

# Investigation of mycobacteria in Crohn's disease tissue by Southern blotting and DNA hybridisation with cloned mycobacterial genomic DNA probes from a Crohn's disease isolated mycobacteria

P D BUTCHER, J J McFADDEN, AND J HERMON-TAYLOR

*From the Department of Surgery, St George's Hospital Medical School, London*

**SUMMARY** A mycobacterial aetiology for Crohn's disease (CD) has been suggested. Slow growing mycobacteria indistinguishable from *M paratuberculosis*, the causative agent of enteritis in ruminants (Johne's disease) have been isolated from CD tissues. We have used cloned genomic DNA probes derived from a CD isolated mycobacteria strain Ben, to investigate the presence of mycobacterial DNA sequences in CD tissues. DNA was extracted from total tissue from 17 CD and four control gut specimens. DNA was digested with restriction endonucleases, electrophoresed and transferred to nylon membranes by Southern blotting and hybridised to radiolabelled DNA probes. No mycobacterial DNA was detected in any tissue sample studied. Reconstitution experiments with known numbers of *in vitro* cultured mycobacteria showed sensitive detection of mycobacterial DNA. DNA extracted from mouse liver, infected with *M lepraemurium* revealed a strong hybridisation signal and showed the applicability of the experimental approach to the detection of mycobacterial DNA in naturally infected tissues. The results do not provide evidence for the involvement of mycobacteria in the pathogenesis of CD but do not exclude the possibility of low levels of infection in subsets of intestinal cells with spheroplast or cell wall deficient forms of mycobacteria.

An infectious aetiology for Crohn's disease (CD) has often been suggested.<sup>1-5</sup> Macroscopic and histological similarities between CD, intestinal tuberculosis in man and paratuberculosis in ruminants (Johne's disease) caused by *Mycobacterium paratuberculosis*<sup>6,7</sup> have led to the suggestion that mycobacteria may also be involved in CD,<sup>8,9</sup> even though earlier studies failed to show the presence of acid fast mycobacteria.<sup>10,11</sup> Burnham *et al*<sup>12</sup> have isolated, after prolonged culture, a mycobacterium similar to *M kansasii* from a lymph node of one out of 27 cases of CD, as well as unidentified pleomorphic, cell wall deficient organisms (spheroplasts) from 22 of these CD cases, and one of 11 non-inflammatory disease controls. They suggested that mycobacterial spheroplasts might be an aetiological agent in CD. Sphero-

plast forms of common bacteria have been found in various inflammatory diseases of the intestine.<sup>13</sup>

A slow growing, mycobactin (an iron chelating growth factor) dependent Mycobacterium species has recently been isolated from three of 14 CD gut tissues, but not controls, after 18 months culture.<sup>14</sup> This organism was similar to *M paratuberculosis* and oral inoculation of neonatal goats with these isolates produced granulomatous, non-caseating tuberculoid ileitis within three to five months<sup>15</sup> from which mycobacteria were reisolated.<sup>16</sup> Subsequently, a further similar isolate has been recovered from CD tissue.<sup>17</sup> These CD mycobacterial isolates were initially recovered in primary culture as slow growing spheroplasts which reverted to bacillary form only after prolonged and repeated subculture. The spheroplast ancestry of the CD bacillary isolates was confirmed by the analysis of restriction fragment length polymorphisms of ribosomal genes by DNA hybridisation.<sup>18</sup> We have also shown that these CD

Address for correspondence: Dr P D Butcher, Department of Gastroenterology, St Bartholomew's Hospital, West Smithfield, London EC1A 7BE.

Received for publication 15 April 1988.

isolated mycobacteria are identical to each other and to *M paratuberculosis* by the use of DNA probes which identify restriction fragment length polymorphisms.<sup>19-20</sup> Unidentified spheroplasts were recovered from 61% of CD tissues but not controls.<sup>17</sup> Several other groups have recently reported independently the isolation of mycobacteria from CD tissues of types similar to *M paratuberculosis* as well as to *M chelonae* and *M avium*.<sup>22,23</sup>

