

Spheroplastic Phase of Mycobacteria Isolated from Patients with Crohn's Disease

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Two strains of an unclassified *Mycobacterium* species were isolated after 18 and 30 months of incubation of media inoculated with resected intestinal tissues from patients with Crohn's disease. These strains represented the third and fourth isolates of this organism from Crohn's disease patients. Ultrastructural examination of this strain and two previously isolated strains revealed the presence of spheroplasts which eventually transformed into the bacillary form of a previously unrecognized *Mycobacterium* species. These cell wall-deficient forms did not stain with conventional dyes and failed to grow on hypertonic media. Restriction polymorphism of the ribosomal DNA genes was used to determine the relationship between the cell wall-deficient and bacillary forms. Identical restriction patterns of the ribosomal DNA genes were found between the spheroplasts and *Mycobacterium* sp. isolates with *EcoRI*, *BamHI*, and *XhoI* restriction endonucleases, thus providing definitive evidence of their origin. Unidentified spheroplasts were isolated from an additional 12 patients with Crohn's disease, of which 7 of 10 seroagglutinated with antiserum prepared against the *Mycobacterium* sp. Spheroplasts were isolated from 16 of 26 (61%) patients with Crohn's disease but not from tissues of 13 patients with ulcerative colitis or 13 patients with other diseases of the bowel. These findings support the role of mycobacteria as etiologic agents in some cases of Crohn's disease.

We have recently reported the isolation of fastidious, mycobactin-dependent, unclassified *Mycobacterium* sp. isolates from two patients with Crohn's disease (5-7). These *Mycobacterium* sp. isolates have characteristics which distinguish them from all previously recognized species, but they most closely resemble *M. paratuberculosis* (5). Incubation periods of up to 18 months may be necessary for primary isolation. Animal studies have shown that the bacillary form of this organism is pathogenic for BALB/c mice and goats, and oral inoculation of one strain into goats resulted in a granulomatous ileocolitis resembling Crohn's disease in 5 to 9 months (7). Primary sites of intestinal infection are the Peyer's patches and lymphoid aggregates of the small and large bowels, the sites where Crohn's disease is thought to begin. Histologic features of the disease in goats include epithelioid-cell granulomas, perilymphatic lymphangitis, ulcerations, and fissures, all important features of Crohn's disease. Immunologic investigations by enzyme-linked immunosorbent assay demonstrated that patients with Crohn's disease have a statistically significant ($P = 0.0003$) increase in antibody titers to the *Mycobacterium* spp. compared with controls and patients with ulcerative colitis (29). Since the original report, two additional strains have been isolated and information has been obtained which suggests that a spheroplast form may account for the long incubation periods required for isolation.

MATERIALS AND METHODS

Microorganisms. Three strains of a previously unrecognized *Mycobacterium* sp. isolated from patients with Crohn's disease were examined. These included *Mycobacterium* sp. Dominic, which was reported previously (5), and two new isolates, strains Ben and Leona, reported herein.

Both spheroplast and bacillary forms of these three strains were propagated and examined. In addition, spheroplasts isolated from 12 patients with Crohn's disease were examined. Spheroplasts were defined as microorganisms which could be cultivated on artificial media but were not characteristic bacteria as determined by light and electron microscopy.

Isolation. Primary isolation techniques have been described previously (5, 7). Briefly, mucosa and submucosa from resected gastrointestinal tissues were digested with 2.5% trypsin, filtered, and centrifuged at $4,340 \times g$. The centrifuge pellet was suspended in 0.1% benzalkonium chloride and allowed to stand at room temperature for 18 to 24 h. Approximately 0.1 to 0.2 ml of the resulting sediment was distributed onto slants of Herrold egg yolk medium (HEYM) containing 2 μg of mycobactin J per ml.

Culture. Subcultures were grown on HEYM, Middlebrook 7H9 broth with oleic acid-albumin-glucose-catalase (OAGC) or Dubos oleic albumin complex, Tween 80, and mycobactin J, and in Middlebrook 7H10 with OAGC, Tween 80, and mycobactin J. Cultures were incubated at 37°C with caps sealed and without CO₂. Broth cultures were grown in 30-ml (25 cm²) tissue culture flasks containing 7 ml of broth (maximum medium depth of 3 mm) and incubated in a horizontal position without agitation (5). Spheroplast growth without transformation was attempted in 7H9 broth, Kirchner base glycerol medium (24), butter agar (2), isolation and holding medium (2), and a modification of Medill-O'Kane medium (18). Media were made hypertonic with 0.34 M sucrose, NaCl, or both. Horse, porcine, sheep, or human sera were added to a volume of 10 or 20%. All media were supplemented with mycobactin J (5). Characteristic bacillary forms obtained from transformed spheroplasts were identified by a modification of standard methods (33) as described previously (5).

