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Mycobacteria in Crohn's Disease: DNA Probes Identify the Wood Pigeon Strain of *Mycobacterium avium* and *Mycobacterium paratuberculosis* from Human Tissue

JOHNJOE McFADDEN,[1] JUDITH COLLINS,[2]* BLAINE BEAMAN,[3] MARIKA ARTHUR,[4] AND GARY GITNICK[4]

School of Biological Sciences, University of Surrey, Guildford, United Kingdom[1]; Department of Medicine, Department of Veterans Affairs, and Oregon Health Sciences University, Portland, Oregon 97207[2]; School of Biological Sciences, University of California, Davis, California 95616[3]; and Department of Medicine, University of California, Los Angeles, California 90024[4]

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***Mycobacterium paratuberculosis* is known to cause Johne's disease, a granulomatous ileitis in ruminants, and may be involved in some cases of Crohn's disease. Like *M. paratuberculosis*, the wood pigeon strain of *Mycobacterium avium* may also show mycobactin dependence on primary isolation that is attenuated on further subculturing. A wood pigeon strain, *M. avium* restriction fragment length polymorphism (RFLP) type A/I, is also capable of causing granulomatous ileitis in experimental animal models but is not known to cause disease in humans. *M. avium* RFLP type A is associated with disease in immunocompromised hosts. Three DNA probes, pMB22 and the two subclones pMB22/S4 and pMB/S12, were found to be capable of distinguishing among *M. paratuberculosis*, *M. avium* type A, and *M. avium* type A/I (wood pigeon strain) on the basis of RFLPs. These DNA probes were used to identify two mycobacterial isolates (*M. paratuberculosis* and *M. avium* type A/I, wood pigeon strain) derived from the intestinal tissues of two patients with Crohn's disease. In addition, the wood pigeon strain of *M. avium* was identified from a patient with ulcerative colitis, and *M. avium* RFLP type A was identified from a patient with colonic carcinoma. This is the first time that *M. avium* A/I (wood pigeon strain) is known to have been isolated from human tissue. There are too few isolates to speculate about the etiological significance of mycobacteria and inflammatory bowel disease, but it is reasonable to conjecture that *M. paratuberculosis* may be responsible for some cases of Crohn's disease and that the wood pigeon strain of *M. avium* may also be an inflammatory bowel disease pathogen in humans.**

Crohn's disease (CD) is a chronic inflammatory disorder of unknown etiology that may involve any segment of the gastrointestinal tract. The original account of this disorder described a chronic granulomatous disease involving the terminal ileum and one that closely resembled intestinal tuberculosis. This similarity suggested that mycobacteria may play a role in CD (1, 7). However, the failure to cultivate specific organisms from the tissues of patients with CD or to demonstrate the presence of acid-fast bacilli led to a retreat from this concept. Subsequently, Burnham and Lennard-Jones (1) isolated a strain of *Mycobacterium kansasii* from a mesenteric lymph node of a patient with CD, and in 1984, Chiodini et al. (3, 4) reported the isolation of a strain resembling *Mycobacterium paratuberculosis* from the intestinal tissues of three patients with CD. At least five different groups of investigators have isolated *M. paratuberculosis* from the tissues of patients with CD (12). The isolation of *M. paratuberculosis* was particularly interesting, because this species is known to cause Johne's disease, a granulomatous ileitis in ruminants that is very similar to Crohn's ileitis in humans (21).

Using similar culture techniques (3, 4), in our laboratory we cultured mycobacterial organisms from the intestinal tissues of five patients with CD, ulcerative colitis (UC), or colonic carcinoma (CC). One of these organisms has been described previously (9) and was a rapid-growing *Mycobacterium chelonei* subsp. *abscessus*. The other four organisms were all identified as the *Mycobacterium avium* complex or *M. paratuberculosis* by conventional identification techniques (2, 13, 22, 23). The distinction of *M. paratuberculosis* from *M. avium* relies heavily on the observation of mycobactin dependence in vitro and the clinical source of the inoculum.