The repeated isolation from CD tissues of a mycobacterial pathogen may be of aetiological significance in CD. The fastidious growth requirements, however, and long term cultures of CD derived organisms, makes their study protracted and very difficult. Their possible presence as spheroplasts or cell wall deficient forms also precludes their detection in CD tissue by microscopy. Direct demonstration of mycobacterial forms in CD tissues must therefore rely on alternative techniques. Detection of mycobacterial genomes by DNA hybridisation would provide a rapid and sensitive approach to this problem. A recent report of DNA-DNA hybridisation in solution of CD tissue total DNA with total DNA from a CD mycobacterial isolate demonstrated mycobacterial DNA sequences in both CD and control gut tissue.<sup>24</sup>

Precise identification of mycobacteria in CD tissue is necessary, however, for their aetiological significance to be evaluated, particularly if closely related non-pathogenic species such as *M avium* may be found in control tissue. We have therefore used a more specific method to detect DNA sequences of mycobacterial origin that makes use of cloned genomic DNA probes derived from a CD mycobacterial isolate, strain Ben identified as *M paratuberculosis*.<sup>14,20</sup> These probes are Mycobacterium specific and have been used to differentiate between mycobacterial species by restriction fragment length polymorphism analysis.<sup>20,21</sup> We report here the use of cloned DNA probes to attempt to detect mycobacterial DNA in tissue derived DNA from 17 CD and four control gut tissues by Southern blotting after restriction endonuclease digestion of test DNA and hybridisation with radiolabelled probes.

## Methods

### TISSUE SOURCE

Specimens of colon and terminal ileum were removed under sterile conditions from Crohn's disease patients undergoing surgical resection at St George's Hospital between September 1983 and June 1986. Full thickness intestinal specimens were taken from involved areas showing typical thickening, cobblestoning and ulceration. All cases of CD were confirmed by clinical, radiological, and histo-

logical criteria. Disease control tissues were from cases of colon carcinoma. Specimens were trimmed of fat, washed in sterile saline and snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . The total number of intestinal specimens screened for all forms of mycobacteria in this report were 17 CD (two colon, one jejunum, 14 terminal ileum) and four controls (one colon, three terminal ileum).

### DNA EXTRACTION

DNA was extracted from tissue homogenates by an enzymic procedure<sup>25</sup> shown to extract DNA from bacillary mycobacteria which are resistant to lysis by conventional DNA extraction procedures. Briefly, tissue in TEN buffer (50 mM Tris-HCl, pH8, 150 mM NaCl, 100 mM EDTA) was sequentially digested with 10 mg/ml Subtilisin (Sigma; protease Type VIII) at  $37^{\circ}\text{C}$  for three hours, 50 mg/ml lysozyme at  $50^{\circ}\text{C}$  for three hours and 3 mg/ml pronase (Calbiochem) at  $37^{\circ}\text{C}$  for 12 hours after the addition of 1% SDS. DNA recovered by phenol/chloroform extraction and ethanol precipitation was redissolved in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) and treated with 10  $\mu\text{g/ml}$  RNA'ase A for two hours at  $37^{\circ}\text{C}$  after heating to  $65^{\circ}\text{C}$  for 15 minutes. DNA was again phenol/chloroform extracted, ethanol precipitated from 0.1 M NaCl and redissolved in TE buffer. DNA concentration was measured by absorbance at 260 nm (1  $A_{260}$  unit = 50  $\mu\text{g/ml}$ ) and its integrity checked by agarose gel electrophoresis. High molecular weight DNA was obtained in all cases.

### PROBES

Cloned mycobacteria specific probes used in this study were from the CD derived *M paratuberculosis* isolate, strain Ben<sup>14</sup>. BamHI fragments of genomic DNA were cloned into the plasmid vector pGEM-1 as previously described.<sup>20</sup> Probes were selected for their ability to detect and discriminate between a range of mycobacteria on the basis of restriction fragment length polymorphisms (RFLP). Three clones were used: (i) pMB22; with a 5 kb insert including a 1.2 kb repetitive element within it that is repeated greater than 10 times in *M paratuberculosis*; (ii) pMB21; this clone hybridises equally well to *M paratuberculosis*, *M avium*, CD isolates, and less to *M kansasii*; (iii) pMB19; this clone hybridises equally well to *M paratuberculosis*, CD isolates, *M avium*, and *M kansasii*. The clone pMB19 has also been shown to hybridise to *M bovis*, *M tuberculosis*, *M intracellulare*, and *M chelonae*. These clones and the RFLP's they generate have been described in detail previously.<sup>20-21</sup>

