profile through to SIM is illustrated. Several traces showed a similar pattern in that apparently no peak was seen on EICP (Fig 6.8b), even when the scale was adjusted similar to the scale in Fig 6.9a; however a definite peak(s) was observed when SIM was used (Fig 6.8c; Fig 6.9a is Fig 6.8c magnified to show the small peaks clearly).

The 9 CD, UC and non-IBD culture extracts which did not grow the PAFOs did not show a TSA peak. Some of the cultures that did grow the PAFOs also failed to show a TSA peak even on SIM. A typical result from a non-IBD lymph node derived extract is shown in Fig 6.10.

Cultures that did not show TSA were similar in appearance to an uninoculated RCM culture on SIM (Fig 6.7c), ie only the $C_{19}:0$ (nonadecanoic acid) peak was seen as in Fig 6.10(b).

Results of mass spectra or SIM done at m/e 312,199 and 167 were not always clear cut. For example the mass spectrum seemed to show ions that were not expected (see discussion) or the relative amounts of the fragments with m/e 312, 199 or 167 were not as expected from the mass spectrum of TSA. Hence, negative ion monitoring with SIM was carried out on four such samples, as an alternative technique to confirm the presence of TSA.

NEGATIVE ION MASS SPECTROMETRY WITH SIM

Results with negative ion-SIM were completely consistent with the presence of TSA. The uninoculated RCM extract did not show a TSA peak The M. kansasii extract (data not shown) and the 4 (data not shown). IBD derived samples showed TSA peaks. Results are shown in Figs 6.11 and 6.12. Both TSA and nonadecanoic acid were detected in the samples. Fig 6.11c shows the TSA peak of M. tuberculosis (H37RV standard). The UC derived culture (a) and the CD derived culture (b) show TSA peaks and the Cl9:0 peak at 8:01/8:02 minutes. It is notable that the other peaks seen in traces (a) and (b) are virtually identical. These peaks could represent fatty acids secreted by the organisms. It is interesting that virtually identical patterns were seen with the two UC (Fig 6.11.a; Fig 6.12.a) and the two CD (Fig 6.11.b; Fig 6.12.b) samples. All peaks were virtually identical except peak heights were different.

m/e	Identity
312 (M)	$\begin{array}{c} CH_3OC \ (CH_2)_8 \ CH(CH_2)_7 CH_3 \\ \ & \\ O & CH_3 \end{array}$
283	M-29
281	M-31
269	M-43
199	CH ₃ OC(CH ₂) ₈ CH— ∥ O CH ₃
171	CH ₃ OC(CH ₂) ₈ — ∥ O
167	loss of CH ₃ OH from m/e 199
149	m/e 167-18
143	CH ₃ OC(CH ₂) ₆ — ∥ O
87	CH ₃ OC(CH ₂) ₂ — ∥ O
74	$CH_3OC = CH_2$ OH

PRINCIPAL PEAKS FROM THE MASS SPECTRUM OF METHYL TUBERCULOSTERATE

M = molecular ion (ionised parent molecule)







Fig 6.2

<u>a, b</u>: TIC data showing a small TSA peak (arrowed) from the extract of <u>M. kansasii</u> grown in RCM(a), compared to a large TSA peak (hatched) from the extract of <u>M. tuberculosis</u> (b).

<u>c</u>: EICP for m/e 312 showing TSA peak (hatched) from <u>M. tuberculosis</u>; data extracted from TIC data shown in (b). The second small peak at 7.6 minutes is due to nonadecanoic acid.



Fig 6.3 Nonadecanoic acid (C_{19} straight chain= C_{19} :0) peak. EICP data: a. Pure sample of nonadecanoic acid as the methyl ester.

b. Extract from <u>M. kansasii</u> grown in RCM, coinjected with the C_{19} straight chain ester. The TSA peak is hatched. Note the two peaks in between the TSA and C_{19} (Cl9:0) peaks. These are peculiar to the RCM cultures and seen in most traces where extracts were made from RCM broth cultures. They were not seen with uninoculated RCM extracts.



extracts of the following cultures at m/e 312, 199 and 167

a. M. tuberculosis grown on LJ medium

b. <u>M. kansasii</u> grown in RCM broth

c. Uninoculated RCM broth.

TSA peaks are hatched. Peaks corresponding to TSA are <u>not</u> seen with RCM broth.



- a. EICP data: peak (arrowed) corresponding to TSA peak of <u>M. kansasii</u> extract, seen in an extract from a CD mesenteric lymph node derived RCM culture.
- b. The peak is additive and superimposes with the TSA peak from <u>M.</u> <u>kansasii</u>. (hatched)

See legend to Fig 6.3 for other peaks visible.



<u>Fig 6.6</u>

- a. EICP from an extract of a CD culture. The presumed TSA peak is hatched (7.373 minutes). For other peaks see legend to Fig 6.3.
- b. Mass spectrum at a retention time of 7.373 minutes. This is similar to the mass spectrum of the methyl ester of TSA from <u>M.</u> <u>tuberculosis</u> (c).



Fig 6.7 GC-MS with SIM at m/e 312.

Representative chromatograms from analyses of extracts of

- a. M. kansasii in RCM broth. The TSA peak is hatched.
- b. ROM culture from a patient with Ulcerative colitis. The TSA peak is hatched.
- c. Uninoculated RCM broth. Only the Clg:0 peak seen.



Fig 6.8

Extract from a CD mesenteric lymph node derived RCM culture: representative chromatograms.

- a. TIC profile
- b. EICP data at m/e 312 false negative for TSA due to lack of sensitivity.
- c. SIM shows TSA peak (arrowed). See Fig 6.9.



Fig 6.9

Same extract as for traces in Fig 6.8

a. A magnified view of Fig 6.8c, showing two peaks at the expected TSA retention time (7.1-7.2 minutes). The second peak (hatched) superimposed with the TSA peak of <u>M. kansasii</u> when the two samples were coinjected (b).



<u>Fig 6.10</u>

Extract from a RCM culture inoculated with a mesenteric lymph node from a patient without IBD (the patient had carcinoma of the bowel). Results do not show a TSA peak either by EICP (a) or SIM(b). This culture extract was therefore negative for TSA.







Fig 6.11

Chromatograms from analyses of RCM cultures from a patient with UC (a) and a patient with CD (b) by using single-ion detection (NICI) with monitoring at m/e 297.3. Hatched peaks represent PFB tuberculostearate. The <u>M. tuberculosis</u> standard is shown for comparison (c). The Cl9:0 peak is at 8:01/8:02 minutes.



Fig 6.12

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Chromatograms from analyses of two further IBD derived samples tested by NICI-SIM at m/e 297.3. Hatched peaks represent PFB tuberculostearate.

- a. Extract from a patient with UC
- b. Extract from a patient with CD
- c. <u>M. tuberculosis</u> standard for comparison

Table 6.2

Presence of TSA indicated in

CD 13/16 cultures

UC 6/10 cultures

Non-IBD 3/8 cultures

All these cultures were positive for growth of the PAFOs.

DISCUSSION

Gas chromatography is a well established technique used to detect mycobacterial lipid constituents in trace amounts. Techniques far more sensitive than GC-FID are now available for TSA detection. Positive ion GC-MS with SIM, and negative ion chemical ionisation with SIM are the main techniques used. Recently, Larsson and colleagues from Sweden, have reported the use of two dimensional gas chromatography with electron capture detection (ECD) as a less expensive but sensitive means for TSA analysis (Larsson *et al.*, 1989). However, this technique MUSIC-ECD (multiple switching intelligent controller column switching system with ECD) is not fully developed as yet in the UK.

The results showed that without doubt GC-MS (positive ion monitoring) with SIM was far more sensitive than conventional GC with a flame ionisation detector. Odham, Larsson and Mardh (1979) suggest that this technique (SIM) is at least 50 times as sensitive as GC with FID. Certainly with the test samples, no TSA peak was likely to be visualised by FID as even the *M. kansasii* extract did not show a clear TSA peak. The PAFOs were present in even smaller quantities than *M. kansasii*, in RCM broth and the proportion of TSA in relation to the total fatty acid content was unknown. Hence only very small amounts of TSA were expected (probably pg levels).

EICP (from TIC data) proved useful (apparent sensitivity increase) by removing the data from irrelevant compounds and ions which are all present in TIC data. However, even EICP data proved insufficient in several cases and SIM needed to be used to detect TSA peaks.

SIM allows extremely high sensitivity as the mass spectrometer scans exclusively at pre-selected ions, maximising the sensitivity of the instrument for the ions of interest. SIM dramatically increased the sensitivity of detection in at least half the samples tested. SIM also has the great advantage of being selective. Analytical difficulties arising due to the complex character of the cultures could be overcome as the technique allows very specific identification of the chosen compound. The retention time of any component peak is unaffected by the presence of other sample components.

For all samples TIC, EICP and SIM data were obtained as it is only from TIC data that mass spectra can be analysed. Ideally, the mass spectra at the correct retention time for TSA, should show if relevant TSA peaks were present or not. However, sometimes the mass spectrum was complicated presumably due to the complex nature of the sample and the presence of peaks apart from those expected for TSA.

NICI with SIM offered even superior sensitivity and selectivity than positive ion monitoring when applied to the diagnosis of pulmonary tuberculosis in sputum (Larsson, Odham, Westerdahl and Olsson, 1987). This is now the preferred diagnostic mode for the analysis of mycobacterial lipid constituents in trace amounts (Larsson *et al.*, 1989).

The sensitivity of the technique was extremely high when applied to the test samples. Quite large peaks of TSA were recorded despite having very small quantities of organisms and small amounts of

derivatised sample as PFB esters. Even though less than 1µl of sample was injected, enough derivatised sample was available for a further 10³ injections.

It was noted that the NICI traces produced were similar in CD and UC samples. It is possible that all the peaks around the TSA peaks are due to fatty acids produced by the organisms, and it is striking that the same peaks occurred in samples originating from different patients with the two different forms of IBD.

Both positive and negative ion-SIM were used at the very limits of sensitivity. Great care was taken to avoid sample contamination inbetween injections and the Hamilton syringe was washed out numerous times after each injection. Although a quantitative assessment was not undertaken, probably pg levels of TSA were detected in this study. It is known that routinely a detection limit of 0.6pg of TSA is acheived with negative ion SIM (Larsson *et al.*, 1987). Hence, the use of GC-MS for TSA was a highly appropriate technique to be applied to cultures with so few organisms. Also the presence of contaminating organisms such as gut bacteria or coryneform organisms or skin derived bacteria introduced during subculture do not interfere with TSA analysis.

Nonadecanoic acid (as the methyl ester) was detected in all samples including the negative and positive controls. This is in agreement with the observations of Larsson *et al* (1987). It is a naturally occurring internal standard and extremely useful as a marker that the derivatisation procedure and analysis was successful. Its absence may indicate either an insufficient sample, a failure of the derivatisation procedure or analytical or instrument pitfalls (Larsson *et al*, 1987). It is notable that the test samples showed this peak and supports good derivatisation and analysis.

The IBD derived samples analysed by NICI-SIM show the TSA peak. This supports the data obtained by positive ion monitoring where sometimes definitive conclusions could not be drawn, although the presence of TSA was strongly indicated. Analysis using the new electron capture detection system may be necessary to further confirm the presence of TSA in these IBD cultures. It is notable that cultures which did not grow the PAFOs showed no TSA peak even on the most sensitive scale.

Tuberculostearic acid (TSA) is present in most mycobacteria, all rhodococci and nocardia, but not in corynebacteria (Collins, Goodfellow and Minnikin, 1982) apart from *C. ammoniagenes*, *C. bovis*, *C. variabilis* and *C. minutissimum* (Collins, 1987; Collins, Goodfellow and Minnikin, 1982; Collins, Smida and Stackebrandt, 1989).

Lipid markers are now being used in the classification and identification of Mycobacteria, Nocardiae, Rhodococci, Coryneform and related taxa. Mycolic acid analysis in mycolic acid-containing strains is now of major chemotaxonomic importance (see Chapter 7, Section A). addition to mycolic acid analysis, analysis for 10-methyl In octadecanoic acid (TSA) has been used as evidence to question classification of various species. Corynebacterium hoagii, C. equi, C. fascians and C. rubrum contain major amounts of 10-methyl octadecanoic acid. This, therefore, does not contradict reclassification of these taxa in the genus, Rhodococcus. The synonym Rhodococcus equi has been suggested for C. hoagii (review by Collins, Goodfellow and Minnikin, 1982). Collins, Goodfellow and Minnikin (1982) suggest that strains within the Arthrobacter, Brevibacterium, Mycobacterium and Corynebacterium group which contain substantial amounts of 10-methyl octadecanoic acid and have mycolic acids with similar chain lengths to most rhodococci should be transferred to the genus Rhodococcus.

However, a clear cut distinction cannot be made between Corynebacterium sensu stricto and Rhodococcus strains on the presence or absence of TSA.

C. minutissimum has mycolic acids similar to corynebacteria (C28 to C36) but contains TSA. C. bowis on the other hand, has TSA but significantly shorter mycolic acids (C22 to C32). Caseobacter polymorphus has short chain mycolic acids (C30 to C36) but resembles the genus Rhodococcus (high G+C%, produces TSA). However, 16S rRNA homology with Corynebacterium variabilis showed 100% sequence homology. Hence, the organism Caseobacter polymorhus should be called C. variabilis (Collins, Smida and Stackebrandt, 1989).

Despite these exceptions, most species of Corynebacteria do not contain TSA. Therefore the suggested presence of TSA in the IBD-derived cultures containing PAFOs, by GC-MS suggests this group of organisms belongs to the genus *Mycobacterium*, or to the very closely related *Nocardia* or *Rhodococcus* species. It seems unlikely that all cultures growing the PAFOs with production of TSA had coryneforms belonging to one of the four TSA producing species of *Corynebacterium*. All these cultures did not grow corynebacteria or any other organisms apart from the PAFOs on bacteriological testing.

Corynebacteria are not only present in the gastrointestinal tract, they are possible contaminants introduced during culture and could not be completely excluded by the use of DNA probes (Chapter 5) due to cross reaction; even if corynebacteria were present (apart from the species listed above) they would not interfere with TSA analysis.

The absence of TSA in corynebacteria (apart from four species) would help exclude the PAFOs from the genus *Corynebacterium*, but this possibility cannot be entirely eliminated.

CHAPTER SEVEN

SECTION A: ANALYSIS FOR THE PRESENCE OF MYCOLIC ACIDS

INTRODUCTION

Mycolic acids are characteristic high molecular weight 3-hydroxy-2alkyl branched fatty acids, which are an integral part of the outer membrane system in mycobacteria. They are attached as esters to position 5 of terminal arabinose residues of the arabinogalactan.Many different structural types of mycolic acids have been characterised from mycobacteria and related actinomycete taxa such as corynebacteria, nocardia and rhodococci (Minnikin, 1982). Mycobacterial mycolic acids are the most complex in structure and possess 60-90 carbon atoms. Nonmycobacterial mycolic acids have relatively simple structures and have 22-66 carbons. Differences in overall mycolic acid, size and chemical structure help in the separation of the genera of the *Corynebacterium-Mycobacterium* axis.

For example, just based on size, the genus *Corynebacterium* has corynomycolic acids of 22-38 carbons, *Nocardia* have nocardomycolic acids of 46-60 carbon atoms and *Rhodococcus* have mycolic acids of 34-66 carbon atoms (Goodfellow and Wayne, 1982).

Mycolic acid analysis should help establish whether the pleomorphic acid fast forms cultivated were mycobacteria.

If mycolic acids were present, they would be present in extremely small quantities. The organisms themselves were present in µg amounts after several months in culture. Tuberculostearic acid was present probably at picogramme levels, hence an extremely sensitive method was needed to analyse for mycolic acids.

i. <u>Samples</u>

0.5-lml of Robertson's cooked meat broth culture was transferred to a separate tube and autoclaved. If meat samples were used a few mg of meat was autoclaved. 17 samples (including 6 samples from subcultures) from 13 CD patients, 11 samples (including 3 samples from subcultures) from 8 UC patients, and 9 samples (including 3 subcultures) from 5 control (non IBD) patients were analysed. 2 CD samples, 2 UC samples and 2 control samples that were analysed were negative for growth of PAFOS. In addition, uninoculated RCM broth and a culture of *M. kansasii* in RCM broth, both at 32°C for 18 months were analysed as the negative and positive control samples respectively. Three standards were also prepared (see part iii).

ii. Mycolic Acid Extraction

Mycolic acids are covalently linked to the wall arabinogalactan and requires vigorous chemical treatment for its release. It is not convenient to purify free mycolic acids, so they are usually isolated as their methyl or other esters. Chloromethyl anthracene derivatives were prepared.

METHOD

A. <u>Alkaline Extraction</u>:

2mls of 15% tetrabutlyammounium hydroxide (TBAH) was added to 1ml of each broth sample to yield tetrabutylammonium salts of the mycolic acids. For samples of meat plus broth, 3mls 15% TBAH was added and ultrasonicated for half an hour after adding TBAH. The samples were heated at 100°C overnight in an oven, then cooled to room temperature. The tubes were centrifuged and the supernatant carefully transferred to Teflon coated screw cap containing tubes.

B. Phase-transfer catalysed esterification of mycolic acids. Chloromethyl anthracene esters were prepared. See Fig 7.A.1. 2mls CH₂Cl₂ (dichloromethane) and 5mg 9-CMA, (9-(chlormethyl) anthracene (Fluka AG, CH-9470 Bucks)) was added. A rotating mixer for 1 hour ensured mixing between the two phases (aqueous TBAH phase and CH2Cl2 phase). The tubes were centrifuged at 2g for 2 minutes. The CMA reacts with the mycolic acids and is transferred into the organic (CH_2Cl_2) phase. The upper aqueous layer was removed and Iml 10% HCl added to the lower layer to neutralise any remaining TBAH. The tubes were shaken vigorously for 1/2 minute, then centrifuged at 2g for 2 The upper layer was removed. Iml distilled H₂O was added to minutes. the lower layer, shaken and centrifuged as above. Again the upper layer was removed and a further distilled water wash done. The lower (CH₂Cl₂) layer was transferred to a small vial and evaporated to dryness under a stream of nitrogen at <37°C.

C. <u>Purification</u>:

The CMA-mycolate derivative is soluble in petrol and the excess CMA reagent is soluble in acetonitrile. Iml acetonitrile and Iml petrol was added, shaken for 1/2 minute and spun at 2000rpm for 2 minutes. (Acetonitrile = methyl cyanide CH₃CN, Petrol = Petroleum spirit 60 to 80°C; both from EDH Limited, Poole). The petrol layer (top) was collected in a small vial. Again Iml petrol was added to the acetronitrile layer, shaken for 1/2 minute and spun at 2g for 2 minutes. The petrol layers were combined and evaporated under nitrogen to dryness. The CMA derivatives remain. These samples were stored at 4°C (in the dark). When ready for use, 50µl CH₂Cl₂ was added to the sample which was then precipitated with 200µl of acetonitrile in a reactivial. The mycolate derivatives were precipitated for 10-30 minutes (in the dark). Further purification was carried out using Bond Elut cartridges.

Fig 7.A.1

(a) Basic mycolic acid structure



- R_1) long carbon
- R_2) chains
- (b) Basis of Phase-transfer catalysed esterification



D. <u>Bond-Elut</u>

Bond Elut Extraction Cartridges

(Analytichem International, Harbor City, California 90 710, USA).

These disposable solid phase extraction cartridges (bonded phase $Si-C_{13}H_{37}$) utilise selective interaction between the compound(s) of interest and a chemically modified silica sorbent. A lml syringe was used to apply positive pressure to the cartridge. Each sample was applied to a different cartridge.