Staining methods for microscopy. Smears were prepared by emulsifying colonies from HEYM in phosphate-buffered

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saline (PBS) or placing a drop of broth on a microscope slide. Smears of spheroplasts and bacilli were heat fixed and stained by the Ziehl-Neelsen and Kinyoun acid-fast techniques (33), intensified Kinyoun stain (18), intensified triple acid-fast stain (1), Gram stain, Giemsa stain, Warthin-Starry silver impregnation technique (26), and the fluorochrome method for mycobacteria (33).

Electron microscopy. Colonies on HEYM were cut out of agar slants and fixed in 4% glutaraldehyde in cacodylate buffer (pH 7.2). After fixation, colonies were dissected from the agar and washed in several changes of fresh cacodylate buffer for 1.5 h. Colonies were postfixed in 2% osmium in cacodylate buffer for 1 h, dehydrated through a series of alcohols (30 to 100%), embedded in Epon A12, and sectioned on an LKB-3 ultratome with a glass knife. Sections were stained with lead acetate and examined on a Philips 300 electron microscope.

Restriction endonuclease polymorphism of rDNA. DNA from the bacillary and spheroplast forms of three isolates from patients with Crohn's disease (strains Leona, Dominic, and Ben) and bacillary forms of isolate Linda (ATCC 43015), *M. paratuberculosis* ATCC 19698, *M. avium-M. intracellulare* serovars 2 and 4, and *M. kansasii* TMC 1201 were examined to determine specific restriction patterns of the ribosomal DNA (rDNA) genes. Organisms were grown in Middlebrook 7H9 broth with Tween 80, Dubos oleic albumin complex or OAGC, and mycobactin J at 37°C without added CO₂. Cultures were incubated until the stationary phase of growth was obtained, and organisms were harvested by centrifugation at 4,340 × g.

Cells were suspended in 10 ml of an isotonic unstable buffer (0.0132 M phosphate buffer [pH 6.8], 0.05 M EDTA, 0.7% NaCl, 2,000 µg of lysozyme per ml) and fractionated in a Hughes press (13) at -80°C and 15,000 to 25,000 lb/in². Disrupted cells were extracted several times with equal volumes of phenol and chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated by the addition of an equal volume of 100% ethyl alcohol and centrifugation at 12,000 × g for 20 min. Pellets were suspended in 2 ml of Tris-EDTA buffer (TE; 0.01 M Tris hydrochloride, 0.001 M EDTA) and RNase (1,000 µg/ml) added to a final volume of 50 µg/ml. The mixture was incubated at 37°C for 30 min and then extracted twice with phenol and chloroform-isoamyl alcohol. DNA was precipitated as described above and suspended in 2,000 µl of TE. Sodium acetate (3 M) was added to a final concentration of 0.3 M, and DNA was precipitated with 2× volumes of cold (-20°C) 95% ethyl alcohol. DNA was suspended in 1,000 µl of TE and standardized to 2 µg/10-µl volume.

Restriction enzymes were obtained from Bethesda Research Laboratories, Gaithersburg, Md., and included *Ava*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RII, *Hinc*II, *Hind*III, *Pst*I, *Pvu*II, *Sst*I, and *Xho*I. Bacillary forms were digested with all of the enzymes, but spheroplasts were digested only with *Eco*RI, *Bam*HI, and *Xho*I. DNA (10 µl) was added to an equal volume of 2× reaction buffer, prepared as directed by the manufacturer, and restriction enzymes were added. Restricted mycobacterial DNAs were subjected to electrophoresis in 1% agarose (ultrapure electrophoresis grade; Bethesda Research Laboratories) in Tris-borate (TBE; 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) buffer. *Hind*III digests of lambda DNA (Bethesda Research Laboratories) were used as a molecular weight standard. Photographs were taken of ethidium bromide-stained gels with a Polaroid camera under UV light (320 nm) with a ruler adjacent to the lambda lane. The migrations of the lambda

fragments were recorded in millimeters. Transfer to hybridization membranes (GeneScreen Plus, New England Nuclear Corp., Boston, Mass.) was performed by the method of Southern (28) as directed by the membrane manufacturer.