Radiolabelled DNA probes were prepared for hybridisation by BamHI excision of the cloned insert and separation by electrophoresis in low temperature

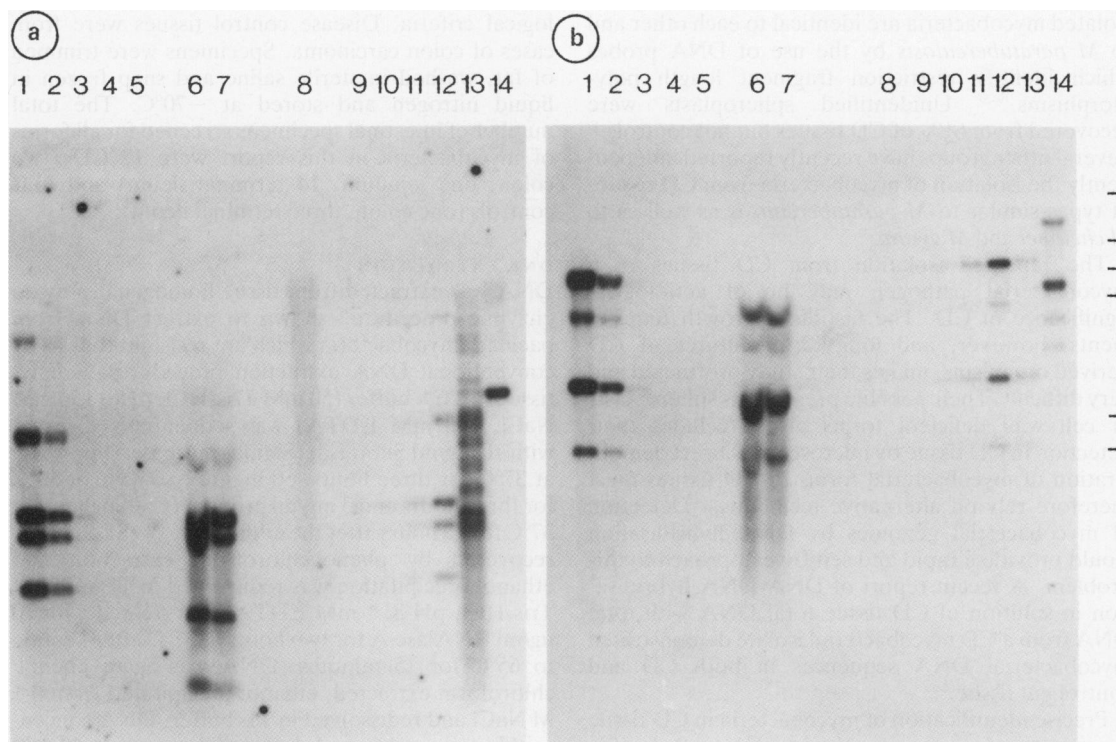


Fig. 1 Specificity and sensitivity of detection of mycobacterial DNA sequences using cloned genomic DNA probes. Lanes 1–5 (respectively): total DNA, 1  $\mu$ g, from normal human gut tissue reconstituted with known numbers of *M. avium* bacilli:  $5 \times 10^6$ ,  $2 \times 10^6$ ,  $2 \times 10^5$ ,  $5 \times 10^4$ ,  $1 \times 10^4$ . Lanes 6 and 7: 10  $\mu$ g DNA from mouse liver infected with *M. lepraemurium*. Lane 8: 10  $\mu$ g normal human leucocyte DNA. Lanes 9–12 (respectively): 1, 5, 20, and 50 ng DNA from *M. avium* bacilli. Lanes 13 and 14: 10 ng of DNA from *M. paratuberculosis* and 200 ng of DNA from *M. phlei*, respectively. DNA was electrophoresed after PVU-11 restriction endonuclease digestion, Southern blotted and hybridised consecutively with the following probes: (a) pMB22 and (b) pMB21 and autoradiographed. Molecular weight size markers are indicated (–) and are from the top of the gel 21, 9.4, 6.6, 4.4, 2.3, and 2.0 kilobase pairs (kb).

gelling agarose; 20–25 ng were radiolabelled with 50  $\mu$ Ci [ $^{32}$ P] deoxy CTP (Amersham; 3000 Ci/mmol) by hexanucleotide random priming and Klenow extension.<sup>26</sup> Double stranded DNA probe was denatured by boiling before hybridisation. Specific activities of  $4.1\text{--}9.8 \times 10^8$  cpm/ $\mu$ g DNA were obtained with approximately 80% incorporation of the label.