The cartridge was rinsed with 0.5ml of 4:1 CH₃CN;CH₂Cl₂. The reactivial which contained the sample was washed out with lml of the selected buffer (4:1 CH₃CN:CH₂Cl₂) and applid to the cartridge. Washing of the reactivial and pushing the sample through the cartridge was repeated three times. Next, 1ml neat CH3 CN was pushed through cartridge. Next the concentrated, purified isolate was collected. The mycolic acid derivative is much more soluble in CH2Cl2; hence 3mls of a 1:4 CH₃CN:CH₂Cl₂ mixture when pushed through the bond elut cartridge removes the mycolic acid derivative retained so far on the column and the derivative is collected by positioning the Bond Elut over a collection vial. The derivative that was collected was evaporated to dryness under nitrogen. Initially, 50µl THF was added (to dissolve the CMA derivative) and 5µls injected. However, samples were probably too dilute to detect the small amounts of mycolates anticipated, hence all samples had 10 μl THF added, and the whole sample was run on reverse HPLC. All samples were injected with a 10µl Hamilton syringe.

E. <u>Use of reverse high performance liquid chromatography to detect the</u> <u>mycolic acids</u>

Instrumentation

A Gilson 305 pump system was used. A Rheodyne 7125 (Cotati, California) 20µl loop injector was used and the detector was an Applied

Biosystems 980 programmable fluorescence detector at a 360nm excitation wavelength and a 418nm emission wavelength.

The column was a Chromsep (Chrompack Ltd) column holder fitted with 2 Lichrosorb RP18 10cm reverse phase cartridges (Catalogue no 028397) of internal diameter 0.3cm.

Solvent

The mobile phase was a 50:60 tetrahydrofuran:acetonitrile mixture (see Appendix) at a flow rate of 0.3-1 mls/min.All solvents used were of HPLC grade. As an alternative, an ACS (Applied Chromatography Systems) 300/02, pump with a Biorad UV monitor model 1305A set at 254nm was used instead of the Gilson pump with fluorescence detector. However, the fluorescence detector being more sensitive, was the detector of choice. Each fraction was collected as the sample passed through the machine - this was extremely useful as the whole sample could be injected, collected and then re-injected. Also relevant fractions (eg where peaks were present) could be collected and fractions alone reinjected.

iii. <u>Standards</u>

Pure mycolates from M. tuberculosis, C. diptheriae and N. otitidiscaviarum (previously N. caviae) were used. These three samples had already been prepared up to the end of stage C by Mr B Bolton (see methods section above). Img of the CMA mycolate of M. tuberculosis was dissolved in 125µl chloroform and run vertically on a TLC (thin layer chromatography plastic backed silica gel precoated F_{254} , 20 x 20cm, 0.2mm thick plate, Merck) plate using Toluene:acetone 99:1 as the solvent. The CMA derivative can be seen under UV light and the appropriate bands on the TLC plate were scraped off.

A similar procedure for C. *diptheriae* and *N. otitidis-caviarum* was carried out. None of these standards needed Bond Elut extraction as the samples were quite pure. To elute the mycolate off the TLC paper,

4ml diethyl ether was added and mixed on a rotary mixer for one hour. The samples were centrifuged at 2000rpm for 2 minutes. The supernatant was removed carefully (the mycolates are soluble in this diethyl ether layer) and evaporated to dryness under nitrogen. 3mls THF was added and lµl samples were analysed by HPLC.

Results

Of 37 CMA derivatives prepared from CD, UC and non IBD culture samples, 15 were run by HPLC. These 15 samples were from 7 CD patients, 5 UC patients and 3 non-IBD controls. A falling baseline was seen with some of the traces which could not be corrected. Increased "noise" on the baseline was seen with increasing sensitivity. Trace 7.A.2.b shows the baseline trace when only the THF/acetonitrile solution was run through, and indicates the "noise" on the baseline these are not peaks.

Fig 7.A.2 a and c shows that pure mycolate prepared from N. otiditis-caviarum (c) runs much closer to the solvent peak compared to pure mycolate from M. tuberculosis (a). This is because Nocardial mycolates are smaller in size compared to mycobacterial mycolates (number of carbon atoms present are outlined in the Introduction to this section). Mycolates from *C. diptheriae* runs even closer to the solvent peak than Nocardia (data not shown) as the number of carbons are even lower than for nocardia. None of the IBD and non-IBD (control node) samples tested were seen to have peaks closer to the solvent peak than the nocardial peaks hence the recorder chart speed was adjusted to a standard rate (2mm/min) as for Fig 7.A.2. At this speed, mycolates from Corynebacteria will not be seen on the trace as they run too close to the solvent peak.

Peaks were seen with 3 CD samples and 1 UC sample (all of which were positive for PAFOs by Ziehl Neelsen stain) at the expected retention time for mycobacterial mycolates. A typical example is seen in figure 7.A.2.d.

This particular trace (d) was from a CD culture which also showed the present of tuberculostearic acid (Chapter 6). The peaks are at the correct distance (measured from the start of the solvent peak) for mycolates of carbon atom numbers belonging to mycobacteria, rather than nocardia (on corynebacteria). Trace e (Fig 7.A.2) shows no peaks and is from a CD culture which was negative (not positive by ZN staining) for the PAFOs. Uninoculated RCM broth gave a trace similar to b and kansasii positive control (grown in RCM) gave the M. peaks corresponding to mycobacterial mycolates (data not shown). Although only a few cultures showed definite peaks at retention times suggestive of mycobacterial mycolates, several other samples showed minor peaks at the correct retention time, but are not included as positive as the traces were not conclusive. The fluorescence detector proved better that the UV detector and traces obtained with the less sensitive UV detector are not shown.



Fig 7.A.2: Mycolic acid analysis by HPLC

- Trace a : Peaks of CMA mycolic acids of *M. tuberculosis* running approx. 20 mm away from solvent peak
- Trace b : Solvent alone. No peaks are seen, only baseline "noise"
- Trace c : Peaks of CMA mycolic acids of *Nocardia otitidis-caviarum*. Nocardomycolate peaks are much closer to the solvent peak than mycolic acid peaks of mycobacteria
- Trace d : CD sample, positive for PAFO growth. Mycolic acid peaks corresponding to <u>mycobacterial</u> mycolates are seen (arrowed)
- Trace e : CD sample where no PAFO growth was obtained. No mycolate peaks are seen. Only baseline "noise"

DISCUSSION

HPLC is a highly sensitive analytical technique. It is therefore sensitive to interference by sample or solvent contaminants. Hence great care was taken to purify samples as much as possible. It did not prove possible to run all the samples prepared as CMA derivatives at the time, as there were some technical difficulties associated with the use of this extremely sensitive equipment.

One of the difficulties was establishing a baseline which did not fall off as the sample was run. Increased noise was noted with higher sensitivity and a compromise had to be made between sensitivity and an acceptable base line. Hence sensitivity of picogramme levels was not achieved for these traces, probably only nanogramme levels were achieved. However, the data obtained was encouraging and peaks corresponding to corynebacteria were not seen. Peaks corresponding to mycobacterial mycolates were seen in a few samples (3/7 CD patients, 1/5 UC patients, 0/3 control patients). The other samples tested did not appear to have definite peaks and possibly this could be due to the fact that we were unable to attain maximum sensitivity. For one CD sample, the whole RCM sample was autoclaved (including meat) and derivatised in an attempt to obtain as much mycolate as possible. When this sample was run through the machine, no mycobacterial mycolate however, the sample took $l^{1}/2$ hours to run right peaks were seen; through before another sample could be injected. This was because the equipment was too sensitive to cope with so much interfering material as must be present in a whole 10ml culture of RCM. Hence, extraordinary sensitivity being valuable on the one hand, may be associated with the method being unusable under different conditions.

There is no doubt that the use of reverse HPLC with a fluorescence detector potentially provided an extremely sensitive means of detecting

the presence of mycolates in the cultures examined. However, a great deal of time and patience is needed to be spent to obtain optimal HPLC conditions for the samples to be tested. The ability to collect fractions of the sample in different test tubes as the sample was run through the machine enalbed repitition and confirmation of results.

Future work to be done includes analysing the rest of the CMA derivatives. This will be carried out at the University of Newcastle-upon-Tyne by Dr D Minnikin and Mr B Bolton. Also gas chromatography-mass spectrometyry (GC-MS) on the HPLC fraction showing the relevant peaks would need to be carried out for exact identification of the mycolic acids. GC-MS on such high molecular weight compounds is technically difficult and this confirmatory work could not be done as the necessary equipment was unavailable.

The direct running of samples from cultures on 2 dimensional TLC was attempted by White in her PhD thesis (1981). She demonstrated corynebacterial mycolic acids as she particularly investigated cultures containing corynebacteria. She was probably able to demonstrate mycolates on TLC as the corynebacteria were present in very large amounts. This would not be possible in the case of the PAFOs. As discussed in Chapter 3, cultures containing corynebacteria. were discarded as contaminants and only cultures with PAFOs were analysed.

The value of mycolic acid analyses in the classification and identification of mycobacteria is definite - the reverse HPLC data is encouraging and it is hoped that if enough growth of the PAFOs can be obtained, then further analysis may prove possible at a future date.

SECTION B: USE OF WESTERN BLOTS DERIVED FROM SODIUM DODECYLSULPHATE POLYACRYLAMIDE GELS TO TRY AND DETECT PROTEIN PROFILES PRODUCED BY THE IBD TISSUE DERIVED ORGANISMS

INTRODUCTION

Aurodye staining of western blots to compare protein profiles of organisms is now a standard laboratory procedure. It was decided to use sonicated culture filtrates, initially of three CD derived cultures which were positive for growth of the acid fast organisms, to try and detect protein patterns and compare them. Also it would be possible to see if any dominant protein band was present.

If protein bands were present on SDS-PAGE, then monoclonal antibodies directed against mycobacteria, or pooled CD sera (sera had been collected from all patients from whom mesenteric lymph nodes had been used for culture) could be used by immunoblotting.

MATERIALS AND METHODS

Samples for SDS-PAGE

- 1. Uninoculated RCM culture (negative control), at 32°C for 18 months
- RCM culture from a CD patient E.B.- culture incubated at 32°C for 24 months
- M. kansassii in RCM (positive control), culture incubated at 32°C for 18 months
- RCM culture from a CD patient A.C.- culture incubated at 32°C for 22 months
- RCM culture from a CD patient D.H.- culture incubated at 32°C for 12 months

Volume of broth samples was 2mls per culture. Some of the surface growth was also included. Samples were sonicated (see Appendix) and culture filtrates were obtained by filtering through low protein

binding Millipore filters (Millex - GV, 0.22µm). Protein concentrates were estimated by spectrophotemetry (see Appendix), after diluting the filtrate 1 in 100 in distilled water.

Sample	Protein (mg/ml)
Uninoculated RCM	8.3
Patient E B	7.0
M. kansassii in l	RCM 9.3
Patient A C	5.7
Patient DH	4.2

The filtrates were kept at -70°C until electrophoresis.

SDS-PAGE:

discussed in detail in the Methods are appendix. Briefly, electrophoresis was carried out according to the discontinuous buffer system of Laemmli (1970) on slab gels which consisted of 12.5% (w/v) acrylamide in the separating gel and 4% (w/v) acrylamide in the stacking gel. Each sample was dissolved in sample buffer (see appendix) to a concentration of 1 $\mu\text{g/ul}$ and boiled for 2 minutes on a boiling water bath before loading 20µl of each sample onto the gel. A mixture of standard protein markers (15 µl of MW-SDS-200, Sigma in 45 μl sample buffer ; boiled) was used as molecular mass markers. 40 μl sample buffer (boiled) was also run on the gel to detect non specific protein bands.

Electrophoresis was then performed on a vertical slab gel unit (Hoefer) at a constant current of 6 mA for 16 hours (overnight).

WESTERN BLOTTING

The sonicated culture filtrates separated by SDS-Page were then transferred to nitrocellulose paper using a semi-dry electroblotter as described by Kyhse-Andersen (1984). Aurodye, a colloidal gold solution (Janssen) was used to localise proteins on the nitrocellulose membrane.
RESULTS



Fig 7.B.1

Western blot analysis of ultrasonicated culture filtrates of two CDderived lymph node cultures (lanes 4 and 6) and appropriate controls (lanes 1, 3, 5). All lanes were stained with Aurodye.

Lane 1: Sample buffer: three non specific bands are seen; Lane 2: Molecular weight markers; Lane 3: Uninoculated RCM broth; Lane 4: CD derived RCM culture (patient E B); Lane 5: *M. kansasii* grown in RCM; Lane 6: CD derived RCM culture (Patient A C)

Apart from the non-specific bands seen also in lane 1, there seems to be one extra protein band (arrowed) in lanes 4 and 6 which is also seen in the protein profile of M. kansasii (lane 5).

SDS-Page profiles were obtained for the three CD cultures analysed. Results from two of the cultures are shown in Fig 7.B.1,lanes 4 and 6. The third culture gave a similar result. One protein band (arrowed) is visible in lanes 4 and 6 which also appears in the protein profile of *M. kansasii*. The 2 bands immediately below the arrowed band are non specific as they also appear in the first lane where sample buffer alone was run. The positive control, *M. kansasii* grown in RCM, (lane 5) did give numerous protein bands as expected; Robertsons cooked meat did not interfere with the ability to obtain a good SDS-Page pattern for *M. kansasii*. The lane with RCM broth alone (lane 3) showed a smear of protein.

The experiment was repeated with all the above samples and the results of the repeat experiment were identical.

DISCUSSION

One protein band was identified per filtrate on Western blotting of three CD derived sonicated culture filtrates, despite dominant protein from RCM broth itself. The presence of a large amount of RCM protein was likely to be the reason that several protein bands were not obtained for the CD cultures, as in an attempt to dilute the RCM protein, any protein produced by the IBD derived organisms was also diluted.

The positive control *M. kansasii* produced a nice protein profile and was grown in RCM for approximately the same length of time (18 months compared to 12, 22 and 24 months for the CD cultures. However, the amount and rate of growth of *M. kansasii* is far in excess of the PAFOS. Aurodye staining of Western blots of culture filtrates would have been expected to be a sensitive method (minimal detection is approximately 0.1µg of protein) for analysis for the presence of protein antigens. However, the organisms were present in extremely small quantities and any proteins present would also be present in

small quantities. Hence small quantities of the organisms together with dilution of proteins may have accounted for the results obtained. As only one protein band was detected, immunoblotting using pooled CD sera was not carried out. SECTION C: INVESTIGATION FOR PROTEINS SECRETED BY THE IBD-TISSUE DERIVED ORGANISMS

INTRODUCTION

Abou-Zeid *et al* (1986) reported the use of brief (4-7 day) culture of small numbers of BCG bacilli in a defined medium (modified Eagle's minimum essential medium) containing [³⁵S] methionine as a way of identifying proteins which are being secreted by actively metabolising organisms rather than leaking from dead ones. They used culture filtrates free of bacteria for protein analysis.

Secreted components were separated by polyacrylamide gel electrophoresis (PAGE) and analysed by autoradiography and in an Ambisbeta scanner. This technique was used to subdivide the strains of BCG studied according to secreted protein patterns. If this technique could be applied to the PAFOs, it would mean they must be actively metabolising as the organisms have to be alive to take up ³⁵S methionine, use it for protein synthesis and then secrete the proteins.

MATERIALS AND METHODS

6 CD cultures, 2 UC cultures and 1 control culture (non IBD), growing the PAFOs were used. In addition an 18 month old culture of *M. kansasii* growing in RCM broth and its subculture (at 4 months incubation) was used. Uninoculated RCM was used as a negative control. All cultures were originally checked for contamination, as previously described (Chapter 3).

1-2mls of RCM surface growth and broth containing the PAFOs were left to settle overnight in eppendorf tubes, to try and sediment the RCM meat. The supernatant was divided into 3 tubes. The tubes were centrifuged at high speed for 10 minutes. The pellet was resuspended in 50µl modified Eagle's minimum essential medium (see Appendix) in

screw capped eppendorfs to which 10µCi of ³⁵S - methionine (Amersham)was added. Gloves were used at all times.

The samples were incubated at 32°C for 2 weeks, 4 weeks and 3 months. As the PAFOs were extremely slow growing, 4-7 days incubation as for BCG bacilli was probably insufficient. At the end of the period, samples were subcultured (using a incubation standard disposable bacteriological loop) onto blood agar plates and incubated aerobically and anaerobically for one week. An equal volume (50µl) of cracking buffer containing SDS and mercaptoethanol was added and samples boiled for 2 minutes. Samples were then stored at -20°C until they were run on a gel. Standard procedures in use in the laboratory were used for separation of secreted components by polyacrylamide gel electrophoresis and analysis by autoradiography and the Ambis beta 8µl of each sample was electrophoresed at 8°C (in an Ambis scanner. electrophoresis unit) using a 12% polyacrylamide gel for $2^{1}/_{2}$ hours. This was run at 20 watts for 20 minutes, then 60 watts for 2 hours and 10 minutes. The gel was dried in a warm air dryer (Ambis dryer) for $1^{1}/_{2}$ hours. It was then either scanned for β -particle emission using the Ambis beta scanner (initially a 30 minute and then an overnight scan) or autoradiographed (gels were exposed to an X-ray film for 1 day or 7 days at room temperature).

RESULTS

97.4

69.0

46.0

33.0

18.4

M

Fig 7.C.1

Autoradiograph showing SDS-Page profiles of ³⁵S-labelled proteins from the following cultures.

M - Molecular weight markers (Dupont): Phosphorylase B 97,400 Da, Albumin (bovine serum) 69,000, ovalbumin 46,000, carbonic anhydrase (33,000), lactoglobulin A (18,367). Lane 1: Uninoculated RCM broth. Lane 2-3, 6-12: IBD derived organisms

The culture shown in lane 11 was later found to be contaminated. Lanes 4 and 5: cultures of *M. kansasii* grown in RCM (culture in lane 5 is a subculture of the culture in lane 4)

The IBD-derived organisms have probably failed to grow in the culture medium used and show no protein bands.

Results proved disappointing. Fig 7.C.1 shows that although protein patterns were detected with *M. kansasii* (lanes 4 and 5), no protein patterns were detected in any other lanes except lane 11. However, this sample must have been contaminated during the procedure, because growth of a gram positive rod (diptheroids) was noted on subculture onto blood agar after incubation with labelled methionine. Lane 1 shows no pattern with uninoculated RCM broth.

DISCUSSION

Since M. kansasii (originally grown in ROM) showed secreted protein patterns, this indicated that labelling of proteins had occurred. The probable explanation for all the test samples showing no trace (except the contaminated sample) was that the PAFOs failed to grow in Eagle's medium, and therefore could not incorporate the label. Another possible explanation is that not enough bacterial cells were used to be able to detect protein profiles after ³⁵S methionine incorporation. Also perhaps the bacterial cells were dormant, metabolising at an extremely slow rate and if these two factors were combined, poor label incorporation and kow protein secretion may have occured.

The inability to grow these organisms on a variety of media (Chapter 3) suggests that the most likely explanation for the lack of protein patterns was that culture conditions were sub-optimal. Details of preferred culture conditions such as availability and exact requirement of oxygen are not known. Screw cap sealed eppendorfs may have been unsuitable.

The reproducability of protein patterns seen when comparing the M. kansasii culture (Fig 7.C.1, lane 4) and its subculture (Fig 7.C.1, lane 5) demonstrates the reliability of the method in identifying similar organisms. Not all gram positive organisms give secreted protein patterns and the trace seen in lane 11 of Fig 7.C.1 may have been due to the PAFOs in the culture. However, in the absence of any other supporting evidence that the PAFOs in other cultures were able to grow and secrete proteins, it has to be assumed that the protein pattern was that of the contaminant.

SECTION D

ANALYSIS FOR THE PRESENCE OF THE 30kDa FIRBROENCTIN-BINDING MYCOBACTERIAL ANTIGEN

INTRODUCTION

Fibronectin (FN)-binding antigens are prominent components of short-term culture supernatants of *Mycobacterium tuberculosis* (Abou-Zeid *et al.*, 1988a): The FN-binding antigen in 3 day old culture supernatants of *M. tuberculosis* was identified by Western blot analysis and shown to be a major protein band of 30kDa. In 21 day old culture supernatants, FN bound to a 30 and 31 kDa double protein band. The 30 and 31kDa proteins are secreted.

The interaction between FN and Mycobacterial receptors is very strong. Eight BCG substrains, three M. tuberculosis strains, M. avium, M. intracellulare, M. vaccae and M. nonchromogenicum were tested and all expressed FN-binding capacity (Ratliff et al., 1988).