In an effort to distinguish *M. paratuberculosis* from the *M. avium* complex on a genetic basis, a 1.5-kb repetitive sequence was identified in *M. paratuberculosis*. This sequence, designated IS*900*, was cloned into the probe pMB22 (5, 10, 12, 20). More recently, a related insertion sequence, designated IS*901* (14, 15), has been discovered in predominantly mycobactin-dependent strains of the *M. avium* complex. It is related to IS*900* and hybridizes to the probe pMB22. These strains have been designated *M. avium* restriction fragment length polymorphism (RFLP) type A/I. The RFLP type A/I banding pattern identifies the wood pigeon strain of *M. avium*, which is described as *M. avium columbae* by some investigators (12). It is often mycobactin dependent on primary culture and has been shown to produce Johne's disease in experimental infections of ruminants (6, 16). Strains of *M. avium* isolated from other sources including disseminated infections in patients with AIDS produced a hybridization pattern designated *M. avium* RFLP type A with the probe pMB22 (11). When using conventional biochemical techniques, the wood pigeon strain is indistinguishable from other *M. avium* complex organisms. The *M. avium* complex strains are frequently

* Corresponding author.

present in the environment and most often account for infection and disease in the immunocompromised host and in those with chronic lung disease (11).

Two subclones of pMB22 were developed and designated pMB22/S4 and pMB22/S12 (15). DNA from the *M. paratuberculosis* genome and wood pigeon bacillus hybridized to give specific banding patterns with pMB22 and pMB22/S4. DNA from *M. paratuberculosis* organisms hybridized only with the more specific subclone pMB22/S12. Using these clones, we sought to identify specifically four mycobacterial isolates derived from the intestinal tissues of two patients with CD and one each with UC and CC in our laboratory. This report describes the use of DNA probes to unambiguously identify these strains.

MATERIALS AND METHODS

Tissue selection and processing for mycobacteria. All patient material originated at the Medical Center of the University of California, Los Angeles. All patients having intestinal resections and from whom fresh unfixed tissue was available from July 1983 until May 1987 were entered into the study. A total of 82 tissue samples were cultured for the presence of mycobacteria. Of these, 27 tissue samples were derived from patients with CD and 55 tissue samples were derived from patients with other intestinal diseases (29 tissue samples were from patients with UC, 2 were from patients with adenomas, 2 were from patients with familial polyposis, 3 were from patients with diverticulosis, and 3 were from patients with diverticulitis; and tissue samples were obtained from patients who required intestinal resection for adenocarcinoma of the colon [$n = 12$], benign small bowel obstruction [$n = 1$], ischemic bowel [$n = 1$], a sigmoid hernia [$n = 1$], and idiopathic megacolon [$n = 1$]). The tissue was washed, homogenized, decontaminated, and cultured on Herrold's egg yolk medium in the presence of myobactin J. Other details about the cultivation of these mycobacteria and the patient population have been described previously (9).

Conventional techniques for mycobacterial identification. The identification of mycobacteria was based on growth rates, colonial morphology, acid-fast staining, growth on Herrold's egg yolk medium, dependence on mycobactin J, and biochemical tests (2, 13, 22, 23).

DNA extraction. The bacterial cells were washed in TEN (100 mM Tris-HCl, 150 mM NaCl, 100 mM EDTA [pH 8]) and treated with subtilisin (10 mg/ml; Sigma Chemical, St. Louis, Mo.) for 3 h at 37°C; this was followed by treatment with lysozyme (1 mg/ml; Sigma) for 3 h at 50°C. Cells were lysed by the addition of 1% sodium dodecyl sulfate (SDS) and pronase (3 mg/ml; Calbiochem, San Diego, Calif.) and were then incubated for 20 h at 37°C. Pronase (5 mg/ml) was added at 18 h. DNA was purified with phenol-chloroform extraction; this was followed by ethanol precipitation. The DNA was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA [pH 7.6]) (8).

Mycobacterial strains. The following standard mycobacterial strains were examined: *M. paratuberculosis* ATCC 19698 (American neotype bovine strain); *M. paratuberculosis* BEN (ATCC 43544) (3, 4); and two mycobactin-dependent *M. avium* strains, WP 1/79 (6, 19), which was isolated from a wood pigeon, and FP 8446 (from the collection of F. Porteals, Institute of Tropical Medicine, Antwerp, Belgium), which was isolated from a deer. These *M. avium* strains were identified as *M. avium* RFLP type A/I (14).

Probes. The derivation of the cloned probes pMB22, pMB22/S4, and pMB22/S12 for *M. paratuberculosis* and the *M. avium* complex has been described previously (10, 12). pMB22 contains a copy of the mycobacterial insertion sequence IS*900* that is present in the genome of *M. paratuberculosis*. Two subclones of pMB22, pMB22/S12 and pMB22/S4, contain only small fragments of IS*900* and were used to distinguish *M. paratuberculosis* from the *M. avium* complex strains. All probes were labeled with [γ-^{32}P]dCTP (specific activity, 3,000 Ci) by Multiprime labeling (Amersham International).