The 5S rDNA gene of *Escherichia coli*, cloned in bacteriophage M13 mp8, was obtained from H. Liebke, Yale University, New Haven, Conn. (15). The bacteriophage was propagated in *E. coli* JM101 cells, and double-stranded phage was isolated by standard methods (16). The rDNA insert was excised by restriction endonucleases and purified by horizontal electrophoresis in 1% low-melting-point agarose (10). Radiolabeled rDNA was prepared with [³²P]dCTP and [³²P]dTTP (3,000 Ci/mmol, 10 µCi/µl) (New England Nuclear) by second-strand synthesis as described by Feinberg and Vogelstein (10). Radiolabeled rDNA was purified by Sephadex G50 chromatography with a spun column (16). Labeled rDNA was suspended in a hybridization solution containing 40% formamide, 0.75 M NaCl, 0.05 M Na₂HPO₄, 0.01 M EDTA, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 1.0% lauryl sulfate, 10% dextran sulfate, and 75 mg of tRNA per ml.

Membranes were prehybridized in hybridization solution (without radiolabeled DNA) overnight at 42°C before hybridization. The hybridization solution was removed, the probe was added, and hybridization was allowed to proceed for 18 to 24 h at 42°C on a rocker platform. The probe was removed and the membranes were washed in hybridization solution (without radionucleotides) and in an additional five solutions, each at 42°C for 30 min. Membranes were gently dried between paper towels and wrapped in plastic wrap. Membranes were placed in metal X-ray cassettes with photographic film and intensifying screens (Du Pont Co., Wilmington, Del.) and stored at -80°C for 3 to 5 days. A semilog graph was constructed by a custom computer program of kilobases versus millimeters of migration of the lambda marker. A graph was prepared for each gel containing a lambda marker. Migration of the mycobacterial rDNA was measured from the autoradiogram, and sizes in kilobases were determined from the lambda graphs.

Seroagglutination. Sera were obtained from New Zealand White rabbits which previously had been immunized with bacillary forms of strain Dominic. Rabbits were inoculated intraperitoneally with approximately 50 mg of heat-killed bacilli suspended in mineral oil. Antibody specificity was determined by enzyme-linked immunosorbent assay (29). Seroagglutination was performed with freshly drawn serum diluted 1:5 with PBS.

Bacterial suspensions were prepared by emulsifying colonies of both forms in PBS, allowing particles to settle for 4 h, and then adjusting the concentration to an optical density of 0.3 at 540 nm with PBS.

Seroagglutination was performed in test tubes (8 by 77 mm) by a modification of the methods described by Schaefer (25, 30) as currently used in the serotyping of mycobacteria (30). To each tube, 0.25 ml of the working dilution of serum was added, followed by 0.25 ml of the bacterial suspension. Control tubes containing 0.25 ml of PBS and 0.25 ml of bacterial suspension and tubes containing 0.25 ml of serum and 0.25 ml of PBS were also included. In addition, seroagglutination was performed with normal rabbit serum obtained before the rabbit was immunized (preimmune serum). Bacterial suspensions of *E. coli*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, and *M. phlei* were used as controls. Tubes were shaken and placed in an incubator at 37°C and examined after 4 and 24 h. Bacillary and spheroplast forms from 2 patients (strains Dominic and Ben) and unidentified

spheroplasts from 10 additional patients with Crohn's disease were examined by seroagglutination.

RESULTS

Isolation. Two unclassified *Mycobacterium* sp. isolates were recovered after 18 and 30 months of incubation of HEYM slants inoculated with a resected intestinal specimen from a 58-year-old male and an 80-year-old female with Crohn's disease. The isolates were identical to mycobacteria isolated from other patients (5) and were designated strains Ben and Leona.