FN is a large glycoprotein found in plasma and extracellular matrix. It binds to many macromolecules including collagen, gelatin and fibrin and participates in cell surface interactions between eukaryotic cells and micro organisms. These FN-binding antigens are now thought to have an important role in mycobacterial disease (Abou-Zeid *et al*, 1988b). These authors also implicate these secreted antigens in the pathogenesis of leprosy. The 30 and 31kDa bands were shown to correspond to components A and B of the BCG85 complex (abundant in culture supernatants of *Mycobacterium bovis* BCG). A polyclonal antibody to the BCG85 complex (designated polyclonal anti-BCG85) bound to the 30/31kDa antigens. A monoclonal antibody HYT27 also bound to the 30/31kDa antigens and BCG85 A and B components. The polyclonal BCG85 inhibits binding of FN to all four antigens (30/31 kDa antigens and BCG85 A and B components). Using the antibodies HYT27

and polyclonal BCG85, an ELISA system was used to try and detect the presence of 30/31kDa antigens in the IBD cultures.

MATERIALS AND METHODS

1 ml of RCM broth (from each sample) was spun at high speed for 5 minutes in a microcentrifuge to precipitate RCM meat. The supernatant was filtered through 0.22μ low protein binding filters(Millex). The following samples including negative and positive controls were investigated by ELISA for the 30/31kDa protein antigens.

EXPERIMENT 1

Samples - culture filtrates.

- Uninoculated RCM broth incubated at 32°C for 10 months negative control.
- 2. Positive control M. kansasii in RCM broth for 18 months at 32°C.
- 3. 2 cultures from non IBD patients.
- 4. 4 CD cultures
- 5. 2 UC cultures
- 6. M. tuberculosis standards diluted in PBS-BSA

7. M.tuberculosis standards diluted in uninoculated RCM broth.

100µl of neat sample was analysed. All samples were tested in duplicate.

METHOD

The ELISA microtitre plate was coated for 48 hours at 4°C, with 100µl of a 1 in 3000 dilution (dilution was in 0.05 M carbonate/bicarbonate buffer, pH 9.6, see appendix) of anti-BCG polyclonal antibody (rabbit immunoglobins to *Mycobacterium bovis* BCG, Dakopatts).

The microtitre plate was washed with PBS-Tween 0.05% (see appendix) three times and incubated with 200µl PBS-Tween BSA (see appendix) for one hour at 37°C. After washing 100µl of neat filtered sample (antigen)

was added and incubated for one hour at room temperature. Plates were washed with PBS-Tween 0.05% three times and 100µl of the mouse monolconal antibody Hyt 27 (from Dr Lene Ljungqvist, Statens Seruminstitut, Copenhagen, Denmark) diluted 1 in 1000 in PBS-Tween-BSA, was added to each well and incubated for one hour at 37°C. The plates were washed again and 100µl of peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts no P260) diluted 1 in 1000 in PBS-BSA Tween was added and the plate incubated for one hour at 37°C.

After washing 100μ l ABTS/H₂O₂ substrate (see appendix) was added and incubated for 15 minutes at room temperature.

ELISA readings were done after 15 minutes, at 630 nm using a microelisa auto reader (Dynatech, MR 580). Readings are shown in Table 7.D.1.

				ELISA reading
<u>Standards</u> M.tuberculosis (diluted in PBS-)	culture BSA:	filtrates		
	10.0 2.0 0.4 0.08	ha/wj ha/wj ha/wj ha/wj		0.662 0.464 0.172 0.047
M.tuberculosis of diluted in RCM 1	culture broth:	filtrates		
	10.0 2.0 0.4 0.08	ha/wj ha/wj ha/wj		0.608 0.428 0.158 0.041
Controls				
uninoculated RC	0.000			
M. kansasii <mark>in H</mark>	RCM			0.038
Dilutions of M.k	ansasii	filtrate:	uninoculated	I RCM
			5:1 1:1 1:5	0.030 0.023 0.018
PBS-BSA (neat)				0.000
<u>CD_samples</u>	1 2 3 4			0.033 0.000 0.043 0.116
<u>UC samples</u>	1 2			0.016 0.026
Non-IBD samples	1 2			0.015 0.008

The *M.tuberculosis* standards give similar results whether diluted in PBS-BSA or in uninoculated RCM broth. Also, ELISA readings with the *M. kansasii* culture filtrate and dilutions in RCM broth suggest that RCM broth itself does not appreciably alter the positive ELISA result. The uninoculated RCM broth ELISA reading is zero. Of the CD samples, sample 4 is a clear positive and certainly samples 1 and 3 give results comparable with that of the *M. kansasii* culture filtrate. The significance of the result obtained with sample 2 of the UC samples, is uncertain. The non-IBD samples gave very low readings.

EXPERIMENT 2

A further 9 samples (CD, UC, non-IBD) were tested using the same methods. Results are seen in Table 7.D.2. One CD sample showed a clear positive (sample 2).

Table 7.D.:	2 : 30 kDa prote	in assay
		ELISA Reading
Standards		
M. tubercul	osis culture filt:	rates
diluted in	PBS-BSA	
	10.0 ug/ml	0.941
	2.0 ug/ml	0.712
	$0.4 \mu g/ml$	0.391
	0.08 ug/ml	0.214
	0.016 kg/m	0.153
	•••••	01200
Controls		
Uninoculate	0.051	
M kansasii	0.116	
		01220
Samples Te	sted	
CD Samples	1	0 072
ob bangies	2	0 410
	2	0.077
	4	0.082
		0.002
UC Samples	1	0 043
oc banpies	± 2	0.040
	2	0.030
	5	0.052
Non-IBD	1	0 041
	⊥ ?	0.041
	<u> </u>	0.002

Results obtained with standards and controls were consistent with the previous experiment, although ELISA readings were on average higher in the second experiment.

DISCUSSION

Only a limited number of species related to mycobacteria have been analysed for the presence of the 30kDa antigen. The antigen was not found in *Nocardia asteroides*, *Corynebacterium pyogenes*, *E. coli* and *Listeria* species (pesonal communication, Abou-Zeid, 1990). However, the antigens were found in the limited number of mycobacterial species examined so far. The sample analysed from the culture of *M. kansasii* in RCM, did not give a very high reading (0.038, Table 7.D.1; 0.116, Table 7.D.2). It could well be that culture conditions in RCM broth at 32°C are not optimal for production of the 30KD antigen. Also the amount of 30kD antigen in the 100µl of sample analysed is unknown.

It is of particular interest that two CD cultures gave high readings (0.116, Table 7.D.1; 0.41, Table 7.D.2), and another two moderately high readings (Table 7.D.1). This is suggestive of the presence of the 30KD antigen in these cultures.

It is not certain whether these FN-binding antigens are secreted and important in all mycobacterial species, however they almost certainly play an important role in two major mycobacterial diseases – tuberculosis and leprosy. These antigens are now considered important markers of mycobacterial disease and its detection in several of the CD cultures is an important observation and has interesting implications.

SECTION E

TISSUE CULTURE

INTRODUCTION

Tissue culture was used to try and improve growth of the organisms. So far, apart from RCM (and Lowenstein Jensen to a lesser extent), no good artificial medium has been found that would give a good growth of the organisms. Perhaps intracellular culture would improve their growth.

Chlamydia have been implicated as possible causative agents in IBD (Schuller *et al.*, 1979). Rectal infection with non-LGV *C. trachomatis* is frequently associated with proctitis in man (Quinn *et al.*, 1983). However, rectal infections with LGV serovars cause a more severe granulomatous proctitis and in severe cases can be indistinguishable clinically and histopathologically from Crohn's disease of the rectum (Levine, Smith and Brugge, 1980).

Furthermore, experimental primate infection with *C. trachomatis* has been shown to mimic rectal infection in humans clinically and histopathologically (Quinn, Taylor and Schachter, 1986). LGV infected cynomolgus monkeys developed severe haemorrhagic ulcerative proctitis. Two of the five monkeys showed crypt abscesses with giant cells and evidence of granuloma formation. Monkeys infected rectally with non-LGV Chlamydia trachomatis showed a mild proctitis with hyperplasia of lymphoid follicles.

Chlamydia are obligate intracellular bacteria and the PAFOs may have a similar life cycle. Hence well established techniques for Chlamydia culture, namely culture in McCoy cells were used, together with two other cell lines in attempts to demonstrate intracellular growth and replication.

MATERIALS AND METHODS

Samples

Three CD cultures (0.25ml of surface growth and broth per tissue culture monolayer), uninoculated RCM broth (as a toxicity control) and *Mycobacterium smegmatis* grown for 2 months in RCM (0.25 ml surface growth and broth per monolayer) as a positive control were used.

Each culture was inoculated in triplicate into three cell lines-McCoy cells, Human Epithelial cells (Hep 2) and Human Embryonic Lung (Hel) cells.

Standard tissue culture methods were used for making cell monolayers (except no antibiotics were used in case these were toxic to the organisms) and cell counting for passaging. Once monolayers were made on glass cover slips in 5ml universal tubes, 0.25mls of each RCM sample was inoculated into each tube. All tubes were centrifuged at 3000 rpm for 45 minutes to aid entry of organisms into the cells.

Each of the cell lines had to be passaged regularly. To passage, half the monolayer was scraped off the cover slip and transferred using a pipette onto a fresh monolayer with maintenance medium. McCoy cells needed to be passaged every three days and other cell lines weekly. Ziehl Neelsen staining was done on the remaining half of the monolayer.

Samples were fixed by adding 70% alcohol to the universal tubes. Hot filtered carbol fuchsin was used for the ZN stain of the cells on the cover slips. After a water wash 12.5% H_2 SO4 was added. A further water wash was followed by malachite green counterstaining. The cover slips were then tapped out of the 5ml universal tubes, mounted onto glass slides and examined.

RESULTS

RCM broth(uninoculated) was not toxic to either of the three cell lines. Plenty of acid fast bacilli were demonstrated with M_{\star} smegmatis in all three cell lines. However, more growth seemed to

occur in the maintenance medium above the monolayer.With the CD cultures, small acid fast clumps were demonstrated intracellularly in Hel cells (Fig. 7.E.1), Hep 2 cells and less so in McCoy cells. Quite often acid fast bacilli were seen, but only in small numbers. However, it was obvious that intracellular replication was not occurring even after five passages.

DISCUSSION

A prolonged incubation time was required for growth of the IBDderived organisms in other culture systems. This made tissue culture unfeasible as a long term culture system due to frequent passage needed for the cell lines, as the cells had to be scraped and passaged every 3-7 days. Staining (using iodine) of Chlamydia in McCoy cell culture is a standard procedure. However, use of the hot ZN stain on cover slips in tubes was technically difficult, although it did prove possible to acheive a good result.

In conclusion, although some small acid fast clumps were demonstrated, intracellular replication was not observed and these cell lines were unsuccessful in improving growth of the CD-derived organisms.



Fig. 7.E.1

Tissue culture using Human Embryonic Lung cells (Hel). ZN stain. Small acid fast clumps seen in Hel cells when CD-derived organisms were passaged. However intracellular replication was not seen.



SECTION F

USE OF A MICROAEROPHILIC CULTURE SYSTEM (REDOX SYSTEM) TO TRY AND IMPROVE GROWTH OF THE PAFOS ISOLATED FROM IBD

INTRODUCTION

Chatterjee claims that mycobacteria recovered from human lepromatous nodules and presumably *H. leprae* have been grown in a medium (called J_7C) that ensured a minimal oxygen tension at initiation of growth and a marginal but increasing availability of oxygen as growth increased (Chatterjee, 1983). This was achieved using strong biological reductants in the medium with a combination of partial vacuum and alkaline pyrogallol in the culture vessel. Chatterjee reported that growth occurred initially as non-acid fast coccoids and bacilli that gradually changed to acid fast bacilli and spherical L-Optimal growth occurred at three months, although initial forms. The growth was shown to growth was noted at 1-2 weeks. be transferrable.

There are several observed similarities between *M. leprae* and the IBD derived organisms (see Chapter 1), not least the difficulty in culturing the organisms. The paucity of bacilli seen in the tuberculoid form of leprosy and a possible life cycle involving alternative forms of mycobacterium (Stanford, Dourmashkin and McIntyre, 1987), namely the Much's granular form, are possible further parallels. The author therefore learnt the use of this rather complicated culture system from Dr Chatterjee on his annual visit to the Middlesex Hospital and used the culture system to try and improve the growth of the IBD-derived forms. Simultaneously, human leproma derived mycobacterial cultures already set up by Dr Chatterjee were passaged by the author.

MATERIALS AND METHODS

1.0ml of three RCM cultures (positive for the PAFOs by ZN staining) were transferred into the culture system described below. All these cultures had previously been confirmed to be contaminant free. Two cultures were originally from two CD patients and one culture was from a patient without IBD (from a control node).

lml of the culture was transferred aseptically into an autoclaved 10ml capacity Thunberg tube and 5mls J₇C medium (see Appendix) was Alkaline pyrogallol (see appendix) was added to the bulbous added. detachable side arm of the tube and a cotton wool plug was firmly placed in the neck of the detachable tube to avoid any spillage of pyrogallol into the culture. Sterile vaseline was applied to the neck of the Thunberg tube and the detachable side arm with pyrogallol and the cotton wool plug was placed over the neck of the tube. This assembled system was incubated at 35°C and examined at 3 months by ZN staining. Passage of lml of material to a further 3mls of fresh medium and repitition of the above procedure was done. Two 3 monthly passages were done.

Another CD culture was provided for Dr Chatterjee who has used this culture system in Jhalda, India.

With the human leproma derived cultures, 1 ml of culture was added to 3 mls fresh medium as above. The only difference was that partial vacuum was applied to the cultures.

RESULTS

With the 3 RCM cultures: Macroscopically no change was seen in the medium (no turbidity was observed). No acid fast organisms were seen on ZN staining, and the medium did not appear to support the growth of the organisms. However Dr Chatterjee (personal communication) reports non acid fast coccoids and short bacillary forms at the sixth transfer of the CD culture taken to Jhalda.

The six human leproma derived mycobacterial strains could be passaged.

DISCUSSION

Although no improvement in growth was observed after 2 passages at 3 months and 6 months, the cultures will be regularly observed 3-6 monthly. It is possible that if the organisms are multiplying extremely slowly or as non acid-fast coccoid forms, then a positive result will not be seen on ZN staining as yet. This work will be continued by Miss Fatima Miranda in the same laboratory to see if more promising longterm results can be observed.

Chatterjee and Roy (1989) do however state that the inoculum for primary culture and transfer always have to be heavy and loop transfers are not possible. Hence original cell (bacterial) density of the IBD cultures may have been insufficient for this culture system. Recently, Chatterjee and Roy (1989) have reported considerable growth enhancement at 10°C incubation. Hence incubation at 10°C and also transfer of cultures using a 1:4 or even a 1:1 dilution may be necessary for growth.

The human leproma derived mycobacteria on the other hand were passaged and were ZN positive in this system. However, the difficulty in this case is to know whether the starting material for the cultures i.e. human leproma derived mycobacteria were indeed M_{\star} leprae and if they are whether a genuine M_{\star} leprae DNA probe is available to identify the passaged organisms as such.

SECTION G

INITIATION OF AN IN VIVO STUDY OF THE PATHOGENICITY FOR NUDE MICE OF THE PAFOS ISOLATED FROM PATIENTS WITH IBD.

INTRODUCTION

Nude mice lack a thymus gland and are unable to mount a T-cell dependent immune response. It is known that the nude mouse can support the growth of *N. leprae* to levels exceeding 10^{10} bacilli per footpad with dissemination and growth throughout the body (Lancaster *et al.*, 1983). This is in comparison to the limited growth obtained in the foot pad of the normal mouse with only 10^4 to 10^6 bacilli per footpad and virtually no dissemination (Shepard, 1960). The similarities already outlined between leprosy and CD (discussed in Chapter 1) prompted the idea that the nude mouse with its severely restricted immune response may be a suitable experimental model for further evaluation and study of the PAFOS isolated from IBD.

White, 1981, inoculated the acid fast material into Balb/C mice: although acid fast bacilli were seen in smears taken from tissues they could not be re-isolated. There was no evidence of replication or progressive disease over an 18 month period. The T cell deprived nude mouse may well show a different disease course.

The use of microbiologically defined nude mice (supplied with a known gut flora but no recognised pathogens) will ensure that any organisms recovered from the animals would have originated from the IBD-derived organisms. Furthermore, the use of athymic nude mice has been put forward as a possible model for the pathogenesis of CD by other workers (Das *et al.*1983,1988; Pena *et al.*1988).

MATERIALS AND METHODS

Samples

Cultures which were growing the PAFOs were chosen for inoculation into the nude mice. These samples had been previously screened for contaminants and PAFO growth. Only contaminant free cultures were used. 13 CD cultures, 5 UC cultures and 2 control cultures were chosen. The cultures were chosen either because they showed a good growth bacteriologically (Chapter 3) or had reacted with mycobacterial probes on dot blotting (Chapter 5), or had TSA detected (Chapter 6). One of the cultures which showed mycobacterial mycolate peaks on HPLC (Chapter 7, Section A) was also used for inoculation. In addition broth from an uninoculated RCM bottle was injected.

All cultures were taken directly from the 32°C warm room at the Middlesex Hospital Medical School to the vinyl isolator at St George's Hospital Medical School, and inoculated directly into the nude mice.

Nude Mice

Microbiologically defined Crl: nu/nu BR (nude, athymic) mice from Charles River Laboratories, 251 Ballardvale Street, Wilmington, Mass 01887, USA, were used. The mice had been screened for viruses (they were virus antibody free) and had had bacterial screens for several organisms including *Staphylococcus aureus*, *p* haemolytic streptococci, Pneumococci, Streptobacillus moniliformis, Salmonella species, and M. pulmonis. This was done by the suppliers. Fig 7.G.1 shows a nude mouse which is now housing some PAFOs inoculated into the foot pads.

Inoculation

Six nude mice were used per culture. 25µl of RCM broth was inoculated per left and right hind foot pad. Unfortunately the ears were too small for inoculation (originally ear pinna biopsies were planned at 6 months).



Fig. 7. Gl.

Microbiologically defined Crl:nu/nu BR (nude athymic) mouse housing CDderived PAFOs : inoculation was into the foot pads. The animal remains well at 4 months. Final results of this study will not be known for 2 years. <u>Plan</u>

The mice will be maintained for up to two years before being sacrificed and examined for PAFOs or acid fast bacilli (AFB) in the tissues. Part of the tissue will be homogenised and examined for viable acid fast organisms. The rest of the tissue will be fixed and used for histological examination.

At the end of the experimental period, blood will be obtained for immunological studies. If antibody production is demonstrated, antibodies can be used for further work.

Also, material from foot pads will be homogenised and inoculated into RCM, Lowenstein Jensen, and other media to check for contaminants. It may be possible to passage the homogenate into another group of nude mice. Histological examination of intestines, mesenteric lymph nodes and other nodes, liver, spleen, lungs and skin will be performed.

RESULTS SO FAR - At 4 months post-inoculation:

Of the 120 mice used for the study, 6 have died so far (one each from each of six different culture sets) - No organisms were demonstrable by ZN or Gram staining. All the other mice remain well. No ill effects were noticed in mice inoculated with uninoculated RCM broth.

DISCUSSION

It was important that no known pathogens were introduced into the animals in the isolators such as Staphylococcus aureus (as the animals can die with multiple abscesses) or *M. kansasii* or *M. tuberculosis*. Hence cultures such as those from the CD patient D. H. (see Chapter 3, Fig. 3.A.3.d) were not used. The culture from D. H. showed on ZN staining the typical appearance of cording seen with *M. tuberculosis* and although it did not grow for final identification to be possible,

was considered unsuitable, to be introduced into nude mice in the isolator.

Exceptional care needs to be taken to maintain nude mice in germ free conditions for two years. If maintained carefully, nude mice will survive 18 months or more although a few may sucumb to natural death or infection before that. The isolates are known to be extremely slow growing. Hence, maximum time is being allocated allowing time for isolates to grow.