Southern blots. DNA (0.5 g) from each mycobacterial isolate was cut with the restriction enzyme *Pvu*II (New England Nuclear), electrophoresed on a 1% agarose gel, and transferred to Hybond N membranes (Amersham International). Membranes were hybridized with probe (10 ng/ml) for 18 h, washed five times (5 min each time) in 3× SSC (0.15 M NaCl plus 0.05 M trisodium citrate [pH 7])–0.1% SDS for 1 h at 65°C and then in 1× SSC–0.1% SDS for 30 min, and autoradiographed (17).

RESULTS

Four mycobacterial isolates were cultured from a total of 82 tissue samples. Two isolates (CD-1 and CD-2) were isolated from patients with CD, one was isolated from a patient with CC, and one was isolated from a patient with UC. All mycobacteria grew on Herrold's egg yolk medium after a period of 3 to 8 months. Isolate CD-1 was identified as a mycobactin-dependent organism characteristic of *M. paratuberculosis* (2). The other three isolates were all slow-growing, nonpigmented bacteria that were not mycobactin dependent after subculture. Biochemical test results and the growth patterns of the isolates suggested that they were all of the *M. avium* complex (13, 22, 23).

These four mycobacterial isolates were then identified on the basis of their RFLPs by using the three mycobacterial probes pMB22, pMB22/S4, and pMB22/S12. Additional mycobacterial DNAs from known sources were digested and hybridized to these same probes. The results of the hybridization pattern for each probe are shown in Fig. 1A through C. Isolate CD-2 hybridized to pMB22, pMB22/S4, and pMB22/S12 and produced banding patterns almost identical to those of the two strains of *M. avium* type A/I; these strains were from a wood pigeon (Fig. 1, lanes 2) and a deer (Fig. 1, lanes 3). Two bands at 2 and 3.2 kb were missing in comparison with the bands obtained from the wood pigeon and deer strains. Isolate CD-1 hybridized to pMB22, pMB22/S4, and pMB22/S12, giving banding patterns identical to that of the American neotype strain of *M. paratuberculosis* (bovine strain) and differing in only one band from the banding pattern for *M. paratuberculosis* BEN isolated by Chiodini et al. (4). Strain BEN was also derived from a patient with CD. The isolate from the patient with UC was identical to CD-2. The DNA from the isolate from the patient with CC hybridized very weakly with the probe pMB22 and gave a faint banding pattern typical for *M. avium* type A. No banding pattern was seen with pMB22/S4 or pMB22/S12. We interpret this as indicating that it was a culture of *M. avium* contaminated with a faster-growing organism. Unfortunately, it was not possible to obtain a pure culture of this strain.

DISCUSSION

We isolated and used IS*900*-specific DNA probes to identify a strain of *M. paratuberculosis* from a single case of CD. The strain is identical to the American neotype (bovine)

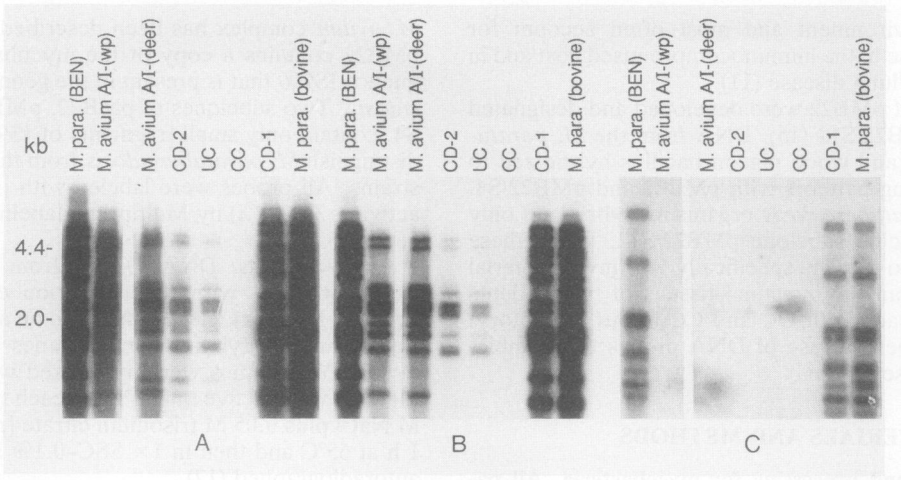

FIG. 1. Restriction fragment patterns after *Pvu*II digestion of DNA extracted from selected mycobacterial strains and mycobacterial isolates from human tissues. DNA (0.5 g) was extracted from each strain or isolate and electrophoresed through 1% agarose; blotted; probed with pMB22 (A), pMB22/S4 (B), and pMB22/S12 (C); and autoradiographed. DNA samples were from *M. paratuberculosis* BEN (ATCC 43544), *M. avium* RFLP type A/I (wood pigeon), *M. avium* RFLP type A/I (deer), a patient with CD (isolate CD-2), a patient with UC, a patient with CC, a patient with CD (isolate CD-1), and *M. paratuberculosis* ATCC 19698 (bovine strain), from left to right, respectively, in each panel.