#### HYBRIDISATION

Extracted DNA was digested with restriction endonuclease *Hinf*I and PVU-11 and 10  $\mu$ g electrophoresed on 1% agarose gels, denatured in 0.4 M NaOH, neutralised and transferred by Southern blotting to nylon filters (Amersham; Hybond-N) following standard protocols.<sup>27</sup> DNA was cross-linked to the filters by ultraviolet irradiation and the filters prehybridised for 18 hours at 65°C in hybridisa-

tion solution: 3 $\times$ SSC (1 $\times$ SSC=0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7, 0.5% SDS, 0.05% BSA, 0.05% polyvinyl pyrrolidone, 0.05% Ficoll, 10% dextran sulphate and 110  $\mu$ g/ml denatured salmon sperm DNA. Hybridisation was continued by the addition of the boiled radiolabelled probe for 18 hours at 65°C. Filters were then removed and washed at 65°C in 3 $\times$ SSC, 0.1% SDS for 3 $\times$ 15 minutes and then 2 $\times$ 30 minutes in 1 $\times$ SSC, 0.1% SDS. Hybridisation of the labelled probe with target DNA was detected by autoradiography at –70°C with intensifying screens for up to 30 days. Filters were reprobbed with a fresh probe after removal of previous probe with 0.4 M NaOH at 45°C for 45 minutes followed by neutralisation in 0.2 M Tris-HCl, pH 7.4, 1 $\times$ SSC. In this way filters can be reprobbed repeatedly without loss of signal.

## Results

### SENSITIVITY AND APPLICABILITY OF THE EXPERIMENTAL APPROACH

In order to quantify the sensitivity of this approach to the detection of mycobacteria in CD tissue, a reconstitution experiment was done in which dilutions of freshly cultured *M avium* ranging from  $10^6$  to  $10^8$  bacilli were added to 0.1 ml of total control gut tissue homogenate (equivalent to 0.03 g of tissue). *M avium* DNA was detected after coextraction with tissue DNA by hybridisation with the DNA probes. Approximately 50  $\mu$ g DNA was recovered from each extraction, and 1  $\mu$ g was loaded in each lane for electrophoresis. The results are shown in Figure 1. The minimal detectable signal was obtained with  $2 \times 10^6$  bacilli (lane 3, Fig. 1a and b). A separate experiment in which known amounts of pure *M avium* DNA, digested with PVU-11, were electrophoresed and hybridised with probes pMB21 and

Table 1 Limits of detection of Mycobacterial DNA using the three cloned genomic DNA probes: expressed as one mycobacterial genome per number of human cells

Mycobacterial species	Probe		
	pMB22	pMB21	pMB19
<i>M paratuberculosis</i>	1/100	1/10	1/10
<i>M avium-intracellulare</i>	1/10	1/10	1/10
<i>M kansasii</i>	1/5	1/5	1/10

pMB22 showed that the minimal detectable amount of DNA was 1 ng after autoradiography; see (Fig. 1a and b lanes 9–12). The signal obtained from 1 ng mycobacterial DNA was routinely visible against the negligible hybridisation background of 10  $\mu$ g human DNA as may be observed in Fig. 1b, lane 8 and Fig. 3. Based on genome sizes of  $3.1 \times 10^9$  daltons for *M avium*<sup>28</sup> and  $3 \times 10^{12}$  daltons for humans, this was