The laboratory at St George's Hospital Medical School have more than ten years experience at maintaining nude mice in isolators using isolates of a potentially pathogenic nature. They have the necessary expertise and skill to make this study feasible. The final results of this study will be known only in approximately two years time and it is hoped that several questions will be answered at the end of this time. It would be of particular interest to note whether the course of any disease which may occur would be different for CD,UC or non-IBD derived organisms.

It may be possible not only to study the pathogenicity of the PAFOs in the nude mouse, but to determine if intracellular growth occurs, and to study reversion to classical acid fast bacilli. Perhaps a second passage into another set of mice may provide similar observations to those observed by Khomenko and othes (discussed in Chapter 2) with demonstration of classical bacilli and disease.

GENERAL DISCUSSION AND FUTURE WORK

The introductory chapter of this thesis presents evidence for an environmental factor in the causation of Crohn's disease. Although not obviously contagious, this factor may be an infectious agent, and evidence is presented that mycobacteria are likely candidates.

possibility exists that an unusual physical form of The mycobacterium is involved. Recent work on tuberculosis from Russia (Khomenko, 1987) and India (Chandrasekar, 1978) supports this possibility for tuberculosis. The fact that acid fast forms have been isolated from CD tissue by several research groups worldwide is clearly intriguing. Some workers (Chiodini et al, 1984 a-d) have isolated acid fast organisms initially as spheroplast forms. However, all isolates described seem to have some sort of a cell wall. Some spheroplast forms have been non-acid fast. However, staining methods used by various research groups differ and a standardised Ziehl-Neelsen staining method may well have given the same results. Burnham et al in 1978 described acid fast forms from IBD cultures. The work done for my thesis was concerned with the isolation and characterisation of these acid fast organisms.

Verification of the presence of PAFOs in IBD derived tissue was achieved in a new study and in the present series, pleomorphic acid fast forms were cultured from mesenteric lymph nodes from 17/25 CD patients, 11/15 UC patients but only 3/17 non-IBD (control patients). The high isolation rate of acid fast forms from ulcerative colitis tissue has not been described by other research groups. The characterisation of these PAFOs was possible as fresh isolates had been made and these organisms could be subcultured. The best medium remains as Robertson's cooked meat broth for primary isolation as well as subculture.

The ability to subculture the organisms showed that they were viable. This was supported by the isoaltion of high molecular weight DNA from them.

As important as the identity of these PAFOs is the question of whether the isolates from CD, UC and non-IBD tissue were the same. Certainly the organisms were extremely pleomorphic, but they were all acid fast. Although only very few cultures in the non-IBD group grew these organisms, they had a similar appearance to the IBD-isolates.

THE ACID FAST STAIN

The mechanism of the staining reaction when using the Ziehl-Neelsen stain is reviewed by Draper (1982). Mycobacteria may be stained with a dye such as carbol fuchsin but cannot be decolourised, basic subsequently with dilute acid $(12.5\% H_2 SO_4 \text{ was used in this study})$. Although the acid fast stain is classically associated with Mycobacteria, Nocardiae, Corynebacteriae and Rhodococci may contain partially acid fast species. The substances in the cell wall that impede the removal of dye by acids are probably lipids especially the mycolic acid esters. Further the way these are bound to the wall appears to be responsible for the degree of acid fastness (Beaman, 1976). Murohashi et al (1969) also attribute acid fastness of mycobacteria to the integrity of the cell wall. Stanford *et al* (1987) postulated a mycobacterial life cycle which included bacilli, granules and globi from which pleomorphic forms could arise. They could revert to classical bacilli occasionally. The presence of occasional acid fast bacili was noted in some of the smears (Chapter 3); hence, this is in agreement with Stanford's hypothesis.

On electron microscopy, the PAFOs were seen to have a cell wall. (Certainly the presence of a cell wall is in agreement with their acid fast appearance).

This (the presence of a cell wall) was contrary to reports by Burnham et al in 1978 on the 1976 culture series. Perhaps the use of hypertonic media used specifically to encourage growth of cell wall defective forms was the reason that this appearance (lack of cell wall) was noted at that time. Hence the PAFOs could belong to any of several genera. The genera Mycobacterium, Nocardia, Rhodococcus and Corynebacterium sensu stricto are a closely related group. In particular, the presence of predominantly straight chain and monosaturated fatty acids in strains of these four genera distinguishes them as a group from Arthrobacter, Brevibacterium, Cellulomonas, Curtobacterium, Microbacterium and Oerskovia all of which contain major amounts of Iso- and ante Iso-methyl branched chain acids (Collins, Goodfellow and Minnikin, 1982). It is known that species belonging to any of these genera (Mycobacterium, Rhodococcus, Nocardia and Corynebacterium) can be pathogenic, hence it is entirely feasible that any of these species is involved in the aetiology of inflammatory bowel disease.

A thorough review of non tuberculous mycobacterioses has been made by Tsukamura (1989). The ecological distribution of mycobacteria, their virulence and conditions of the host all influence lung disease due to mycobacteria other than tubercle bacilli. The *M. avium* complex, *M. kansasii* and *M. malmoense* are among those implicated.

There is a wide range of infections documented due to many different species of mycobacteria, such as *M. marinum*, *M. ulcerans*, *M. chelonei* and *M. leprae*. Extremely rare infections are also described; for example, *M. scrofulaceum* has been associated with meningitis and osteomyelitis and *M. szulgai* has been implicated in lung disease.

Most rhodococci are saprophytic and considered non pathogens, however infections in animals and humans are well documented. *Rhodococcus equi* causes infections in animals and *Rhodococcus*

aurantiacus causes infections in humans (lung infections, Tsukamura and Kawakami, 1982; Meningitis, Prinz *et al.*, 1985). Mesenteric lymphadenitis of swine by *P. sputi* has recently been reported (Tsukamura *et al.*, 1988). *R. sputi* has also been observed in sputa of patients with chronic pulmonary disease (Tsukamura and Yano, 1985).

Similarly, nocardiae have been implicated in disease. *N.* asteroides sensu stricto and *Nocardia farcinica* have been implicated in infections of the lung (Tsukamura, 1982). Nocardial mycetoma due to *N.* brasiliensis is well described. Exceptionally *N. caviae* (now *N.* otitidis-caviarum) can cause pulmonary infection or mycetoma (Beaman, 1976). Also, human nocardiosis is well documented in immunodeficiency states.

Hence, it is possible that any species belonging to any of these genera (*Mycobacterium*, *Nocardia* or *Rhodococcus*) are capable of pathogenicity, given the correct host and immunological setting.

The possibility of the PAFOs belonging to the genus Corynebacterium was not favoured. Deliberate attempts were made to grow corynebacteria. Cultures containing coryneforms were discarded; these organisms are present in the GI tract and on skin and may well have been introduced when culturing mesenteric lymph nodes or during handling and subculturing of the specimens.

Unfortunately, the use of mycobacterial DNA probes could not eliminate corynebacterial cross reactions when used to test PAFO DNA. This was not surprising in view of the presence of common antigens between corynebacteria and mycobacteria. However, analysis for tuberculostearic acid is more specific for mycobacteria, rhodococci and nocardiae species. Although four species of corynebacteria are known to contain TSA, in general coryneforms do not contain this fatty acid. The data obtained was suggestive of the presence of TSA in

several of the cultures known to be growing the PAFOs. Together with the acid fast appearance and evidence from dot blot hybridisation, this is suggestive of the presence of mycobacteria(or a very closely related genus) in the cultures. It is notable that cultures not growing the PAFOs showed no trace of TSA.

TSA has been demonstrated in lymph nodes from patients with sarcoidosis (Hanngren *et al*, 1987). Sarcoidosis is a multisystem chronic granulomatous disease of unknown aetiology with many similarities to CD. Also, Graham *et al* (1988) claim to have isolated cell wall defective acid fast bacteria from skin biopsies of sarcoidosis patients. Here again attention is called to mycobacteria and findings show many parallels with CD.

Further evidence to suggest that the PAFOs may belong to the genus Mycobacterium, came from mycolic acid analysis. This analysis is now used for the minimum description of a mycobacterial species and is extensively used in taxonomical studies of mycobacteria and related genera. Mycolic acid analysis is an investigation of prime importance when assigning a strain to one of the genera Mycobacterium, Nocardia and Rhodococcus. Broadly the total number of carbon atoms in mycobacterial mycolic acids is 60-88, in Rhodococcus is 34-66 and Nocardia is 46-60 (Minnikin, 1982; Tsukamura, 1989). Corynomycolic acids have 23-38 carbon atoms (Collins, Goodfellow and Minnikin, 1982), although there are exceptions to these broad groups.Even though final identification was not possible, a few cultures showed peaks on reverse HPLC at the retention time expected for mycobacterial mycolates.

Other markers of mycobacterial disease such as the 30kD fibronectin binding antigen was demonstrated in a few CD cultures even though optimum culture conditions for expression of this antigen may not have been used. This was an important observation.

Unfortunately, the amount of growth could not be increased despite trying various media including tissue culture systems and a redox system used for the cultivation of human leproma derived mycobacteria.

Techniques available were stretched to the very limits of sensitivity. Analysis for TSA was at pg levels; so much so that great care was needed to avoid trace contamination of samples. Reverse HPLC used for mycolic acid analysis could not be exploited to maximum sensitivity at the time but this may be achieved in due course in Dr Minnikin's laboratory and further samples can be analysed then.

One of the most crucial achievements of the work done was to make available enough DNA for nucleotide sequencing. The numerous dot and Southern blots done were evidence enough of the technical problems associated with working with such small quantities of DNA, although some answers were obtained by these methods.

Probe hybridisation with DNA from primary cultures and subcultures was evidence supporting the ability to passage the organisms and therefore of viability. Hybridisation between DNA from different IBDderived isolates was evidence of relatedness between these organisms.

There were also several unexplained findings. In particular the appearance of cording (on ZN staining) seen with a CD derived culture (from patient D.H.), its hybridisation with mycobacterial probes but inability to grow for further identification. Cloning of PAFO DNA from D.H. and subsequent hybridisation did not give clear cut results although some clones did hybridise with other PAFO derived DNA.

It can be conclusively stated that the genome of the organisms is <u>not</u> that of \mathcal{M} . *paratuberculosis*. Although several research groups are implicating this organism, the PAFOs are not mycobactin dependent and do not have the genome of \mathcal{M} . *paratuberculosis*.

DNA amplification has now opened up the possibility of genomic analysis at the nucleotide level and it will reveal whether CD and UC derived isolates are similar or not. Various investigations done so far could not differentiate between CD and UC derived organisms. There is no doubt that the PAFOs are viable organisms and are associated with IBD. They are significantly more often isolated from IBD than control tissue. The first step in satisfying Koch's postulates is to demonstrate an organism in all cases of the disease. The isolation rate from IBD tissue is however not 100% but there may be several possible explanations for this. If the organisms do exist as Much's granules, they may not be easily cultivable; whatever the form of the organism, techniques of culture may not be sensitive enough or the optimal medium may not yet have been found for their culture. In some patients, only very few organisms may have been present at the time of culture or the organisms may be dormant, and are not in a state of active growth and do not replicate well.

If these organisms are indeed involved in the aetiology of IBD, it could be due to direct infection or due to the immune reponse associated with the presence of the antigen. Another possibility is that a cell mediated autoimmune phenomenon could be initiated as a response to a cross reacting mycobacterial antigen. CD may be multifactorial and may have multiple causes and the PAFOs may only be associated in some cases of the disease. Other mycobacteria may be involved in other cases. However, with the possibility of the use of antimycobacterial drugs, it is important to be able to choose those cases of CD which are treatable by chemotherapy and the isolation of mycobacteria may favour this choice.

The "leaky mucosa" of Crohn's disease however complicates the interpretation of the role of bacteria isolated from CD tissue. The

organisms isolated may only have a secondary role and may not be initiators of the disease. However, their isolation from UC tissue as well and low isolation rate from controls (non-IBD tissue) tempts the hypothesis that the organisms establish themselves in hosts who have an appropriate immunogenetic background and thereby cause different disease manifestations.

Mycobacterial diseases are essentially immunologically mediated and this may well be the case with these organisms. This was also an important factor when choosing an animal model, as each species may respond differently to a given antigen. The choice of bacteriologically defined nude mice was made with the background of its success in leprosy. Hopefully, multiplication of organisms will occur. Future work is aimed at completely identifying the acid fast organisms and fulfilling Koch's postulates.

Once the DNA sequencing work is complete, suitable primers could be used (from PAFO DNA) for PCR analysis of organisms directly from CD tissue.

Bacteriologically, improvements need to be made to enable more prolific growth of these organisms in a shorter time. Antigens can then be prepared for immunological studies. If organisms of this kind are to be cultured from IBD tissue, prolonged culture for months or even years must be planned. Perhaps then, a higher proportion of research workers will be able to isolate these organisms from IBD tissue.

It is concluded that viable extremely slow growing pleomorphic acid fast organisms can be isolated from a high proportion of cases of Crohn's disease and ulcerative colitis, but only from a few non-IBD control nodes. The organisms from both CD and UC appear homogenous and they all belong to the *Mycobacterium-Nocardia-Rhodococcus* group but are

probably not corynebacteria. Although DNA work was limited due to small quantities of DNA isolated from small numbers of organisms, the work is at a stage where DNA from these organisms has been amplified by polymerase chain reaction and sequencing of the DNA has started. Also a long term animal study has been initiated but results of this study will not be known for two years. This may provide some answers on the pathogenicity of these unusual organisms. It is postulated that these PAFOs are an unusual form of mycobacterium as they show many mycobacterial markers, but their role in the causation of CD is still unknown.

REFERENCES

Abou-Zeid C, Smith I, Grange J, Steele J and Rook G (1986). Subdivision of daughter strains of Bacille Calmette-Guerin (BCG) according to secreted protein patterns. J. Gen. Microbiol., <u>132</u>; 3047-3053.

Abou-Zeid C, Smith I, Grange J M, Ratliff T I, Steele J and Rook G A W (1988a). The secreted antigens of *Mycobacterium tuberculosis* and their relationship to those recognised by the available antibodies. J. Gen. Microbiol., <u>134</u>; 531-538.

Abou-Zeid C, Ratliff T L, Wiker H G, Harboe M, Bennedsen J and Rook G A W (1988b). Characterisation of fibronectin-binding antigens released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. Infection and Immunity, 56; 3046-3051.

Acheson E D (1960). The distribution of ulcerative colitis and regional enteritis in Unitied States veterans with particular reference to the Jewish religion. Gut, $\underline{1}$; 291-293.

Allan R N and Hodgson H J (1986). Inflammatory Bowel Disease. In: Recent Advances in Gastroenterology, (Pounder R Ed) Longman, London.

Allgayer H and Stenson W F(1987). Treatment of an animal model of colitis with 16,16' dimethyl prostaglandin E2. Gastroenterol.,92; 1295.

Aluwihare A P (1971). Electron microscopy in Crohn's disease. Gut, <u>12</u>; 509-518.

Ambrose N S, Johnson M, Burdon D W and Keighley M R B (1984). Incidence of pathogenic bacteria from mesenteric lymph nodes and ileal serosa during Crohn's disease surgery. Br.J.Surg., <u>71</u>;623-625.

Aronson M D, Phillips C A and Beekan W L (1974). Isolation of a viral agent from intestinal tissue of patients with Crohn's disease and other intestinal disorders. Gastroenterol., <u>66</u>; 661.

Aronson R A, Cook S L and Roche J K (1983). Sensitization to epithelial antigens in chronic mucosal inflammatory disease. I. Purification, characterisation and immune reactivity of murine epithelial cell-associated components (ECAC). J. Immunol., <u>131</u>; 2796-2804.

Armstrong D, Gold JW, Dryjanski J, Whimbey E, Polsky B, Hawkins C, Brown A E, Bernard E and Kiehn T E. (1985). Treatment of infections in patients with the acquired immunodeficiency syndrome. Ann. Intern. Med., <u>103</u>; 738-743.

Asakura H, Tsuchiya M, Aiso S*et al* (1982). Association of human lymphocyte-DR2 antigen with Japanese ulcerative colitis. Gastroenterol, <u>82</u>; 413-418.

Bapat C V and Modak M S (1978). Growth of the ICRC bacilli in the foot-pad of mice. Lepr. Ind., 50; 144-155.

Barksdale L and Kim K S (1977). *Mycobacterium*.Bact. Reviews, <u>41</u>; 217-372.

Bass J B (1986). *Mycobacterium avium intracellulare* rational therapy of chronic pulmonary infection? (editorial). Am. Rev. Respir. Dis., 134; 431-432.

Beaman B L (1976). Possible mechanisms of Nocardial pathogenesis. In: The Biology of the Nocardiae (Goodfellow M, Brownell G H and Serrano J A, Eds), pp386-417. Academic Press Inc (London) Ltd.

Beeken W L, Goswami A K K and Mitchell D N (1975). Studies of a viral agent isolated from patients with Crohn's disease and other intestinal disorders. Gut <u>16</u>; 401.

Belsheim M R, Darwish R Z, Watson W C and Schieven B (1983). Bacterial L form isolation from inflammatary bowel disease patients. Gastroenterol., 85; 364-369.

Bennett R, Rubin P H and Present D H (1988). Inflammatory bowel disease in husbands and wives - frequency of Inflammatory Bowel Disease in 35 offspring of 19 couples. Gastroenterol., 5; A611.

Best W R, Becktel J M, Singleton J W and Kern F (1976). Development of a Crohn's disease activity index. Gastroenterol., <u>70</u>; 439-444.

Binder V, Both H, Hansen P K et al (1982). Incidence and prevalence of ulcerative colitis and Crohn's disease in the county of Copenhagen, 1962 to 1978. Gastroenterol., <u>83</u>, 563-568.

Blaser M J, Miller R A, Lacher J and Singleton J W (1984). Patients with active Crohn's disease have elevated serum antibodies to antigens of seven enteric bacterial pathogens. Gastroenterol., <u>87</u>; 888-894.

Bolton P M, Owen E, Heatley R V, Jones Williams W and Hughes L E (1973). Negative findings in laboratory animals for a transmissible agent in Crohn's disease. Lancet \underline{ii} ; 1122-1124

Bousfield I J, Smith G L, Dando T R and Hobbs G (1983). Numercial analysis of total fatty acid profiles in the identification of Coryneform, Nocardioform and some other bacteria. J. Gen. Microbiol., <u>129</u>; 375-394.
Brown W R and Lee E M (1974). Radioimmunologic measurements of bacterial antibodies. II. Human serum antibodies reactive with Bacteroides fragilis and enterococcus in gastrointestinal and immunologic disorders. Gastroenterol., 66; 1145-1153.

Budde W L and Eichelberger J W (1977). The mass spectrometer as a substance selective detector in chromatography. J. Chromatography, <u>134</u>; 147-158.

Burnham W R, Lennard-Jones J E, Stanford J L and Bird R G (1978). Mycobacteria as a possible cause of Inflammatory Bowel Disease. Lancet, <u>ii</u>; 693-696.

Burnham W R (1980). Mycobacteria and Crohn's disease. MD thesis, University of Dublin.

Calkins B M, Lilienfeld A M, Garland C F et al (1984). Trends in the incidence rates of ulcerative colitis and Crohn's disease. Dig. Dis. Sci, <u>29</u>; 913-920.

Calkins B M and Mendeloff A I (1986). Epidemiology of Inflammatory Bowel Disease. Epidemiologic Reviews, <u>8</u>; 60-91.

Cave D R, Mitchell D N, Kane S P and Brooke B N (1973). Further animal evidence of a transmissible agent in Crohn's disease. Lancet, <u>ii</u>; 1120-1122.

Cave D R, Mitchell D N and Brooke B N (1975). Observations on the trasmissibility of Crohn's disease and ulcerative colitis. Gut 16; 401.

Chadwick V S, Schlupp M, Cooper B, Barbezat G, Morris K, Friend G and Chadwick D.(1987). Gastroenterol., <u>92</u>;1341.