strain and differs in only one band from strain BEN, which was isolated from a patient with CD by Chiodini et al. (3, 4). This single-band polymorphism occurs randomly in strains of *M. paratuberculosis* isolated from human and animal sources (18). *M. paratuberculosis* has now been isolated from the tissues of patients with CD by numerous independent laboratories and has been isolated only from either the intestinal tissue of patients with CD or animals with Johne's disease. This organism has not been isolated from any control tissues, nor has it been isolated from persons with AIDS (11), in whom infections with other common environmental mycobacteria are common. It has been isolated from primates with a disease similar to Johne's disease (17).

We isolated two strains of *M. avium* RFLP type A/I that appeared, by DNA probing, to contain repetitive DNA elements related to IS*901* and to be very closely related to the wood pigeon strain of *M. avium*. These strains were isolated from the intestinal tissue of a patient with CD and from the colon of a patient with UC. The significance of the banding pattern differences found between the strains from humans and animals is unknown; however, strains like the wood pigeon bacillus containing IS*901* show some polymorphism with these probes. To our knowledge, this is the first description of the isolation of strains of this group of *M. avium* from human clinical material. The use of DNA typing makes this distinction possible. Ordinarily, this pathogen infects wood pigeons (*Columba palumbus*) and is also found in other birds and livestock such as deer (18). Like *M. paratuberculosis*, it is usually mycobactin dependent, but unlike *M. paratuberculosis*, this dependence is usually lost on repeated subculturing. Examination of previously published data on *M. avium* strains isolated from patients with AIDS (11) failed to identify any *M. avium* RFLP type A/I strains. However, approximately 10% of strains that were described as *M. paratuberculosis* and that were isolated from ruminants with Johne's disease were found to be *M. avium* RFLP type A/I (14). The ability of *M. avium* RFLP type A/I (also known as the wood pigeon strain) to cause Johne's disease in experimental infections of calves is particularly provocative in view of its isolation from two patients with inflammatory bowel disease. A third strain was isolated from a patient with cecal carcinoma and was identified as *M. avium* RFLP type A. This is not surprising, since *M. avium* is commonly isolated from patients with AIDS, and the route of infection is thought to be the gastrointestinal tract (11).

The etiological significance of the isolation of mycobacteria from patients with inflammatory bowel disease is still uncertain since specific mycobacteria could be isolated only from a small number of the specimens examined. The low rate of recovery of pathogenic mycobacteria could be due to their presence or possible involvement in only a small number of cases of inflammatory bowel disease, or alternatively, the success rate of isolation may be very low. Techniques that are being developed and that demonstrate the presence of specific types of mycobacterial DNA in the tissues of patients with inflammatory bowel disease without the need for culture should resolve this issue.

REFERENCES

1. **Burnham, W. R., and J. E. Lennard-Jones.** 1978. Mycobacteria as a possible cause of inflammatory bowel disease. Lancet **ii:**693–696.
2. **Chiodini, R. J.** 1986. Biochemical characteristics of various strains of *Mycobacterium paratuberculosis*. Am. J. Vet. Res. **47:**1442–1445.
3. **Chiodini, R. J., H. J. VanKruiningen, R. S. Merkal, W. R. Thayer, and J. A. Coutu.** 1984. Characteristics of an unclassified *Mycobacterium* species isolated from patients with Crohn's disease. J. Clin. Microbiol. **20:**966–971.
4. **Chiodini, R. J., H. J. VanKruiningen, W. R. Thayer, R. S. Merkal, and J. A. Coutu.** 1984. Possible role of mycobacteria in inflammatory bowel disease: an unclassified *Mycobacterium* species isolated from patients with Crohn's disease. Dig. Dis. Sci. **29:**1073–1079.
5. **Collins, D. M., D. M. Gabric, and G. W. DeLisle.** 1989. Identification of a repetitive DNA specific to *Mycobacteria paratuberculosis*. FEMS Microbiol. Lett. **60:**175–178.
6. **Collins, P., P. R. J. Matthews, A. McDiarmid, and A. Browne.** 1983. The pathogenicity of *Mycobacterium avium* and related mycobacteria for experimental animals. J. Med. Microbiol. **16:**27–35.