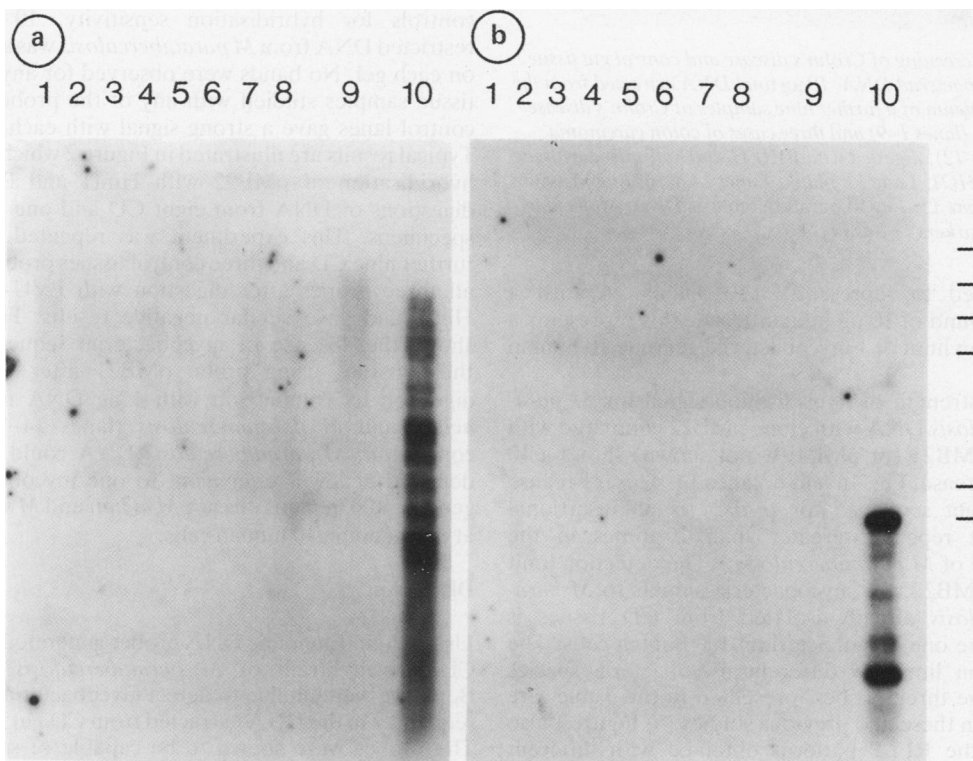


Fig. 2 Screening of Crohn's disease and control gut tissue for mycobacterial DNA. 10  $\mu$ g total DNA from terminal ileum of eight cases of Crohn's disease (lanes 1–8) and 1 control (lane 9), digested with PVU-11 (a) or with *Hinf*I (b) restriction endonuclease and hybridised with probe pMB22. Lane 10: 10 ng *M paratuberculosis* DNA. Molecular weight markers (–) as in Fig. 1.

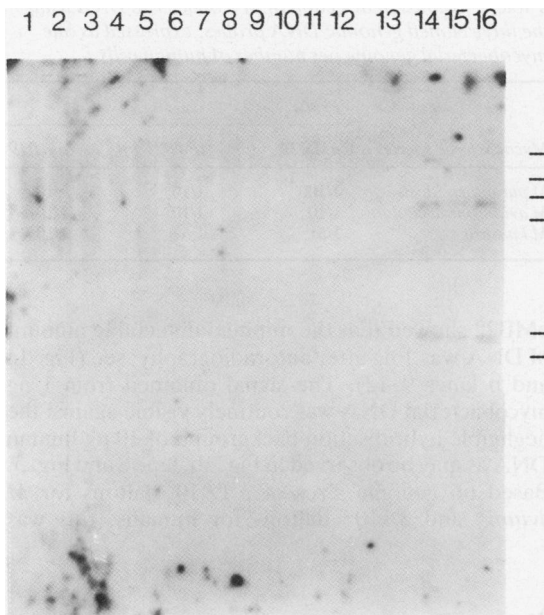


Fig. 3 Screening of Crohn's disease and control gut tissue for mycobacterial DNA. 10 µg total DNA extracted from the terminal ileum of a further nine samples of Crohn's disease gut tissue (lanes 1–9) and three cases of colon carcinoma (lanes 10–12), digested with PVU 11 and hybridised with the probe pMB21. Lane 13: blank. Lanes 14 & 16: 1 ng *M avium* DNA. Lane 15: 1 ng *M paratuberculosis* DNA. Molecular weight markers (–) as Fig. 1.

calculated to represent  $2 \times 10^5$  bacilli. Against a background of 10 µg human DNA, this represents a detection limit of 1 mycobacterial genome/10 human cells.