Chandrasekhar S (1978). Cell wall deficient forms of tubercle bacilli. Review. Indian J of Chest disease, <u>22</u>; 114-122.

Chao L P , Steele J, Rodrigues C, Lennard-Jones J, Stanford J L, Spiliadis C and Rook G A W (1988). Specificity of antibodies secreted by hybridomas generated from activated B cells in the mesenteric lymph nodes of patients with inflammatory bowel disease. Gut, 29; 35-40.41.

Chapman R W, Cottone M, Selby W S, Shepherd H A, Sherlock S and Jewell D P (1986). Serum autoantibodies, ulcerative colitis and primary sclerosing cholangitis. Gut, <u>27</u>; 86-91.

Chatterjee B R (1980). Cultivable precursors of *M. leprae*. Lepr. India, <u>52</u>; 513-526.

Chatterjee B R (1983). Growth of *Mycobacterium leprae* in a Redox system. Lepr. India, <u>55</u>; 426-449.

Chatterjee B R and Roy R D (1989). Growth of *Mycobacterium leprae* in a Redox system: III. evidence of growth at low temperatures (psychrophilia) and further refinement of growth medium. Indian J. Lepr., <u>61</u>; 458-466.

Chiodini R J, Van Kruiningen H J, Thayer W R, Merkal R S and Coutu J (1984a). Possible Role of Mycobacteria in Inflammatory Bowel Disease. I. An Unclassified *Mycobacterium* species isolated from Patients with Crohn's Disease. Dig. Dis. Sci., <u>29</u>; 1073-1079.

Chiodini R J, Van Kruiningen H J, Merkal R S, Thayer R and Coutu J (1984b). Characteristics of an unclassified *Mycobacterium* species isolated from patients with Crohn's disease. J. Clin. Microbiol., <u>20</u>; 966-971.

Chiodini R J, Van Kruiningen H J, Thayer W R, Coutu J A and Merkal R S (1984c). In vitro antimicrobial susceptibility of a *Mycobacterium* sp. isolated from patients with Crohn's disease. Antimicrob. Agents and Chemother. <u>26</u>; 930-932.

Chiodini R J, Van Kruiningen H J, Thayer W R, Merkal R S and Coutu J (1984d). An unclassified *Mycobacterium* species isolated from patients with Crohn's disease. Gastroenterol., <u>86</u>; 1048.

Chiodini R J, Van Kruiningen H J, Thayer W R and Coutu J A (1986a). Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease. J. Clin. Microbiol., 24; 357-363.

Chiodini R J, Van Kruiningen H J, Thayer W R and Coutu J A (1986b). Differentiation of the mycobacteium species from Crohn's disease by restriction fragment length polymorphism of the ribosomal genes. Gastroenterol., <u>90(ii)</u>; 1372.

Chiodini R J (1988) Identification of mycobacteria from Crohn's disease by restriction polymorphism of the 55 ribosomal DNA genes. In: Inflammatory bowel disease: Current status and future approach, (MacDermott R P Ed) pp 509-514, Elsevier Science Publishers, Amsterdam.

Chiodini R J (1989). Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. <u>2</u>; 90-117.

Cho S N, Brennan P J, Yoshimura H H, Korelitz B I and Graham D Y (1986). Mycobacterial aetiology of Crohn's disease: serologic study using common mycobacterial antigens and a species-specific glycolipid antigen from *Mycobacterium paratuberculosis*. Gut, <u>27</u>; 1353-1356.

Colemont L J, Pattyn S R, Michielsen P P, Pen J H, Pelckmans P A, Van Maercke Y M and Portaels F (1988). Acid fast bacilli in Crohn's disease. Lancet, <u>i</u>; 294.

Collins J, Beaman B, Arthur M and Gitnick G (1986). Isolation of mycobacteria from intestinal tissues. Gastroenterol.,<u>90(ii)</u>, 1377.

Collins J, Beaman B, Brooks D, Arthur M and Gitnick G (1987). A prospective controlled study of mycobacteria in Crohn's disease. Gastroenterol., <u>92(ii)</u>; 1352.

Collins J F, Strickland R G, Kekahbah E L, Arthur M H, Naeim F and Gitnick G (1988). Histological response and antigen transmission in the lymph nodes of athymic nu/nu mice inoculated with Crohn's disease tissue filtrates. Gut, <u>29</u>; 983-989.

Collins M D, Goodfellow M and Minnikin D E (1982). Fatty acid composition of some mycolic acid containing coryneform bacteria. J. Gen. Microbiol., 128; 2503-2509.

Collins M D (1987). Transfer of Arthrobacter variabilis (Muller) to the genus Corynebacterium, as Corynebacterium variabilis, comb. nov. Int. J. System Bacteriol., <u>37</u>; 287-288.

Collins M D, Smida J and Stackebrandt E (1989). Phylogenetic evidence for the transfer of *Caseobacter polymorphus* (Crombach) to the genus Corynebacterium. Int. J. System Bacteriol., <u>39</u>; 7-9.

Crohn B B, Ginzburg L and Oppenheimer G D (1932). Regional Ileitis - A Pathological and Clinical Entity. JAMA, <u>99</u>; 1323-1329.

Dalziel T K (1913). Chronic interstitial enteritis. Br. Med. J. <u>ii</u>; 1068-1070.

Das K M, Valenzuela I, Williams S E, Soeiro R, Kadish A S and Baum S G (1983). Studies of the etiology of Crohn's disease using athymic nude mice. Gastroenterol., <u>84</u>; 364-374.

Das K M, Zuckerman M, Shafritz A and Vecchi M (1988). Athymic nude (nu/nu) mice: an experimental animal for studies of the etiology of Crohn's disease. In: Inflammatory Bowel Disease: Current Status and Future Approach. (MacDermott R P Ed), pp 461-467. Elsevier Science Publishers, Amsterdam.

De Groote G, Desmyter J, Vantrappen G and Phillips C A (1977). Rotavirus antibodies in Crohn's disease and Ulcerative Colitis. Lancet, \underline{i} ; 1263-1264.

Draper P (1982). The anatomy of mycobacteria In: The Biology of the Mycobacteria, Volume 1. (Ratledge C and Stanford J. Eds), pp15-17, Acad. Press.

Elliot P R, Lennard-Jones L E, Burnham W R, White S and Stanford J L (1980). Further studies on skin testing with mycobacterial antigens in Inflammatory Bowel Disease. Lancet, \underline{i} ; 483-484.

Elmgreen J, Berkowicz A and Sorensen H (1983). Defective release of C5a related chemo-attractant activity from complement in Crohn's disease. Gut, <u>24</u>; 525-531.

Elmgreen J, Both H and Binder V (1985). Familial occurrence of complement dysfunction in Crohn's disease: correlation with intestinal symptoms and hypercatabolism of complement.Gut, <u>26</u>;151-157.

Evans J G and Acheson D E (1965). An epidemiological study of ulcerative colitis and regional enteritis in the Oxford area. Gut, $\underline{6}$; 311-324.

Eyer S, Spadaccini C, Walker P, Ansel H, Schwartz M and Summer H W (1980). Simultaneous Ulceratis colitis and Crohn's Disease: a report of a case. Am. J. Gastroenterol., <u>73</u>; 345-349.

Farmer G W, Vincent M M, Fuccillo D A, Horta-Barbosa L, Ritman S, Sever J L and Gitnick G L (1973). Viral investigations in Ulcerative Colitis and regional enteritis. Gastroenterol., <u>65</u>; 8-18.

Farmer R G, Michener W M and Mortimer E A (1980). Studies of family history among patients with inflammatory bowel disease. Clin. Gastroenterol., <u>9</u>; 271-278.

Farmer R G, Michever W M and Sivak D S (1981). Studies of family history in inflammatory bowel disease. In: Recent advance in Crohn's disease. (Pena A S, Weterman I T, Booth C C, Strober W, Eds), pp213-218, Martinus Nijhoff Publishers.

Feinberg A P and Vogelstein B (1984). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem., <u>137</u>; 266-267.

Fiocchi C, Battisto J R and Farmer R G (1981). Studies on isolated gut mucosal lymphocytes in inflammatory bowel disease. Detection of activated T-cells and enhanced proliferation to staphylococcus aureus and lipopolysaccharides. Dig. Dis. Sci., <u>26</u>; 728-736.

French G L, Chan C Y, Cheung S W and Oo K T (1987a). Diagnosis of pulmonary tuberculosis by detection of tuberculostearic acid in sputum by using gas-chromatography-mass spectrometry with selected ion monitoring. J. Inf. Dis., <u>156</u>; 356-362.

French G L, Chan C Y, Cheung S W, Teolt R, Humphries M J and Mahony G O (1987b).Diagnosis of tuberculous meningitis by detection of tuberculostearic acid in cerebronspinal fluid. Lancet, <u>ii</u>; 117-119.

Garland C F, Lilienfeld A M, Mendeloff A I *et al* (1981). Incidence rates of ulcerative colitis and Crohn's disease in fifteen areas of the United States. Gastroenterol., <u>81</u>;1115-1124.

Gibson P R and Jewell D P (1986). Local immune mechanisms in Inflammatory bowel disease and colorectal carcinoma. Gastroenterol.,<u>90</u>; 12-19.

Gilat T and Rozen P (1980). The epidemiology of Crohn's disease, trends and clues. In: Recent advances in Crohn's disease. Pena A S, Weterman I T, Booth C C, Strober W, Eds., pp153-157. Martinus Nijhoff Publishers 1981.

Gitnick G L, Arthur M H and Shibata I (1976). Cultivation of viral agents from Crohn's Disease. Lancet, <u>ii</u>; 215-217.

Gitnick G L and Rosen V J (1976). Electron microscopic studies of viral agents in Crohn's disease. Lancet, <u>ii</u>; 217-219.

Gitnick G, Rosen V J, Arthur M H and Hertweck S A (1979). Evidence for the isolation of a new virus from ulcerative colitis patients - comparison with virus derived from Crohn's disease. Dig. Dis. Sci., <u>24</u>; 609-619.

Gitnick G (1984). Is Crohn's disease a mycobacterial disease after all? Dig. Dis. Sci., <u>29</u>; 1086-1088.

Gitnick G, Collins J, Beaman B, Brooks D, Arthur M and Imaeda T (1988). Prospective evaluation of mycobacterial infection in Crohn's disease: isolation and transmission studies. In: Inflammatory Bowel Disease: Current status and future approach. (MacDermott R P Ed), pp527-533. Elsevier Science Publishers, Amsterdam.

Goodfellow M and Wayne L G (1982). Taxonomy and Nomenclature. In: The biology of the mycobacteria, Volume 1; Physiology, identification and classification. Chapter 10 (Ratledge C and Stanford J L, Eds), pp471-521, Acad Press.

Graham D Y, Markesich D C and Yoshimura H H (1987a). Mycobacteria and Inflammatory Bowel Disease; results of culture. Gastroenterol., <u>92</u>; 436-442.

Graham DY, Markesich DC, Kalter DC and Yoshimura H H. (1987b). Isolation of Spheroplasts of Acid-Fast Bacteria from Sarcoid Tissue and not from Controls (Abstract). 11th World Congress on Sarcoidosis, Milan.

Graham D Y, Markesich D C, Kalter D C and Yoshimura H H (1988). Isolation of cell wall-defective acid-fast bacteria from skin lesions of patients with sarcoidosis. In: Sarcoidosis and other granulomatous disorders, (Grassi C, Rizzato G, Pozzi E Eds) pp161-163. Elsevier Science Publishers, Amsterdam.

Grange J M, Gibson J and Nassau E (1980). Enzyme-linked immunosorbent assay. A study of antibodies to *Mycobacterium tuberculosis* in the IgG, IgA and IgM classes in tuberculosis, sarcoidosis and Crohn's disease. Tubercle, <u>61</u>; 145-152.

Granger D N, Grisham M B, Kvietys P R and Sekizuka E (1987). Neutrophils mediate the increased intestinal vascular permeability induced by luminal perfusion with N-formylmethionylphenylalanine(FMLP). Gastroenterol., <u>92</u>; 1413.

Green E P, Moss M T, Hermon-Taylor J and McFadden J J (1989a). Insertion elements in mycobacteria. Acta. Leprol. Geneve., <u>7</u>; Supplement 1; 239-242.

Green E P, Tizard M L, Moss M T, Thompson J, Winterbourne D J, McFadden J J and Herman-Taylor J (1989b). Sequence and characteristics of IS900 an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res., 17; 9063-9073.

Greenberg H B, Gebhard R L, McClain C J, Soltis R D and Kapikian A Z(1978). Antibodies to viral gastroenteritis viruses in Crohn's disease. Gastroenterol., <u>76</u>; 349-350.

Haagsma J, Mulder C J J, Eger A, Bruins J, Ketel R J and Tytgat G N J (1988). Mycobacterium species isolated from patients with Crohn's disesase. In: Inflammatory bowel disease: Current status and future approach. (MacDermott R P, Ed) pp535-537. Elsevier Science Publishers, Amsterdam.

Hampson S J, McFadden J J and Hermon-Taylor J (1988). Mycobacteria and Crohn's disease. Gut, <u>29</u>; 1017-1019.

Hampson S J, Parker M C, Saverymuttu S H, Joseph A E, McFadden J-J and Hermon-Taylor J (1989a). Quadruple antimycobacterial chemotherapy in Crohn's disease: results at 9 months of a pilot study in 20 patients. Aliment. Pharmacol. Therap., <u>3</u>; 343-352.

Hampson S J, Thompson J, Moss M T, Portaels F, Green E P, Hermon-Taylor J and McFadden J J (1989b)DNA probes demonstrate a single highly conserved strain of Mycobacterium avium infecting AIDS patients. Lancet, \underline{i} ; 65-68.

Hanngren A, Odham G, Eklund A, Hoffner S, Stjernberg N and Westerdahl G (1987). Tuberculostearic Acid in Lymph Nodes from Patients with Sarcoidosis. Sarcoidosis, <u>4</u>; 101-104.

Heatley R V, Bolton P M, Owen E, Jones-Williams W and Hughes L E (1975). A search for a transmissible agent in Crohn's disease. Gut, <u>16</u>; 528-532.

Helphingstine C J, Hentges D J, Campbell B J, Butt J and Barrett J T (1979). Antibodies detectable by counterimmunoelectrophoresis against Bacteroides antigens in serum of patients with inflammatory bowel disease. J. Clin. Microbiol., $\underline{9}$, 373-378.

Hibi T, Aiso S, Yoshida T, Watanabe M, Asa K H and Tsuehiya M (1982). Anti-colon antibody and lymphocytophilic antibody in ulcerative colitis. Clin. Exp. Immunol., <u>49</u>; 75-80.

Hodgson H J F, Potter B J and Jewell D P (1977). C3 metabolism in ulcerative colitis and Crohn's disease. Clin. Exp. Immunol., <u>28</u>; 490-495.

Hopwood et al (1985). Genetic Manipulation of Streptomyces. The John Innes Foundation, Norwich.

Hornick D B, Dayton C S, Bedell G N and Fick R B (1988). Non tuberculous mycobacterial lung disease. Substantiation of a less aggressive approach. Chest, <u>93</u>; 550-555.

Hunt P S and Trotters S (1973). Lymphoblastic response to autologous colon epithelial cells in ulcerative colitis in vitro. Gut, <u>14</u>; 875-879.

Hunter S W, Gaylord H and Brennan P J (1986). Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. J. Biol. Chem., <u>261</u>; 12345-12351.

Ibbotson J P, Pease P E and Allan R N (1987). Cell-Wall Deficient Bacteria in Inflammatory Bowel Disease. Eur. J. Clin. Microbiol., <u>6</u>; 429-431.

Jones V A, Dickinson R J, Workman E, Wilson A J, Freeman A H and Hunter J O (1985). Crohn's disease: maintenance of remission by diet. Lancet, <u>ii</u>; 177-180.

Jiwa N M, Mulder C J J, Van den Berg F M, Tytgat G N J, Haagsma J and Walboomers J M M (1988). Elevated IgG to mycobacterial PPDs in Crohn's disese. From: Inflammatory Bowel Disease, Current Status and Future Approach. (McDermott R P, Ed) pp 543-546, Elsevier Science Publishers, Amsterdam.

Kapikian A Z, Barile M F, Wyatt R G, Yolken R H, Tully J G, Greenberg H B, Kalica A R and Chanock R M (1979). Mycoplasma contamination in cell culture of Crohn's disease material. Lancet <u>ii</u>; 466-469.

Keighley M R, Arabi Y, Dimock F, Burdon D W, Allon R N and Alexander-Williams J (1978). Influence of inflammatory bowel disease on intestinal microflora. Gut, <u>19</u>; 1099-1104.

Kewenter J, Hulten L and Kock N G (1974). The relationship and epidemiology of acute terminal ileitis and Crohn's disease. Gut, 15; 801-804.

Khomenko A G and Golyshevskaya V I (1984). Filtrable forms of *Mycobacterium tuberculosis*. Z. Erkrank Atm. Org, <u>162</u>; 147-154. J A Barth, Leipzig.

Khomenko A G (1987). The variability of *Mycobacterium* tuberculosis in patients with cavitary pulmonary tuberculosis in the course of chemotherapy. Tubercle, <u>68</u>; 243-253.

Kirsner J B (1973). Genetic aspects of inflammatory bowel disease. Clin. Gastroenterol., <u>2</u>; 557-575.

Kirsner J B (1975). Problems in the differentiation of Ulcerative colitis and Crohn's Disease of the colon: the need for repeated diagnostic evaluation. Gastroenterol., <u>68</u>; 187-191.

Kirsner J B and Shorter R G (1982). Recent developments in non specific Inflammatory Bowel Disease. N. Engl. J. Med., <u>306</u>; 775-785.

Kirsner J B (1982). Later development of inflammatory bowel disease in the healthy spouse of a patient. N. Engl. J. Med., <u>307</u>; 1148.

Kirsner J (1984). Crohn's disease. JAMA, 251; 80-81.

Kobayashi K, Brown W R, Brennan P J and Blaser M J (1988). Serum antibodies to mycobacterial antigens in active Crohn's disease. Gastroenterol., 94; 1404-1411.

Kobayashi K, Blaser M J and Brown W R (1989). Immunohistochemical examination for mycobacteria in intestinal tissues from patients with Crohn's Disease. Gastroenterol., <u>96</u>; 1009-1015.

Kyhse-Andersen J (1984). Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods, 10; 203-209.

Kyle J (1971). An epidemiological study of Crohn's disease in north east Scotland. Gastroenterol, <u>61</u>; 826-833.

Kyle J and Stark G (1980). Fall in the incidence of Crohn's disease. Gut, <u>21</u>; 340-343.

Laemmli U K (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature, <u>227</u>; 680-685.

Lancaster R D, Hilson G R F, McDougall A C and Colston M J (1983). *Mycobacterium leprae* infection in nude mice: bacteriological and histological responses to primary infection and large inocula. Infect. Immun., <u>39</u>; 865-872.

Larsson L, Mardh P A, and Odham G (1979). Detection of tuberculostearic acid in mycobacteria and nocardiae by gas chromatography and mass spectrometry using selected ion monitoring. Chromatogr. Biomed. Appl., <u>163</u>; 221-224.

Larsson L, Mardh P A, Odham G and Westerdahl (1980). Detection of tuberculostearic acid in biological specimens by means of glass capillary gas chromatography/electron and chemical ionisation mass spectrometry utilising selected ion monitoring. J. Chromatogr. Biomed. Appl., <u>182</u>; 402-408.

Larsson L, Odham G, Westerdahl G and Olsson B (1987). Diagnosis of pulmonary tuberculosis by selected ion monitoring: improved analysis of tuberculosterate in sputum using negative-ion mass spectrometry. J. Clin. Microbiol., <u>25</u>; 893-896.

Larsson L, Jimenez J, Sonesson A and Portaels F (1989). Twodimensional gas chromatography with electron capture detection for the sensitive determination of specific mycobacterial lipid constituents. J. Clin. Microbiol., 27; 2230-2233.

Lashner B A, Alison A E, Kirsner J B and Hanauer S B (1986). Prevalence and incidence of inflammatory boewl disease in family members. Gastroenterol., <u>91</u>; 1396-1400. 156.