7. **Crohn, B. B., L. Ginzburg, and G. Oppenheimer.** 1932. Regional ileitis—a pathologic and clinical entity. JAMA **99**:1323–1329.
8. **Davis, L. G., M. D. Dibner, and J. F. Battey (ed.).** 1986. DNA extraction, p. 47–50. *In* Basic methods in molecular biology. Elsevier, New York.
9. **Gitnick, G., J. Collins, B. Beaman, D. Brooks, M. Arthur, T. Imaeda, and M. Palieschesky.** 1989. Preliminary report on isolation of mycobacteria from patients with Crohn's disease. Dig. Dis. Sci. **34**:925–932.
10. **Green, E. P., M. L. V. Tizard, M. T. Moss, J. Thompson, D. J. Winterbourne, J. J. McFadden, and J. Hermon-Taylor.** 1989. 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.
11. **Hampson, S. J., F. Portaels, J. Thompson, M. T. Moss, E. P. Green, J. Hermon-Taylor, and J. J. McFadden.** 1989. DNA probes demonstrate a single highly conserved strain of *Mycobacterium avium* infecting AIDS patients. Lancet **ii**:65–68.
12. **Hermon-Taylor, J., M. Moss, M. Tizard, Z. Malik, and J. Sanderson.** 1990. Molecular biology of Crohn's disease mycobacteria. Bailliere's Clin. Gastroenterol. **4**:23–42.
13. **Kubica, G. P.** 1984. Clinical microbiology, p. 133–175. *In* G. P. Kubica and L. G. Wayne (ed.), The mycobacteria, a sourcebook (part A). Marcel Dekker, Inc., New York.
14. **Kunze, Z. M., F. Portaels, and J. J. McFadden.** 1992. Biologically distinct subtypes of *Mycobacterium avium* differ in possession of insertion sequence IS*901*. J. Clin. Microbiol. **30**:2366–2372.
15. **Kunze, Z. M., S. Wall, R. Appelberg, M. Silva, F. Portaels, and J. McFadden.** 1991. IS901, a new member of a widespread class of atypical insertion sequences is associated with pathogenicity in *Mycobacterium avium*. Mol. Microbiol. **5**:2265–2272.
16. **Mathews, P. R. J., and A. McDiarmid.** 1979. The production in bovine calves of a disease resembling paratuberculosis with a *Mycobacterium sp.* isolated from a wood pigeon (*Columbia palumbus L*). Vet. Rec. **104**:286.
17. **McClure, H. M., R. J. Chiodini, D. C. Anderson, R. B. Swenson, W. R. Thayer, and J. A. Couti.** 1987. *Mycobacterium paratuberculosis* (Johne's disease) in a colony of stump-tail macaques (Macaca arctoides). J. Infect. Dis. **155**:1011–1019.
18. **McFadden, J. J.** Unpublished data.
19. **McFadden, J. J., J. Thompson, E. Hull, S. Hampson, J. Stanford, and J. Hermon-Taylor.** 1988. The use of cloned DNA probes to examine organisms isolated from Crohn's disease tissue, p. 515–520. *In* R. P. Macdermott (ed.), Inflammatory bowel disease: current status and future approach. Excerpta Medica, Amsterdam.
20. **Moss, M. T. G., E. P. Green, M. L. Tizard, Z. P. Malik, and J. Hermon-Taylor.** 1991. Specific detection of *Mycobacterium paratuberculosis* by DNA hybridisation with a fragment of the insertion element IS900. Gut **32**:395–398.
21. **Patterson, D. S. P., and W. M. Allen.** 1972. Chronic myobacterial enteritis in ruminants as a model of Crohn's disease. Proc. R. Soc. Med. **65**:998–1001.
22. **Vestal, A.** 1976. Procedures for the isolation and identification of *Mycobacteria*. U.S. Department of Health, Education and Welfare Publication No. (CDC) 76-8230. Center for Disease Control, Atlanta.
23. **Wayne, L. G.** 1984. Mycobacterial speciation, p. 25–65. *In* G. P. Kubica and L. G. Wayne (ed.), The mycobacteria, a sourcebook (part A). Marcel Dekker, Inc., New York.