The strength of hybridisation signal for *M paratuberculosis* DNA with clone pMB22 compared with clone pMB21 (or pMB19 – not shown) shows a 10 fold increase, Fig. 1a and b, lanes 13. This is because the target sequence for pMB22 is an insertional element repeated greater than 10 times in the genome of *M paratuberculosis*.<sup>20</sup> The detection limit using pMB22, for mycobacteria similar to *M paratuberculosis* already isolated from CD tissues is therefore one Mycobacterium/100 human cells. The detection limit for different mycobacteria species using the three probes, presented in the Table, are based on these and previous studies.<sup>20,21</sup> Figure 1 also shows the RFLP patterns obtained with different species and shows that *M avium*, *M paratuberculosis*, and *M phlei* are clearly and precisely distinguishable.

To show the applicability of this experimental approach to detecting naturally occurring mycobacterial infections we applied these techniques to 40 mg liver from a mouse infected with *M*

*lepraemurium*. Hybridisation analysis with clones pMB22 and pMB21 showed a very strong signal after autoradiography, Fig. 1a and b, lanes 6 and 7. The signal obtained was at least as strong as that from  $5 \times 10^6$  *M avium* bacilli, which against a background of 10 µg mouse DNA represents an infection level of approximately 2.5 organisms/mouse liver cell. Clearly from Figure 1, lanes 1–3, at least a 25-fold reduction in signal would still be detectable, indicating a level of sensitivity of detection for *M lepraemurium* of one organism/10 cells.

#### DETECTION OF MYCOBACTERIAL DNA WITHIN CD INTESTINAL DNA EXTRACTS

DNA was extracted from full thickness involved intestinal specimens from a total of 17 cases of CD and four normal control colon carcinoma cases. A maximum of 10 µg restriction endonuclease digested DNA (equivalent to DNA from approximately 6 mg of tissue) was electrophoresed in each lane, blotted onto nylon filters and hybridised consecutively with the three cloned probes, as described. As internal controls for hybridisation sensitivity, 10 ng of restricted DNA from *M paratuberculosis* was also run on each gel. No bands were observed for any of the tissue samples studied with any of the probes. The control lanes gave a strong signal with each probe. Typical results are illustrated in Figure 2 which shows hybridisation of pMB22 with Hinf1 and PVU-11 digestions of DNA from eight CD and one control specimens. This experiment was repeated with a further nine CD and three control tissues probed with all three probes after digestion with PVU-11 and Hinf1 and gave similar negative results. Figure 3 shows the absence of mycobacterial sequences in these tissues using probe pMB21 after PVU-11 digestion by comparison with 1 ng DNA from *M avium* and *M paratuberculosis* (lanes 14–16). In conclusion, *M paratuberculosis* DNA could not be detected at levels equivalent to one mycobacterial genome/100 human cells nor *M avium* and *M kansasii* at one genome/10 human cells.

#### Discussion

Using cloned genomic DNA probes generated from a CD-derived strain of *M paratuberculosis*, strain Ben<sup>14</sup>, we were unable to detect mycobacterial DNA sequences in the DNA extracted from CD gut tissues. The probes were shown to be capable of sensitive detection of mycobacterial DNA and of discriminating between closely related mycobacteria by restriction fragment length polymorphism analysis<sup>20,21</sup> (RFLP). The combination of the three probes used in this study would have detected DNA related to *M paratuberculosis*, *M avium-intracellulare*, and *M*

*kansasii*, all species that have been variously isolated from CD tissues. The maximum levels of sensitivity achieved for the detection of *M paratuberculosis* was one organism/100 human cells and for the other species one organism/10 cells. Whether such levels might be similar to natural infections by these organisms is unknown. Certainly acid fast bacilli at these levels have not been detected by staining.<sup>10 11</sup> Cell wall deficient or spheroplast forms of mycobacteria which have been recovered from cultures of Crohn's disease tissue by several groups<sup>12 14 22 23</sup> may be the natural intracellular form of mycobacteria in CD, as previously suggested.<sup>12</sup> There are, however, no data available for the intracellular abundance of these forms of mycobacteria. We suggest that if these forms do occur in CD then they persist at levels beyond our present methods of detection in subsets of cells permissive to invasion by and persistence of mycobacteria, such as macrophages or giant cells within granulomata.