LeDuc L E, Nast C C, Patterson J B and Zipser R D (1987). Chemotactic peptides induce colon inflammation: A new model and mechanism of colitis. Gastroenterol., <u>92</u>; 1496.

Lee F I and Costello F T (1985). Crohn's disease in Blackpool: incidence and prevalence 1968-1980. Gut, <u>26</u>; 274-278.

Levine J S, Smith P D and Brugge W R (1980). Chronic proctitis in male homosexuals due to lymphogranuloma venereum. Gastroenterol., <u>79</u>; 563-565.

Lockhart-Mummery H E and Morson B C (1960). Crohn's Disease (Regional enteritis) of the large intestine and its distinction from Ulcerative colitis. Gut, $\underline{1}$; 87-105.

Maniatis T, Fritsch E F and Sambrook J (1982). Molecular cloning: A laboratory manual. Cold Spring Harbour Laboratories.

Mardh P A, Larsson L, Hoiby N, Engbaek H C and Odham G (1983). Tuberculostearic acid as a diagnostic marker in tuberculous meningitis. Lancet, \underline{i} ; 367.

Marmur J. (1961). A procedure for the isolation of deoxy ribonucleic acid from micro-organisms. J. Mol. Biol., <u>3</u>; 208-218.

Matthews N, Mayberry J F, Rhodes J, Neale L, Munro J, Wensinck F, Lawson G H K, Rowland A C, Berkhoff G A and Barthold S W (1980). Agglutinins to bacteria in Crohn's disease. Gut, <u>21</u>; 376-380.

Mayberry J, Rhodes J and Hughes L E (1979). Epidemiology Incidence of Crohn's disease in Cardiff between 1934 and 1977. Gut, 20; 602-608.

Mayberry J F, Rhodes and, Newcombe R G (1980). Familial prevalence of inflammatory bowel disease in relatives of patients with Crohn's disease. Br. Med. J., <u>280</u>; 84.

McClure H M, Chiodini R J, Anderson D C, Swenson R B, Thayer W R and Coutu J A (1987). *Mycobacterium paratuberculosis* infection in a colony of stump-tail macaques (Macaca arctoides). J. Inf. Dis., <u>155</u>; 1011-1019.

McFadden J J, Butcher P D, Chiodini R J and Hermon-Taylor J (1987a). Determination of genome size and DNA homology between an unclassified mycobacterium species isolated from patients with Crohn's disease and other mycobacteria. Gen. Microbiol., <u>133</u>; 211-214.

McFadden J J, Butcher P D, Chiodini R J and Hermon-Taylor J (1987b). Crohn's disease - isolated mycobacteria are identical to *Mycobacterium paratuberculosis* as determined by DNA probes that distinguish between mycobacterial species. J. Clin. Microbiol., <u>25</u>; 796-801.

McFadden J J, Butcher P D, Thompson J, Chiodini R J and Hermon-Taylor J (1987c). The use of DNA probes identifying restriction fragment length polymorphisms to examine the *Mycobacterium* avium complex. Mol. Microbiol., <u>1</u>; 283-291.

McFadden J J and Houdayer C (1988). No evidence for antibodies to mycobacterial A60 antigen in Crohn's disease sera by enzyme-linked immunoabsorbent assay (ELISA). J. Med. Microbiol., <u>25</u>; 295-298.

McFadden J J, Thompson J, Green E, Hampson S J, Stanford J, Haagsma J, Chiodini R and Hermon-Taylor J (1988). Identification and characterisation of further bacteria isolated from Crohn's disease and control tissues. Gastroenterol., <u>94</u>; A294.

McIntyre G and Stanford J L (1986a). The relationship between immunodiffusion and agglutination serotypes of *Mycobacterium avium* and *Mycobacterium intracellulare*. Eur. J. Respir. Dis. <u>69</u>; 135-141.

McIntyre G, Stanford J L (1986b). Immunodiffusion analysis shows that *Mycobacterium paratuberculosis* and other mycobactin dependent mycobacteria are variants of *Mycobacterium avium*. J. Appl. Bacteriol, <u>61</u>; 295-298.

McLaren L C, Gitnick G (1982). Ulcerative colitis and Crohn's disease tissue cytotoxins. Gastroenterol., <u>82</u>, 1381-1388.

Minnikin D E (1982). Lipids: complex lipids, their chemistry, biosynthesis and roles. In: The biology of the mycobacteria, Volume 1; Physiology, identification and classification. Chapter 4 (Ratledge C and Stanford J L, Eds), pp95-184, Acad Press Inc Ltd, London.

Minnikin D E, Dobson G, Goodfellow M, Draper P and Magnusson M (1985a). Quantitative comparison of the mycolic and fatty acid compositions of Mycobacterium leprae and Mycobacterium gordonae. J. Gen. Microbiol., <u>131</u>; 2013-2021

Minnikin D E, Minnikin S M, Parlett J H and Goodfellow M (1985b). Mycolic acid patterns of some rapidly growing species of mycobacterium. Zbl. Bakt. Hyg. A., <u>259</u>; 446-460.

Mitchell D N and Rees R J W (1969). A Transmissible Agent from Sarcoid Tissue. Lancet, <u>ii</u>; 81-84.

Mitchell D N and Rees R J W (1970). Agent transmissible from Crohn's disease tissue. Lancet, <u>ii</u>; 168-171.

Moll J M (1985). Inflammatory bowel disease. Clin. Rheum. Dis., <u>11</u>, 87-111.

Monk M, Mendeloff A I, Siegel C I and Lilienfeld A (1967). An epidemiological study of ulcerative colitis and regional enteritis among adults in Baltimore. I. Hospital incidence and prevalence, 1960-1963. Gastroenterol., <u>53</u>; 198-210.

Monk M, Mendeloff A I, Siegel C I et al (1969). An epidemiological study of ulcerative colitis and regional enteritis among adults in Baltimore. II. Social and demographic factors. Gastroenterol., <u>56</u>; 847-857.

Moráin C O, Prestage H, Harrison P, Levi A J and Tyrrell D A J (1981). Cytopathic effects in cultures inoculated with material from Crohn's disease. Gut, <u>22</u>; 823-826.

Morgan K L (1987). Johne's and Crohn's: Chronic Inflammatory Bowel Diseases of Infectious Actiology? Lancet,<u>i</u>; 1017-1019.

Morris T and Rhodes J (1984). Incidence of ulcerative colitis in the Cardiff region 1968-1977. Gut, <u>25</u>, 846-848.

Much H (1907). Uber die granulare, nach Ziehl nicht farbbare Form des Tuberkulose virus. Beitrage Klinisches Tuberkulose,<u>8</u>; 85. Murohashi T, Kondo E and Yoshida K (1969). The Role of Lipids in Acid-Fastness of Mycobacteria. Am. Rev. Resp. Dis., <u>99</u>; 794-798.

Nagai T and Das K M (1981). Detection of colonic antibody in tissues from ulcerative colitis using purified colitis colon tissuebound IgG (CCA-IgG). Gastroenterol., <u>81</u>; 463-470.

Odham G, Larsson L and Mardh P A (1979). Demonstration of tuberculostearic acid in sputum from patients with pulmonary tuberculosis by selected ion monitoring. J. Clin. Invest., <u>63</u>; 813-819.

Owen R J, Beck A, Dayal P A and Dawson C (1988). Detection of genomic variation in *Providencia stuartii* clinical isolates by analysis of DNA restriction fragment length polymorphisms containing rRNA cistrons. J. Clin. Microbiol., <u>26</u>; 2161-2166.

Owen R J (1989). Chromosomal DNA fingerprinting - a new method of species and strain identification applicable to microbial pathogens. J. Med. Microbiol., <u>30</u>; 89-99.

Parent K and Mitchell P D (1976). Bacterial variants: etiologic agent in Crohn's disease?. Gastroenterol., <u>71</u>; 365-368.

Parent K and Mitchell P D (1978). Cell wall-defective variants of Pseudomonas-like (Group Va) bacteria in Crohn's disease. Gastroenterol., <u>75</u>; 368-372.

Parker M, Hampson S, Saverymuttu S, Cawthorn S, McFadden J J, Thomspon J, Green E, Rutter K and Hermon-Taylor J (1987). Combination antimycobacterial chemotherapy with major clinical response in established Crohn's disease. Gut, <u>28</u>; Al390.

Patel R, Kvach J and Mounts P (1986). Isolation and restrition endonuclease analysis of mycobacterial DNA. J. Gen. Microbiol., <u>132</u>; 541-551.

Pena A S, Wolvoort H C, Lamers C B H W and Ruitenberg E J (1988).Seroreactivity of Crohn's disease patients sera against lymphoid cells from nude mouse lymph nodes primed with intestinal tissue. In: Inflammatory Bowel Disease: Current status and Future Approach (MacDermott R P Ed) pp471-481.

Phillpotts R J, Hermon-Taylor B and Brooke B N (1979). Virus isolation studies in Crohn's disease: a negative report. Gut, <u>20</u>; 1057-1062.

Phillpotts R J, Hermon-Taylor J, Teich N M and Brooke B N (1980). A search for persistent virus infection in Crohn's disease. Gut, <u>21</u>; 202-207.

Picciotto A, Gesu G P, Schito G C, Testa R, Varagona G and Celle G (1988). Antimycobacterial chemotherapy in two cases of inflammatory bowel disease. Lancet, <u>i</u>; 536-537.

Prantera C, Argentieri R, Mangarotti R and Levenstein S (1988). Dapsone and remission of Crohn's disease. Lancet, <u>i</u>; 536. 208.

Price W H (1979). A high incidence of chronic inflammatory bowel disease in patients with Turner's syndrome. J. Med. Genet., <u>16</u>; 263-266.

Prinz G, Ban E, Fekete S and Szabo Z (1985). Meningitis caused by *Gordona aurantiaca (Rhodococcus aurantiacus*). J. Clin. Microbiol., <u>22</u>; 472-474.

Quinn T C, Stamm W E, Goodell S E, Mkritchian E, Benedetti J, Corey J, Schuffler M D and Holmes K K (1983). The polymicrobial origin of intestinal infections in homosexual men. N. Engl. J. Med., 309; 576-582.

Quinn T C, Taylor H R and Schachter J (1986). Experimental proctitis due to rectal infection with *Chlamydia trachomatis* in non-human primates. J. Inf. Dis., <u>154</u>; 833-841.

Rastogi N, Levy-Frebault V and David H L (1983). Spheroplast formation for nine rapidly growing mycobacteria. Curr. Microbiol., <u>9</u>; 201-204.

Ratliff T L, McGarr J A, Abou-Zeid C, Rook G A W, Stanford J L, Aslanzadeh J and Brown E J (1988). Attachment of mycobacteria to fibronectin-coated surfaces. J. Gen. Microbiol., <u>134</u>; 1307-1313.

Ratnam S and Chandrasekhar S (1976). The pathogenicity of spheroplasts of *Mycobacterium tuberculosis*. Am. Rev. Resp. Dis. 114; 549-554.

Rhodes J and Mayberry J (1986). Ulcerative Colitis. Medicine International, 26; 1056-1062.

Roche J K, Fiocchi C and Youngman K (1985). Sensitisation to epithelial antigens in chronic mucosal inflammatory disease. Characterisation of human intestinal mucosa-derived mononuclear cells reactive with purified epithelial cell-associated components in vitro. J. Clin. Invest., <u>75</u>; 522-530.

Roth M, Petersen G, McElree C, Vadheim C M, Panish J F and Rotter J I (1989). Familial empiric risk estimates of inflammatory bowel disease in Ashkenazi Jews. Gastroenterol., <u>96</u>; 1016-1020.

Rozen P, Zonis J, Yekutiel P et al (1979). Crohn's disease in the Jewish population of Tel Aviv-Yafo: epidemiologic and clinical aspects. Gastroenterol., <u>76</u>; 25-30.

Ruseler-Van Embden J G H and Both-Patoir H C (1983). Anaerobic gram negative faecal flora in patients with Crohn's disease and healthly subjects. Antonie Van Leeuwenhoek, <u>49</u>; 125-132.

Rutgeerts P, Vantrappen G, Van Isveldt J and Geboes K (1988). Rifabutin therapy in patients with recurrent Crohn's disease after ileocolonic resection. Gastroenterol., <u>94</u>; A391.

Saverymuttu S H, Camilleri M, Rees H, Lavender P, Hodgson H J and Chadwick V S (1986). Indium-111 granulocyte scanning in the assessment of disease extent and disease activity in inflammatory bowel disease. Gastroenterol., <u>90</u>; 1121-1128.

Schinella R A, Greco A, Cobert B L, Denmark L W and Cox R P (1980). Hermansky-Pudlak syndrome with granulomatous colitis. Ann. Intern. Med., <u>92</u>; 20-23.

Schuller J L, Piket-Van Ulsen J, Veeken I V D, Michel M F and Stolz E (1979). Antibodies against Chlamydia of lymphogranuloma-venereum type in Crohn's disease. Lancet, \underline{i} ; 19-20.

Schultz M G, Rieder H L, Hersh T and Riepe S (1987). Remission of Crohn's disease with antimycobacterial chemotherapy. Lancet, <u>ii</u>: 1391.

Sedlack R E, Whisnant J, Elveback L R et al (1980). Incidence of Crohn's disease in Olmsted County, Minnesota, 1945-1975. Am. J. Epidemiol., <u>112</u>; 759-763.

Selby W S, Janossy G, Bofill M and Jewell D P (1984). Intestinal lymphocyte subpopulations in inflammatory bowel disease an analysis by immunohistological and cell isolation techniques. Gut, <u>25</u>; 32-40.

Shaffer J L, Hughes S, Linaker B D, Baker R D and Turnberg L A (1984). Controlled trial of rifampicin and ethambutol in Crohn's disease. Gut, <u>25</u>; 203-205.

Shepard C C (1960). The experimental disease that follows the injection of human leprosy bacilli into the footpads of mice. J. Exp. Med., <u>112</u>; 445-454.

Shield M J (1983). The importance of immunologically effective contact with environmental mycobacteria. In: The Biology of the Mycobacteria, Volume 2. Immunological and Environmental Aspects (Ratledge C and Stanford J L, Eds), pp346, Academic Press.

Shorter R G, McGill D B and Bahn R C (1984). Cytotoxicity of mononuclear cells for autologous colonic epithelial cells in colonic diseases. Gastroenterol., <u>86</u>; 13-22.

Skinsnes O K, Matsuo E, Chang P H C and Andersson B (1975). In vitro cultivation of leprosy bacilli on hyaluronic acid based medium. Preliminary Report. Intern. J. Lepr., <u>43</u>; 193-208.

Somerville K W, Logan R F A, Edmond M and Langman M J (1984). Smoking and Crohn's disease. Br. Med. J., <u>289</u>; 954-956.

Southern E M (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol., <u>90</u>; 503-517.

Stanford J L (1980). Acid fast organisms in Crohn's disease and Ulcerative colitis. From Recent Advances in Crohn's Disease. (Pena A S, Weterman I T, Booth C C and Strober W, Eds), pp274-277. Martinus Nijhoff Publishers.

Stanford J L (1983). Immunologically important constituents of mycobacteria: Antigens. From The Biology of the Mycobacteria, Volume 2; Immunological and environmental aspects, Chapter 2, (Stanford J L and Ratledge C, eds), pp92-93, Academic Press.

Stanford J L, Dourmashkin R and McIntyre G (1987). An investigation into the role of mycobacteria in Crohn's disease. In Inflammatory Bowel Disease - Frontiers of Actiology. Proceedings of the Eighth BSG SK and F International Workshop (Allan R N and Hodgson H J F, Eds), pp24-28. Smith Kline and French Labs Ltd.

Stanford J L (1987). Much's granules revisited. Tubercle, 68; 241-242.

Stanford J L, Dourmashkin R, McIntyre G and Visuvanathan S (1988). Do mycobacteria exist in alternative physical forms and what part may they play in the aetiology of inflammatory bowel disease. Inflammatory Bowel Disease: current status and future approach, p503-508.

Tabaqchali S, O'Donoghue D P and Bettelheim K A (1978). Escherichia coli antibodies in patients with inflammatory bowel disease. Gut, <u>19</u>; 108-113.

Tanner A C R, Badger S, Lai C-H, Listgarten M A, Visconti R A and Socransky S (1981). *Wolinella* gen. nov.,*Wolinella* succinogenes (*Wibrio* succinogenes Wolin et al.) comb. nov., and Description of *Bacteroides* gracilis sp. nov., *Wolinella* recta sp. nov., *Campylobacter* concisus sp. nov., and *Eikenella* corrodens from humans with Periodontal Disease. Int. J. Syst. Bacteriol., <u>31</u>; 432-445.

Taub R N and Siltzbach L E (1972). Induction of granulomas in mice by injection of human sarcoid and ileitis homogenates. In Iwaik ans Hosada Y (eds). Proc. VIth Int. Conference on Sarcoidosis, Univ. of Tokyo Press, Tokyo.

Taylor-Robinson D, O'Morain C A, Thomas B J and Levi A J (1979). Low frequency of Chlamydial antibodies in patients with Crohn's disease and Ulcerative colitis. The Lancet, \underline{i} ; 1162-1163.

Thayer W R, Coutu J A, Chiodini R J, Van Kruiningen H J and R S Merkal (1983). *Mycobacterium paratuberculosis* antibodies in Crohn's disease. Gastroenterol., <u>84</u>; 1334.

Thayer W R, Coutu J A, Chiodini R J , Van Kruiningen H J and Merkal R S (1984). Possible role of mycobacteria in Infalammatory Bowel Disease.II. Mycobacterial antibodies in Crohn's Disease. Dig. Dis.Sci., <u>29</u>; 1080-1085.

Thayer W R, Coutu J A, Chiodini R J and Van Kruiningen H J (1988). Use of rifabutin and streptomycin in the therapy of Crohn's disease-preliminary results. In: Inflammatory bowel disease: Current status and future approach. (MacDermott R P, Ed), pp565-568. Elsevier Science Publishers, Amsterdam.

Thornton J R, Emmett P M and Heaton K W (1979). Diet and Crohn's disease: characteristics of the pre-illness diet. Br. Med. J., <u>2</u>; 762-764.

Thornton J R, Emmett P M and Heaton K W (1985). Smoking, sugar and inflammatory bowel disease. Br. Med. J., <u>290</u>; 1786-1787.

Tsukamura M and Kawakami K (1982). Lung infection caused by *Bordona aurantiaca (Rhodococcus aurantiacus*). J. Clin. Microbiol., <u>16</u>; 604-607.

Tsukamura M (1982). Nocardiae that recently caused lung infection in Japan - *Nocardia asteroides* and *Nocardia farcinia*. Microbiol. Immunol., <u>26</u>; 341-345.

Tsukamura M and Yano I (1985). *Rhodococcus sputi* sp. nov., nom. rev., and *Rhodococcus aurantiacus* sp. nov., nom. rev. Int. J. Syst. Bacteriol., <u>35</u>; 364-368.

Tsukamura M, Komatsuzaki C, Sakai R, Kaneda K, Kudo T and Seino A (1988). Mesenteric lymphadenitis of swine caused by *Rhodococcus sputi*. J. Clin. Microbiol., <u>26</u>; 155-157.

Tsukamura M (1988). Evidence that antituberculosis drugs are really effective in the treatment of pulmonary infection caused by Mycobacterium avium complex. Am. Rev. Resp. Dis., <u>137</u>; 144-148.

Tsukamura M (1989). Reviews of non-tuberculous mycobacterioses. A collection of reviews (in Japanese and English).

Tytgat G N J and Mulder C J J (1986). The aetiology of Crohn's disease. Review. Int. J. Colorect. Dis., $\underline{1}$; 188-192.

Van De Merwe J P (1984). The human faecal flora and Crohn's Disease. Antonie van Leerwenhoek, <u>50</u>; 691-700.

Van den Berg-Loonen E M, Kekken-Saeys B J, Meuwissen S G M et al (1977). Histocompatibity antigens and other genetic markers in ankylosing spondylitis and inflammatory bowel disease. J. Immunogenet., <u>4</u>; 167-175.