Although extraction methods and sensitivity calculations were based on *in vitro* cultured *M avium* bacilli, *M lepraemurium* infected mouse liver provided a positive control for the application of our methods to the detection of intracellular forms of mycobacteria. Our DNA extraction method has also been successfully applied to an *in vitro* cultured spheroplast like organism recovered from CD tissue.<sup>29</sup> We therefore believe that spheroplasts, if present in CD tissue, would have been susceptible to the extraction methods used. Nevertheless, the absence of mycobacterial DNA at these levels of sensitivities does not exclude a mycobacterial aetiology for CD because some mycobacterial infections (lepomatous leprosy in man or *M paratuberculosis* infections in ruminants) produce large numbers of bacilli, while others are associated with very few undetectable organisms in tissues (tuberculosis and tuberculous leprosy). In this regard it is relevant to report that attempts by us to detect *M tuberculosis* DNA in several human tuberculous cervical lymph nodes were unsuccessful (unpublished observations).

A recent report has shown the presence of mycobacterial DNA in CD and control tissue derived DNA using solution hybridisation with CD isolated mycobacteria strain Linda total DNA as probe.<sup>24</sup> The thermal stability data for their DNA hybrids indicated that these sequences were neither identical to each other nor to strain Linda and they concluded that one mycobacterial species may not be specifically associated with CD. The levels of mycobacteria they found ranged between one organism/two human cells to one/700 cells. The total absence of mycobacterial DNA sequences observed in our present study may reflect our slightly lower sensitivity range or the specificity of cloned probes which readily

distinguish a positive banding pattern from background hybridisation. A previous study by us also failed to show mycobacterial DNA in mesenteric lymph nodes from 21 cases of CD.<sup>30</sup>

Clearly even more sensitive techniques than those described here providing precise information on the organisms detected are required. Gene amplification techniques using the polymerase chain reaction have recently been described that may enrich a specific DNA sequence more than a million fold.<sup>31 32</sup> With the DNA sequence data for specific mycobacterial target DNA at present being obtained in this laboratory, these methods combined with Southern blotting and RFLP analysis should provide crucial information concerning the possible mycobacterial aetiology of Crohn's disease.

This work was supported by grants from Action Research for The Crippled Child and The Anthony and Elizabeth Mellows Charitable Trust to whom we would like to express our sincere thanks.

#### References

- 1 Mayberry JF, Rhodes J. Epidemiological aspects of Crohn's disease: a review of the literature. *Gut* 1984; **25**: 886-99.
- 2 Allan A. Inflammatory bowel disease: Aetiology and epidemiology. *Curr Opin Gastroenterol* 1985; **1**: 461-7.
- 3 Mitchell DN, Rees RJW. Possible role of infectious agents in Crohn's disease. *Z Gastroenterol* 1979; **17**: 98-100.
- 4 Beeken WL. Transmissible agents in inflammatory bowel disease. *Med Clin N Am* 1980; **64**: 1021-35.
- 5 Pena AS, Weterman IT, Booth CC, Strober W, eds. *Recent advances in Crohn's disease. Developments in Gastroenterology 1*. London: Martinus-Nijhoff, 1981.
- 6 Patterson DSP, Allan WM. Chronic mycobacterial enteritis in ruminants as a model of Crohn's disease. *Proc R Soc Med* 1972; **65**: 998-1001.
- 7 Chiodini RJ, van Kruiningen HJ, Merkal RS. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Vet* 1984; **74**: 218-62.
- 8 Golde DW. Aetiology of regional enteritis. *Lancet* 1968; **i**: 1144-5.
- 9 Gitnick G. Is Crohn's disease a mycobacterial disease after all? *Dig Dis Sci* 1984; **29**: 1086-8.
- 10 Wilensky A, Moschowitz E. Non specific granuloma of the small intestine. *Am J Med Sci* 1927; **173**: 374-80.
- 11 Crohn BB, Ginsberg L, Oppenheimer GD. Regional ileitis. A pathological and clinical entity. *JAMA* 1932; **99**: 1323-9.
- 12 Burnham WR, Lennard-Jones JE, Stanford JL, Bird RG. Mycobacteria as a possible cause of inflammatory bowel disease. *Lancet* 1978; **ii**: 693-6.
- 13 Belsheim MR, Darwish RZ, Watson WC, Schieven B. Bacterial form isolation from inflammatory bowel disease patients. *Gastroenterology* 1983; **85**: 364-9.
- 14 Chiodini RJ, van Kruiningen HJ, Thayer WR, Merkal RS, Coutu JA. Possible role of mycobacteria in inflammatory bowel disease. 1 An unclassified mycobacterium