Van Dyke T E, Dowell V R, Offenbacker S, Snyder W and Hersh T (1986). Potential role of micro-organisms isolated from peridontal lesions in the pathogenesis of inflammatory bowel disease. Infect. and Immun., <u>53(3)</u>; 671-677.

Van Kruiningen H J, Thayer W R, Chiodini R J, Meuton D J and Coutu J A (1988). An immunoperoxidase search for mycobacteria in Crohn's Disease. In: Inflammatory Bowel Disease. Current status and future approach (MacDermott R P, Ed), pp547-552 Elseiver Science Publishers, Amsterdam.

Verspaget H W, Mieremet-Ooms M A C, Weterman I T and Pena A S (1984). Partial defect of neutrophil oxidative metabolism in Crohn's disease. Gut, <u>25</u>; 849-853.

Wakefield A J, Dhillon A P, Rowles P M, Sawyer A M, Pittilo R M, Lewis A M M and Pounder R E (1989). Pathogenesis of Crohn's disease: multifocal gastrointestinal infarction. Lancet, <u>ii</u>; 1057-1062.

Wandall J H and Binder V (1982). Leucocyte function in Crohn's disease. Gut, <u>23</u>; 173-180.

Ward M and McManus J P A (1975). Dapsone in Crohn's Disease. Lancet, <u>i</u>, 1236-1237.

Warren J B, Rees H C and Cox T M (1986). Remission of Crohn's disease with tuberculosis chemotherapy. N.Eng.J.Med., <u>314</u>; 182.

Wensinck F, Custers-Van Lieshout L M, Poppelaars-Kustermans P A and Schroder A M (1981). The faecal flora of patients with Crohn's disease. J. Hyg. Camb., <u>87</u>; 1-12.260.

Weterman I T and Pena A S (1984). Familial incidence of Crohn's disease in the Netherlands and a review of the literature. Gastroenterol., <u>86</u>; 449-452.

White S A, Nassau E, Burnham W R, Stanford J L and Lennard-Jones J E (1978). Further evidence for a mycobacterial aetiology of Crohn's disease. Gut <u>19</u>; A443-444.

White S A (1980). Investigation into the identity of acid fast organisms isolated from Crohn's disease and Ulcerative colitis. From Recent Advances in Crohn's disease (Pena A S, Weterman I T, Booth C C and Storber W, Eds), pp278-282. Martinus Nijhoff Publishers.

White S A (1981). An investigation into a possible mycobacterial aetiology for inflammatory bowel disease. PhD thesis, University of London.

Whorwell P J, Phillips C A, Beeken W L, Little P K and Roessner K D (1977). Isolation of reovirus-like agents from patients with Crohn's disease. Lancet, \underline{i} ; 1169-1171.

Whorwell P J, Davidson I W, Beeken W L and Wright R (1978a). Search by immunofluorescence for antigens of Rotavirus, *Pseudomonas maltophilia* and *Mycobacterium kansasii* in Crohn's disease. Lancet, <u>ii</u>; 697-698.

Whorwell P H, Eade O E and Hossenbocus A et al (1978b).Crohn's disease in a husband and wife. Lancet, <u>ii</u>, 186-187.

Whorwell P J, Holdstock G, Whorwell G M et al (1979). Bottle feeding, early gastroenteritis and inflammatory bowel disease. Br. Med. J., 1, 382.

Williams W J (1965). A study of Crohn's syndrome using tissue extracts and the Kveim and Mantoux tests. Gut, $\underline{6}$; 503-505.

Wright R and Truelove S C (1965). A controlled therapeutic trial of various diets in ulcerative colitis. Br. Med. J., 2; 138.

Yoshimura H H, Estes M K and Graham D Y (1984). Search for evidence of a viral aetiology for inflammatory bowel disease. Gut <u>25</u>; 347-355.

Yoshimura H H, Graham D Y, Estes M K and Merkal R S (1987). Investigation of association of mycobacteria with Inflammatory Bowel Disease by nucleic acid hybridisation. J. Clin. Microbiol., <u>25</u>; 45-51.

Yoshimura H H, Markesich D C and Graham D Y (1988). Studies of mycobacteria isolated from patients with inflammatory bowel disease. In: Inflammatory bowel disease. Current status and future approach. (MacDermott R P Ed), pp 521-525. Elsevier Publishers, Amsterdam.

Zetsel L (1978). Crohn's disease in a husband and wife (letter). Lancet, <u>ii</u>; 583.

APPENDIX

CHAPTER THREE

Lowenstein-Jensen Medium

Mineral salt solution	
Potassium dihydrogen phosphate (anhydrous)	2.4g
Magnesium sulphate	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol	12ml
Distilled water	600ml
Malachite green (2% aqueous solution)	20ml

Add all the constituents and shake to mix. Sterilise solutions by autoclaving (121°C,25 minutes) and when cool,add 19 eggs (approx 1 litre).

Sterilise egg shells (with methylated spirits) and flame. Crack egg and whisk. Filter through a sieve. Mix beaten eggs with solution. Distribute aseptically into sterile containers.Slope and inspissate at 75-80°C for one hour.

Robertson's Cooked Meat Broth

This was obtained commercially from Southern Group Laboratories Ltd. Fresh bullock's heart 500 g Distilled water 500 ml Sodium hydroxide lN NaOH 1.5 ml Mince heart, place in alkaline boiling water and simmer for 20 mnts. Drain off liquid through a muslin filter. Introduce into bottles. Peptone infusion broth

Liquid filtered from cooked meat	500 ml
Peptone	2.5 g
NaCL	1.25 g

Steam at 100°C for 20 mnts, add 1 ml pure HCL and filter. Bring reaction of filtrate to pH 8.2, steam at 100°C for 30 mnts, and adjust reaction to pH7.8.Cover meat in bottles with broth.Autoclave at 121°C for 20 mnts. <u>CA Agar</u> (Corynebacterium agar)

Tryptone soya broth	30g
Yeast extract	10g
Agar No l	12g
Tween 80	5ml
Distilled water up to	1L
Melt down and pour into pe	etri dishes when ready for use.

<u>Columbia Blood Agar</u> - Oxoid <u>Chocolate Agar</u> -Gibco . Heat to 60°C <u>Camplylobacter medium</u> - Oxoid

Sauton Medium

Asparagine	6g
Glycerol	30ml
Citric Acid	2g
K2 HPO4	1.5g
MgSO4	0.25g
FeNH4 citrate	0.05g
Aq dest	1L

Adjust ph to 6.2 using 1.4ml concentrated ammonia. For solid medium, add 12g/litre Bactro agar oxoid number 3. Bottle and autoclave 15 lbs/15 minutes. When cool add 25mls sterile 40% glucose/litre.

Herrold's Egg Yolk Medium (HEYM)

Nutrient agar base:-Beef extract 3g Bacto peptone 10g Sodium chloride 5g Distilled water 1L Agar No 1 15g Glycerol 60ml

Make up nutrient agar base and divide into 150ml portions and autoclave 151bs/30 minutes. Sterilise eggs (wash, soak in alcohol). Add 1 egg yolk to each 150ml of agar when it has cooled to 55°C.Dispense aseptically into bottles. Allow to set in sloping position.

<u>Mycobactin</u> (lmg/ml stock) Filter through 0.2μ filter to ensure sterility. Add to a final concentration of 3 $\mu\text{g}/\text{ml}$ to HEYM (and Mycobactin to nutrient agar base and put in a 55-60°C water bath). Large RCM bottle (add 45µl to the 15ml broth volume) Small RCM bottle (add 15µl to the 5ml broth volume) Mycoplasma medium Base: mycoplasma agar base 19.9g (CM_{401}) yeast extract 20g distilled water 640mls Autoclave Supplements - horse serum 40ml 1% thallous acetate 20ml 10% urea 5.6ml 2% manganese sulphate 8.4ml 0.2% phenol red indicator 3.0ml 60µg ml⁻¹ penicillin 1.6ml HCl (to give pH 7.00) 4ml

Ziehl Neelsen stain

Reagents:	Carbol	Fuchsin	- Basic fuchsin	5g
			Phenol	25g
			Ethanol	50ml
			Aq dest to	500ml

12.5% H₂SO4

0.5% aqueous malachite green

Filter carbol fuchsin through two layers Whatman 12.5cm filter paper before use, to avoid stain deposit.

Heat fix smear in gently heat.Apply carbol fuchsin and heat until steam rises. Leave for 15 minutes. Wash with tap water. Add 12.5% sulphuric acid for 5 minutes. Rinse in tap water.Counterstain with malachite green solution for 30 seconds. Wash with water and air dry.

<u>Gram stain</u> Heat fix smear. Ammonium oxalate-crystal violet - one minute.Water wash. Lugol's iodine - one minute. Water wash. Acetone iodine - 10 seconds. Water wash. Saffranin counterstain - two minutes. Wash and dry.

CHAPTERS FOUR AND FIVE

<u>Subtilisin</u> :Sigma No P-5380 Protease, Subtilisin Carlsberg; subtilopeptidase A, Type VIII from Bacillus subtilis.

Lysozyme :Sigma No L-6876 Muramidase, mucopeptide Nacetylmuramoylhydrolase; EC 3.2.1.17 from chicken egg white, grade 1.

<u>SDS</u>: 20% stock solution of sodium dodecyl sulphate was prepared. SDS (BDH Chemicals, No 44215) 20g; distilled water 100mls.

<u>Pronase</u> Calbiochem brand 53702, from Streptomyces griseus, Behring Diagnostics, La Jolla, CA 92037 Hoechst.

<u>RNAase</u>

Ribonuclease, Sigma product no. R-5503 from bovine pancreas.5 X crystallised. Type 1-AS. Essentially protease free. Boiled and prepared as a stock solution of 20mg/ml and stored at -20°C. Before use, thaw and add to TE buffer. RNAase stock solution 0.75µl TE buffer 1.5ml

Ammonium acetate Ammonium acetate (BDH Chemicals no 10013) 7.7g Distilled water 10mls

TEN Buffer

50mm Tris base (Sigma no T-1503)6.05g100mm EDTA (ethylene diamine tetra acetic acid37.2gdisodium salt, BDH Chemicals Ltd no 10093)150mm NaCl (BDH Chemicals Ltd)8.76gVolume made up to 1L with distilled water (adjust to pH8 with HCl).Autoclave to sterilise.

<u>TE Buffer</u>

10mm tris base (Sigma No T-1503),1.21g1mm EDTA (BDH Chemicals Ltd, no 10093)0.37gVolume made up to 1L with distilled water, adjust to pH 8 with HClAutoclave.

<u>Phenol</u>

Crystalline phenol was redistilled at 160°C to remove contaminants that cause breakdown or cross-linking of DNA and RNA. Gloves were worn and redistillation was carried out in a fume cupboard. The phenol was then stored at -20°C in aliquots. When needed, the phenol was warmed to roam temperature and melted at 68°C in a water bath. 8-Hydroxyquinoline was added to a final concentration of 0.1%. This compound is an antioxidant and a partial inhibitor of RNase. It also imparts a yellow colour facilitating phase identification. Preparation of phenol was as recommended in Molecular Cloning, A Laboratory Manual, Maniatis, Fritsch and Sambrook, 1982, p438. Phenol (BDH Product no 10188) 100ml 8-Hydroxyquinolone (Sigma product no H6878) 0.1g Then saturate with TEN buffer and store at 4°C.

Chloroform: Isoamyl Alcohol 24:1 v/v

Chloroform (BDH) 48 mls; Iso amyl alcohol 2mls

Chloroform denatures proteins. Isoamyl alcohol reduces foaming during the DNA extraction procedure and facilitates separation of the aqueous and organic phases.Preparation and use recommended in Molecular Cloning, A Laboratory Manual by Maniatis, Fritsch and Sambrook, 1982 pg438.

Optical Density (OD) Measurements

10µl of DNA in TE (with RNase) buffer was added to lml distilled water and mixed. The Unicam SP1800 UV spectrophotometer was zeroed using two cuvettes with distilled water. The sample cuvette was filled with each sample and OD measurements (absorbance) were read at 260 and 280nm. A ratio of OD 260/280 > 1.7 indicated the DNA was pure with litte protein contamination. This was found to be the case for all samples tested. DNA yield was determined spectrophotometrically. A260 = 1 = 50 μ g/ml⁻¹ DNA. Hence DNA concentration = OD₂₆₀ x 50 x 100 μ g/ml. <u>Agarose</u> : Agarose No A-6013, Sigma <u>Agarose Gel Electrophoresis</u> Tris Borate EDTA (TBE) buffer; pH 8.0 Stock solution (20x) 2.0M Tris base 242.2g 2.0M Boric acid 123.7g 40mm EDTA 14.9g ------1 litre

Agarose Gel

Dissolve 2g agarose in 200mlof TBE buffer (1x) in microwave for 3 minutes. Add ethidium bromide to 0.5 μ g/ml whilst gel is cooling. When cooled to hand hot, pour into a 10 x 15cm gel mould. Place comb with 20 μ l spacers for each well to take a 20 μ l sample. After the gel is set, place in gel tank containing 1 x TBE buffer with 0.5 μ gml⁻¹ ethidium bromide.

Agarose Gel Electrophoresis with TAE Buffer

Make a 1% agarose gel by dissolving 1g agarose in 100ml distilled water in a microwave oven for 3 minutes. Add 5 μ l of a 10mg/ml solution of ethidium bromide. Add TAE buffer to give a 1 x solution, cool to hand hot and pour slowly into a 10 x 15cm gel mould, containing a comb with 20 μ l spacers. Avoid bubbles especially near the wells. After the gel is set, place in a gel tank and submerge with a solution of 1 x TAE buffer with 0.5 μ g/ml ethidium bromide.

TAE buffer - stock solution, pH8.0

Trizma base	242g
Glacial acetic acid	57.lml
EDTA (0.5M)	100ml
Distilled water up to	lL

PVU II restriction endonuclease

Northumbria Biologicals Ltd (NBL), Cramlington, Northumberland.

<u>Hind III restriction endonuclease</u> (NBL) Stock = 1000 units/83µl

<u>Sst I restriction endonuclease</u> Stock = 2000 units/400µ1 20 x SSC stock solution pH7 (standard saline sodium citrate buffer) 3.0M Nacl (sodium chloride) 0.3M Na₃ Citrate (trisodium citrate) ABC solution is a mixture of oligonucleotides composed from 4 solutions 0, A, B and C. Solution O: 1.25M Tris HcL pH8 0.125M Mgcl₂ Solution A: Iml Solution O 5µl each dATP, dGTP and dTTP 18µl B-mercaptoethanol Solution B: 2M Hepes buffer pH6 (Sigma) Solution C: Hexadeoxyribonucleotides (Pharmacia) A, B and C are mixed in the ratio 100:250:150

Sodium Phosphate Buffer for Southern Blotting Stock solution: adjusted to pH 7.2 16mls 0.5M Na₂HPO₄ 34mls 0.5M NaH₂PO₄ Add 25mls stock solution to 475mls distilled water (1 in 20 dilution)

CHAPTER SIX

<u>Alcoholic KOH</u> (ethanolic KOH) Use ethanol containing hydroquinone 4µg/ml Ethanol 94 ml : 33% KOH 6 ml

Diazomethane

Prepared from Diazald (Aldrich Chemical Co, P O Box 355, Milwaukee, Wisconsin 53201, USA). This highly toxic substance should always be handled in a fume cupboard.

CHAPTER SEVEN

Section A

Tetrahydrofuran: acetonitrile (50:60) reservoir: 120mls acetonitrile and 100ml THF were filtered through an 0.2µ filter and the mixture was then sonicated for 3 minutes to remove any absorbed gases.

Section B

Western Blotting

Sonication

Harvest organisms in RCM broth culture (culture volume used = 2ml) into 3 mls distilled water in a glass sonication vessel. Put rubber cap on vessel and place in sonicator. Insert probe to approximately 1/8" below the surface of the material to be sonicated. Sonicate for 15 minutes.

Transfer sonicate into tubes and centrifuge for 1hr at 10,000g at 4°C. After centrifugation, filter with 0.22µm low protein binding filter (Millex-GV, Millipore). Then estimate protein concentration.

<u>Protein Estimation</u> (Method of Warburg and Christian, Biochemische Zeitschrift (A41) <u>310</u>, 384).

Dilute filtrate 1 in 100 in distilled water. Use spectrophotometer (Unicam SP1800UV) and measure absorbance at 260 and 280nm. Calculate ratio of A280/A260 and read corresponding chart factor (protein) μ g/ul = chart factor x A280 x 100.

Buffers/Solutions used for SDS-Page

12.5% (w/v) acrylamide resolving gel30% acrylamide monomer solution12.5ml1.5M resolving gel buffer, pH8.87.5ml10% (w/v)SDS0.35ml10% Ammonium persulfate0.15ml(0.05-0.1g in 0.5-1mL distilled water)

distilled water 9.55ml per gel

Mix all components in a 100ml volumetric cylinder by inversion. Avoid bubbles. Add 15µl TEMED (tetra methylethylenediamine) and mix again. TEMED is toxic and should be handled carefully. Once TEMED is added, pour quickly or gel will set.

Butanol saturated with distilled water

Add butanol to water in a universal. Shake to mix. Leave to separate at room temperature for one hour. Butanol is in the top layer.

Sample Buffer 0.0625M - Tris Hcl ph 6.8 2% (w/v) SDS 10% (v/v) glycerol 0.001% bromophenol blue 5% (v/v) 2-mercaptoethanol As 2-mercaptoethanol is toxic, handle in class 1 safety cabinet.

Resolving gel overlay solution 0.375 M-Tris HCl pH8.8 0.1% (w/v) SDS

Monomer Solution

Acrylamide grade 1 (BDH)	58.4g
Bisacrylamide (BDH)	1.6g
Distilled water	200ml

<u>Stacking Gel Buffer</u> 0.5 M Tris HCl pH6.8 <u>Resolving Gel Buffer</u> 1.5M Tris Hcl pH 8.8

4% (w/v) acrylamide stacking gel

Acrylamide monomer solution	1.3ml
Stacking gel buffer	2.5ml
10% (w/v) SDS	0.lml
10% ammonium persulphate	0.lml
(0.05-0.1g in 0.5-1ml	
distilled water)	
Distilled water	6.Oml
0.04% Bromophenol blue	0.05ml

Mix all components by inversion in a volumetric cylinder. Avoid bubbles. Add 10µl tetramethylethylene diamine (TEMED) and mix. Pour quickly once TEMED is added or gel will set. <u>Cathode Buffer</u> 25mM Tris, 40mM 6-amino-n-hexanoic acid, 20% Methanol, pH 9.4. Add methanol just before use.

Anodic buffer No 10.3MTris, 20% methanol pH 10.4Anodic Buffer No 225mMTris, 20%Methanol, pH 10.4

Electrode Buffer ph 8.3 Make up 10 x stock solution Using Trisma base 30g Glycine 144g Distilled Water 1 litre When ready to use: Stock solution 500ml Distilled water 4450ml 10% (w/v) SDS <u>50ml</u> 5L

PBS-Tween 0.01M PBS pH7.2	
KH2 PO4	0.2g
NA ₂ HPO ₄ 2H ₂ O	1.135g
Kcl	0.2g
NaCl	8g
Dissolve in distilled water	lL
Add Tween	0.3%

<u>Aurodye</u> Use 0.2ml/cm² of blot; Use 60ml Aurodye solution.

SDS-Page Method

The apparatus is thoroughly cleaned in deionised and then distilled water. Gloves are worn for the whole procedure. Prepare a 12.5% acrylamide resolving gel and pour in between the two glass plates of the gel apparatus. Gently, layer lml of butanol saturated with water onto gel surface. Leave for 45-60 minutes for gel to polymerise. Pour off the butanol layer and rinse (by rocking) the surface of the gel x3 with resolving gel overlay solution. Add approximately 3mls of resolving gel overlay solution and leave at room temperature for not longer than 2-3 hours. Pour away this solution and add 1.2ml of stacking gel solution to rinse (by rocking) the surface of the resolving gel. Pour stacking gel over the resolving gel to a height of approximately 2.5cm. Insert a methanol cleaned comb for 30-45 minutes. The comb is removed carefully, taking care not to break the gel. Each sample was dissolved in sample buffer and boiled To remove bubbles, each well has distilled water added for 2 minutes. and then removed using a 50µl Hamilton syringe. 10µl of electrode buffer is added to each well using a Gilson pipette. Apply samples to wells using a Hamilton syringe which is washed well in between sample applications. The sample sinks below the buffer in the well.