- species isolated from patients with Crohn's disease. *Dig Dis Sci* 1984; **29**: 1073-9.
- 15 Chiodini RJ, van Kruiningen HJ, Merkel RS, Thayer WR, Coutu JA. Characteristics of an unclassified *mycobacterium* species isolated from patients with Crohn's disease. *J Clin Microbiol* 1984; **20**: 966-71.
  - 16 van Kruiningen HJ, Chiodini RJ, Thayer WR, Coutu JA, Merkel RS, Runnels PL. Experimental disease in infant goats induced by a mycobacterium isolated from a patient with Crohn's disease. *Dig Dis Sci* 1986; **31**: 1351-60.
  - 17 Chiodini RJ, van Kruiningen HJ, Thayer WR, Couteau JA. Spheroplastic phase of mycobacteria from Crohn's disease. *J Clin Microbiol* 1986; **24**: 357-63.
  - 18 Chiodini RJ, van Kruiningen HJ, Thayer WR, Coutu JA. Differentiation of the mycobacterium sp. from Crohn's disease by restriction fragment length polymorphism of the ribosomal genes [Abstract]. *Gastroenterology* 1986; **90**: 1372.
  - 19 McFadden JJ, Butcher PD, Chiodini RJ, Hermon-Taylor J. Determination of genome size and DNA homology between an unclassified *mycobacterium* species isolated from patients with Crohn's disease and other mycobacteria. *J Gen Microbiol* 1987; **133**: 211-4.
  - 20 McFadden JJ, Butcher PD, Chiodini RJ, Hermon-Taylor J. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between Mycobacterial species. *J Clin Microbiol* 1987; **25**: 796-801.
  - 21 McFadden JJ, Butcher PD, Thompson J, Chiodini R, Hermon-Taylor J. The use of DNA probes identifying restriction fragment length polymorphisms to examine the *Mycobacterium avium* complex. *Mol Microbiol* 1987; **1**: 283-91.
  - 22 Graham DY, Markesich DC, Yoshimura HH. Mycobacteria and inflammatory bowel disease. Results of culture. *Gastroenterology* 1987; **92**: 436-42.
  - 23 Collins JB, Beaman B, Arthur M, Gitnick G. Isolation of mycobacteria from intestinal tissues [Abstract]. *Gastroenterology* 1986; **90**: 177.
  - 24 Yoshimura HH, Graham DY, Estes MK, Merkel RS. Investigation of association of mycobacteria with inflammatory bowel disease by nucleic acid hybridisation. *J Clin Microbiol* 1987; **25**: 45-51.
  - 25 Patel R, Kuach JT, Mounts P. Isolation and restriction endonuclease analysis of Mycobacterial DNA. *J Gen Microbiol* 1986; **132**: 541-51.
  - 26 Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction fragments to high specific activity. *Anal Biochem* 1984; **137**: 226-7.
  - 27 Maniatis T, Fritsch EF, Sambrook J. *Molecular cloning - A laboratory manual*. New York: Cold Spring Harbor Laboratory Publications, 1982.
  - 28 Baess I. Determination and re-examination of genome sizes and base ratios on deoxyribonucleic acid from Mycobacteria. *Acta Pathol Microbiol Immunol Scand* 1984; **B92**: 209-11.
  - 29 McFadden JJ, Thompson J, Hull E, Hampson S, Stanford J, Hermon-Taylor J. The use of cloned DNA probes to examine organisms isolated from Crohn's disease tissue. 1987 In: MacDermott, ed. *Inflammatory bowel disease: current status and future approach*. Amsterdam: Elsevier, 1988.
  - 30 Butcher PD, McFadden JJ, Hermon-Taylor J. The use of cloned mycobacterial DNA probes for the detection of Mycobacteria in Crohn's disease tissue. *Biochem Soc Trans* 1987; **15**: 547-9.
  - 31 Saike RK, Scharf S, Faloona F, et al. Enzymatic amplification of beta globin genomic sequences and restriction site analysis for diagnosis of sickle cell disease. *Science* 1985; **230**: 1350-4.
  - 32 Saike RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988; **239**: 487-91.