The gel tank should be thoroughly cleaned with de-ionised and then distilled water prior to use. The gel is placed carefully in the tank at 4°C and the tank is filled up to the top with tank (electrode) buffer. Electrophoresis was performed on a vertical slab gel unit (Hoefer Instruments, Biotech, Beds) at 4°C, at a constant current of 6mA for 16 hours (overnight), until the dye reached the bottom of the gel.

Western Blotting

Cut a nitrocellulose membrane of dimensions 14 x 14cm. Soak in distilled water until ready for use. Also cut 2 dialysis membranes of the same dimensions, to prevent any protein transfer onto the membrane. Twenty-four sheets of Whatman no. 1 filter paper are cut to the same dimensions as the membranes. Once electrophoresis is complete, drain tank buffer and lay flat the gel inbetween the two glass plates of the gel apparatus. Lift off one glass plate. Remove as much stacking gel as possible. The bottom left hand corner of the gel is cut with a scalpel for orientation.

Place gel slab in cathode buffer. The gel is ready for blotting. A semi-dry multi gel electroblotter was used (Ancos, Denmark (Dako Ltd)). Place 6 layers of filter paper soaked in anode buffer 1 onto the anode. Use a clean glass rod to eliminate bubbles. Next place 3 layers of fitler paper soaked in anode buffer 2 and eliminate bubbles using a glass rod. The nitrocellulose membrane is placed above the filter paper and next the gel with care taken to avoid bubbles. Three layers of filter paper soaked in cathode buffer, a dialysis membrane and finally

six layers of filter paper soaked in cathode buffer is placed above the gel. Cover with the cathode (lid) and apply a current of 140mA for 100 minutes.

Once transfer to the membrane is complete, mark the nitrocellulose membrane in a similar manner to the gel (cut using a scalpel) for orientation.

Aurodye Staining

Wash nitrocellulose membrane in PBS-tween for 2 hours at 32°C (use a shaker). Next, wash in PBS tween (x4) for 30 minutes, each at room temperature. Next, wash in distilled water for 5 minutes, then 10 minutes on a shaker. Place membrane in a polythene bag in Aurodye stain (Janssen) and rock on a shaker overnight at room temperature. When stained, rinse in distilled water and air dry or dry between filter paper.

SECTION C

Eagle's minin	num essential medium (modified)
Al	5 ml
В	5 ml
ID	5 ml
E	50jul
F	500µl
G	5 ml
Н	5 ml
J	l ml
H ₂ O	70ml
NaOH to pH 7	3

Amino acids mg/50ml

<u>Al</u>

Arginine. HCl 50; Histidine.HCl.H₂O 10;Isoleucine 26; Leucine 26; Lysine 20; Phenylalanine 16; Threonine 23; Tryptophan 5; Tyrosine 20; Valine 23; Cysteine 12. <u>B</u>

Proline 10; Asparagine 20; Glutamic acid 12; Aspartic acid 10; Serine 10; Glycine 10.

<u>H</u> Glutamine 292 D (bulk salts mg/50ml) NaCl 6800, KCl 400, CaCl₂ 2 H₂O 265, NH₄Cl 50, phenol red 17; NaH₂PO₄ 158, glucose 1000. $\underline{\mathbf{E}}$ (Trace elements mg/50ml) CuAc₂ 3.3, KI 1, FeCl₃ 20, MnCl₂35, NH₄ molybdate 20, ZnCl₂ 33, H₃BO₃ 5. <u>F</u> (Vitamins) Biotin, Ca pantothenate, choline. HCl, folic acid, nicotinamide, nicotinic acid, pyridoxal, pyridoxine, pyridoxamine, thyamine. HCl and ascorbic acid all at 10 mg/50ml; inositol 20; riboflavin 1; PABA 5. <u>G</u> Hepes buffer 4.76g/50ml. J Nucleosides all at 10 mg/10ml. A,T,G,C,U,deoxy A,C,G. SECTION D <u>Coating Buffer</u> (0.05M carbonate buffer pH9.6) Na₂Co₃ 1.59g NaHCo3 2.93g Distilled water 1LPBS (Phosphate buffered saline) pH 7.4 NaCl 8g KH2 PO4 0.2g Na₂HPO₄ 1.135g Kcl 0.2g Tween 20 0.5ml PBS Tween-BSA Tween 20: BDH Limited, Poole, England Polyoxyethylene (20) - sorbitan monolaurate Product 66368 Albumin bovine fraction V 96-98% albumin. Sigma No A-9647 Add 100ml PBS-Tween 0.05% and 1g BSA (1% BSA) and use magnetic stirer

to dissolve.

ABTS substrate (Sigma cat no A-1888) 2,2' - Azino-di- 3-ethyl benzthiazoline sulfonic acid For use: 12.5mg ABTS + 25ml citrate/phosphate buffer 0.1M, pH 4.1 + 87.5µl H₂O₂ (BDH, Poole, Product no 10128) SECTION F Medium used for Thunberg tube culture system a. Basal medium: Formula for 1L L-asparagine 5g $MgSO_4$, $7H_2O$ lg Ammonium citrate 2g Nacl 2g D-glucose 1.5g Glycerol 20ml Ferric Am citrate 75mg $ZnSo_4$, $7H_2O$ 10mg CaCl₂ 1.9mg K2 HPO4 738mg KH2 PO4 2.4g Tissue culture medium 199: dehydrated powder base for 1L Trypticase Soy broth base 5g Bacto yeast extract (Difco) 0.5g Bacto Fildes enrichment (Difco) 10ml Distilled water to 1L Sterilised by filtration through a Seitz filter b. Redox-amine mixture (all Sigma biochemicals) ATP, Ascorbic acid, Nicotinamide, reduced Glutathione, Cysteine,

Spermine, Spermidine, Putrescine, Cadaverine, Dimerecaptopropanol all added at 20μ g/ml final concentration and sterilised by filtration through 0.22μ m Millipore filters. Also Vitamin Kl and Vitamin E added at 20μ g/ml final concentration.

- c. Cholesterol (Merck) 100mg and Lecithin (BDH) 100mg together with ntetradecane (10ml) and Tween 80 (0.5ml). 0.5% of this liposome mixture is used.
- d. Filter sterilised sheep serum added at 0.5% final concentration.
- e. Bacto gelatin (Difco) autoclaved. Added at 0.5% final concentration.
- f. Crystalline penicillin at a final concentration of 100 units/ml.

Pyrogallol (Sigma No P-0381) Weigh 2g.

<u>NaOH</u> pellets (Analar) Weigh 1.6g. Dissolve in 20mls distilled water. Add to 2g pyrogallol prior to use. Elsevier

MIMET 00323

Simple enzymic method for isolation of DNA from diverse bacteria

Shico Visuvanathan¹, Michael T. Moss², John L. Stanford¹, John Hermon-Taylor² and John J. McFadden²

¹Department of Medical Microbiology, University College and Middlesex School of Medicine, London, UK; ²Department of Surgery, St. George's Hospital Medical School, London, UK

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Summary

An enzymic method for the extraction of high molecular weight DNA from a wide range of diverse bacteria, including mycobacteria, is described. The method is simple, rapid, requires few manipulations and is suitable for extracting quantitative yields of DNA from as few as $\approx 3 \times 10^{7}$ bacterial cells. The DNA produced is of good quality and is suitable for molecular biological manipulations.

Key words: Bacterium: DNA extraction; Enzymic

Introduction

Various protocols have been described for the extraction of DNA from bacterial cells [1-4]. The critical step is lysis of the bacterial cells, to release DNA whilst avoiding its degradation or shearing. Enzymic methods of lysis [1, 2] produce high molecular weight DNA; however, many bacteria, such as mycobacteria, have cell walls that are resistant to enzymic lysis by the commonly described methods.

Physical and chemical methods of lysis, such as grinding in liquid N, grinding with glass beads, passage through a Hughes press [3] or other cell disrupters or treatment with agents such as sodium triisopropylnaphthalene sulphonate and sodium 4-amino salicylate [4] are often employed but the DNA produced is often of low molecular weight due to physical shearing or chemical degradation during extraction, and therefore unsuitable for many molecular biological protocols. Additionally, large volumes

Correspondence to: M. T. Moss, St George's Hospital Medical School, Cranmer Terrace, London SW170RE, UK

are often necessary, resulting in low yields when only small numbers of bacteria are available.

We have developed a simple method to extract high molecular weight DNA from mycobacteria [5], modified from that described by Patel et al. [2]. We now show that this method can be successfully applied to a wide range of diverse bacterial species, producing good yields of high molecular weight DNA, suitable for restriction endonuclease digestion and cloning. The method is simple, requires few manipulations, is rapid and can be applied to very small numbers of bacterial cells. We suggest its use as a general method of DNA extraction from bacteria for which specific protocols have not been developed and/or only very small quantities of bacterial cells are available.

Methods

Bacterial strains and culture conditions

A total of 68 bacterial strains are listed in Table 1.

Mycobacterial species. The strains examined were obtained from J. L. Stanford. The original respective sources are listed in Table 1. All mycobacteria were subcultured onto Lowenstein – Jensen medium and incubated at $35 \,^{\circ}C$ for the appropriate incubation periods and then harvested as described below. M. avium was grown in Middlebrook 7H9 broth with OADC enrichment.

Other bacterial species. All NCTC, NCIB, IAM, ATCC and VPI strains were obtained freeze-dried. Clinical isolates were subcultured onto Columbia blood agar. Corynebacterium, Propionibacterium and Rhodococcus species were first cultured on Corynebacterium agar (Tryptone soya broth 30 g, yeast extract 10 g, agar no. 1 12 g and Tween 80 5 ml, (1^{-1}) at 37 °C. All other isolates were grown on Columbia blood agar at 37 °C.

DNA extraction

Cells ($\approx 200 \text{ mg}$ wet weight) were harvested from liquid culture by centrifugation at 5000×g for 5 min and resuspended in 1 ml TEN buffer (50 mM Tris – HCl, pH 8.0; 150 mM NaCl; 100 mM EDTA) in Eppendorf tubes; or scraped off solid media, into Eppendorf tubes containing TEN buffer (1 ml). Cells were recovered by centrifugation for 5 min (5000×g) in a benchtop microcentrifuge and resuspended in 0.4 ml TEN. To minimise biological hazard, the bacteria were incubated at 70 °C for 1 h. Fifty µl subtilisin (100 mg·ml⁻¹, Sigma type VIII) was then added and the mixture was incubated at 37 °C for 18 h after which 100 µl lysozyme (200 mg·ml⁻¹, Sigma) was added and the mixture was incubated at 50 °C for 5 h. Lysis was completed by the addition of 50 µl 20‰ (w/v) SDS and 50 µl pronase (30 mg·ml⁻¹, Calbiochem). The mixture was further incubated for 20 h, with the addition of a further 50 µl pronase after 12–15 h. The sample was spun for 5 min in a benchtop microcentrifuge to pellet the cell debris. The supernatant was recovered with a fine-tipped pipette with care being taken not to disturb the pellet.

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An equal volume phenol : chloroform : isoamyl alcohol (50 : 48 : 2; v/v) saturated with TEN was added and the mixture was shaken by hand for 10 min. The phases were separated by centrifugation and the aqueous phase, containing the DNA, was carefully recovered. DNA was precipitated with 0.2 vol. 10 M ammonium acetate and 2 vols. 100°_{0} ethanol. After thorough mixing (by hand), the DNA could often be spooled. The spooled DNA was washed in 70°_{0} ethanol, dried briefly and resuspended (by gentle pipetting) in 100 μ l TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) containing $10 \ \mu\text{g} \cdot \text{ml}^{-1}$ boiled ribonuclease A (Sigma). Alternatively, the mixture was stored overnight at -20° C, the DNA recovered by centrifugation (10 min), washed in 70°_{0} ethanol and then dried and resuspended in TE as before. The DNA was stored at 4° C for at least 24 h before use to ensure it had fully dissolved.

DNA yield was determined spectrophotometrically [A260=1=50 μ g·ml⁻¹DNA]. When extracting DNA from very small quantities of bacteria, the volumes and amounts were all reduced to one-fifth of the above and 5 μ l carrier glycogen (5 μ g·ml⁻¹, Boehringer) was added to the resuspended cells before the addition of enzymes.

DNA manipulations

The approximate molecular weight of intact DNA was determined by electrophoresis of 200 ng DNA aliquots, in 0.8% agarose gels in TAE-running buffer (10 mM Tris – HCl: 5 mM sodium acetate; 0.5 mM EDTA, pH 7.8) containing ethidium bromide (0.5 μ g·ml⁻¹), with *Hind* III-digested λ phage DNA as a size marker. Restriction endonuclease digestions of DNA (0.2 – 5.0 μ g) were performed in 20- μ l vols., in accordance with the manufacture is recommendations, but in buffers that additionally contained spermidinc hydrochloride (4 μ g·ml⁻¹). If the volume of test DNA exceeded 5 μ l, then the digestion was performed in 100–200 μ l and the DNA was then precipitated (0.1 vol. 2 M NaCl, 2 vols. 100% ethanol), pelleted by centrifugation, dried briefly and resuspended in 20 μ l TE. Digested DNA was electrophoresed through 1% agarose gels as above.

Results

DNA of high molecular weight was extracted from all the bacteria examined (Table 1), with variable yields, and DNA samples digested with most restriction endonucleases (Fig. 1). When complete digestion was not obtained with one restriction endonuclease, another invariably produced complete digestion, indicating incomplete digestion may have been due to methylation.

Discussion

This method is generally applicable to a wide range of bacterial species. *All* aerobic and anaerobic gram-positive and gram-negative bacteria and mycobacteria investigated produced high yields of good quality DNA suitable for molecular biological manipulations. The protocol requires few manipulations and is rapid. The method is also highly reproducible.
TABLE 1

DNA YIELDS FROM BACTERIAL STRAINS

Organism	Source	Yield* (µg DNA+g ⁻¹ wet wt cells)
Aeromonas hydrophila	Porton Down 13109	625
Alcaligenes odorans	NCTC 10388	294
Arthrobacter globiformis	NCIB \$907	333
Bacillus subtilis	CI	115
Bucteroides asaccharolyticus	NCTC 9337	192
Bacteroides fragilis	NCTC 8343	225
Bacteroides vulgatus	NCTC 10583	582
Brevibacterium lipolyticum	IAM 1413	149
Cellulomonas biazotea	NCIB 8077	275
Cellulomonas fimi	NCTC 7547	150
Campylobacter jejuni	CI	267
Campvlobacter ovlori	CI	156
Clostridium difficile	CI	108
Clostridium perfringens	CI	134
Clostridium septicum	CI	110
Corvnehacterium 1K	NCTC 11915	167
Corvnebacterium minutissimum	NCTC 10284	370
Enterohacter cloacae	CI	200
Escherichia coli	NCTC 10418	215
Fubactorium spratacions	NCTC LI838	115
Eubactorium Iontum	NCTC 11813	250
Eusphacterium varium	NCTC 10560	£11
Gardinarolla vaginalis	CL	03
Klahsialla nnaumonina	CI	66
Lactobacillus casai var rhamnosus	NCIB 8010	182
Listaria monopytoganas	CL	172
Musshactorium asiaticum	Locituta Postaur	52
Mycobacterium usium	NCTC 2550	-4
Mycobacterium boyis PCC	Clava strain	125
Mycobacterium obvis BCG	ATCC 19620	51
Mycobacterium childe	E1	2.4
Mycobacterium diernhöjen	LI NCTC 359	50
Mycobacterium duvalli	NCTC 10270	34
Muchaetarium formitum	NOTO 1010	07
Mycobacterium gostalium	NETC 3575	91 63
Mycobacterium gastri	NSCI CI	03
Mycobacterium gilvum		64
Mycobacterium goruonae	NCTC 9822	24
Nivcobacterium intracettulare	A1CC 13950	13
Mycobacterium kunsasti	CI NOTO LLOOD	104
Mycobacterium maimoense	NCTC 11299	208
Mycobacterium marinum		9,
Augebastation neodurum	NCTC 10439	130
Nycobacterium nonchromogenicum	El	100
Nycobacterium paraluberculosis	VI	18
Wycobacierium rhodesiae	ATCC 27024	128
Nycobacterium sinegmatis	NC1C 333	78
Mycobacterium thermoresistibile	ATCC 19528	116
Mycobacterium xenopi	NSCI	99
Mycoplasma species	CI	76

TABLE 1 Continued

Organism	Source	Yield* (ug DNA-g) wet wit cells)
Nocardia brasiliensis	IMRV 800	111
Peptococcus asaccharolyticus	NCTC 11461	185
Peptostreptococcus magnus	CI	159
Propionibacterium acnes	VPI 0162	58
Propionibacterium avidum	VPI 0589	50
Proteus mirabilis	Cl	189
Pseudomonas aeruginosa	NCTC 10662	182
Rhodococcus corallinus	NCTC 10668	44
Rhodococcus species	NCIB 9784	29
Rothia dentocariosa	NCTC 10207	233
Salmonella enterstidis	CI	263
Staphylococcus aureus	NCTC 6571	84
Staphylococcus epidermidis	CI	63
Streptococcus faecalis	Cl	84
Streptococcus milleri	Cl	35
Streptococcus pneumoniae	CI	55
Wolinella recta	NCTC 11489	149
Yersinia enterocolítica	CI	383

* Corrected to nearest µg. CI, clinical isolate: NSCI, nonsignificant clinical isolate; EI, environmental isolate: VI, veterinary isolate.



Fig. 1. DNA samples electrophoresed through a 1^{σ0} agarose gel and stained with ethidium bromide.
Lanes 1-5, 200-ng aliquots of undigested DNA samples. Lanes 7-11, 5-μg aliquots of Pvull-digested DNA samples, showing complete digestion. Bacteriophage λ DNA digested with HindIII was used as a size marker (Lane 6). Lanes: (1, 7) Staphylococcus epidermidis, (2, 8) Fusobacterium varium, (3, 9) Clostridium perfingens, (4, 10) Mycobacterium kansasii, (5, 11) Nocardia asteroides.

DNA was also extracted by this method from *Candida albicans* (a yeast) and similar results were obtained.

Further work with mycobacteria has shown that the enzyme incubation steps (18 h subtilisin, 5 h lysozyme, 20 h pronase) can be considerably reduced (i.e., 2 h subtilisin, 4 h lysozyme, 12 h pronase) without loss of yield (data not shown) and it is likely that this would also be found for most other bacteria.

Since this simple method works for this diverse range of bacteria, including some thought resistant to lysis (i.e., mycobacteria), it should prove useful in extracting DNA from bacterial species for which specific extraction methods have not been described. The method is also suitable for extracting DNA from very small quantities of bacteria; we have used this method to extract 100 ng DNA from $\approx 3 \times 10^7$ *M. avium* cells (representing a theoretical yield of 65%). The method may also be a simple alternative where previously published methods are complicated, produce low yields or result in DNA of poor quality.

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References

- 1 Marmur, J. (1961) A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3, 208-218.
- 2 Patel, R., Kvach, J. T. and Mounts, P. (1986) Isolation and restriction endonuclease analysis of mycobacterial DNA. J. Gen. Microbiol. 132, 541-551.
- 3 Hughes, D.E. (1951) A press for disrupting bacteria and other micro-organisms. Br. J. Exp. Pathol. 32, 97-109.
- 4 Hopwood, D. A., Bibb, M. J., Chater, K. F., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. and Schrempf, H. (1985) In: Genetic Manipulation of *Streptomyces*: a Laboratory Manual, John Innes Foundation, Norwich.
- 5 McFadden, J. J., Butcher, P. D., Chiodini, R. and Hermon-Taylor, J. (1987) Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. J. Clin. Microbiol. 25, 796-801.

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