The isolates were first detected as several small (0.5 by 0.5 mm) translucent colonies developing on HEYM slants after approximately 8 to 15 months of incubation. Smears prepared from these primary translucent colonies and stained by Ziehl-Neelsen and Kinyoun acid-fast techniques, intensified Kinyoun stain, fluorochrome, intensified triple acid-fast stains, Gram stain, Giemsa stain, and the Warthin-Starry silver impregnation technique failed to reveal characteristic bacterial forms. Inoculation of primary colonies onto slants of fresh HEYM resulted in the appearance of small opaque colonies after approximately 4 months of incubation, but again organisms could not be discerned by stained smears. Small white colonies grew in approximately 3 months on the third passage to HEYM, though again no cells could be observed in stained smears. Material from colonies was inoculated into 7H9 broth, and after 2 weeks of incubation broth turbidity developed. Smears prepared from the broth again failed to reveal any forms resembling microorganisms. Continued incubation resulted in increased turbidity and the presence of non-acid-fast pleomorphic material (Fig. 1) which adhered poorly to glass slides and was indistinguishable from cellular debris or other material which may have been present on slants or glass slides. Approximately 5 to 6

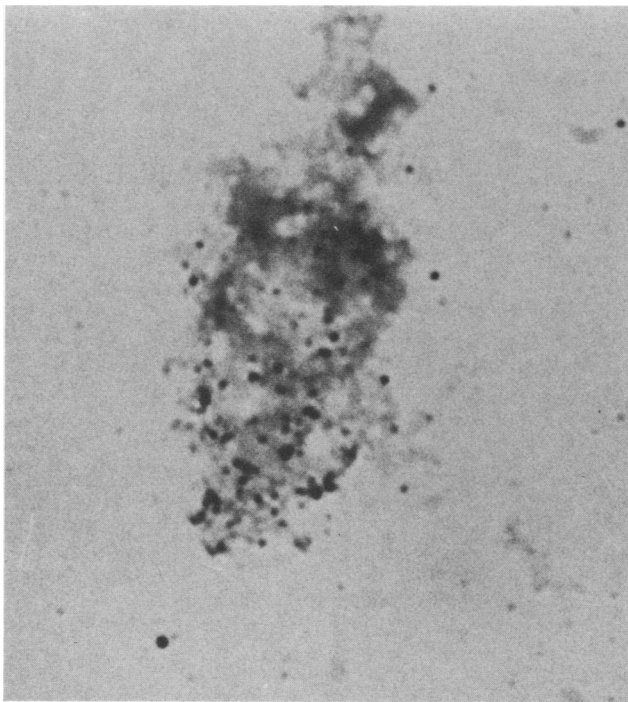


FIG. 1. Earliest form of visible growth after incubation of spheroplasts in broth. There are multiple coccoid bodies among amorphous material. (Ziehl-Neelsen; magnification, $\times 1,200$.)

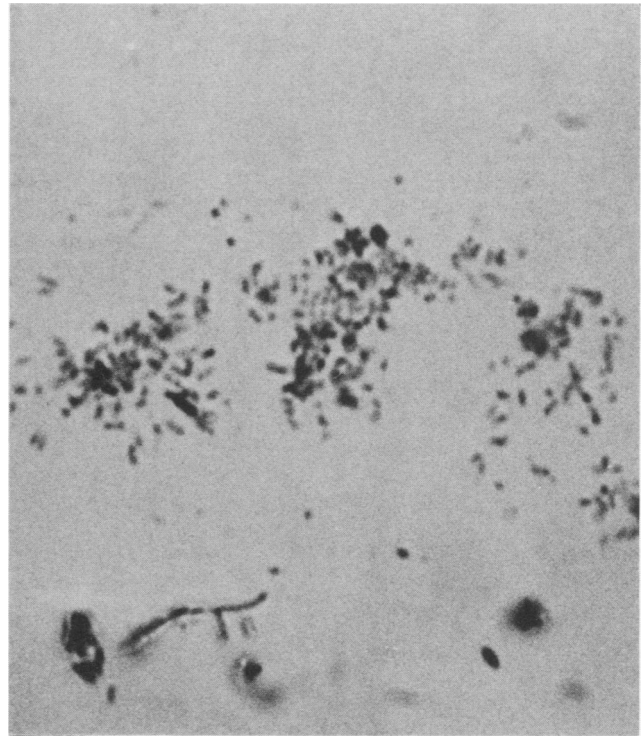


FIG. 2. Non-acid-fast coccobacilli developed in broth during transformation from spheroplast to bacillary state. (Ziehl-Neelsen; magnification, $\times 1,200$.)

weeks following inoculation into broth, large gram-positive bacilli and coccobacilli were present within the pleomorphic material (Fig. 2). Continued incubation (7 to 9 weeks following inoculation) resulted in the appearance of a few acid-fast bacilli, usually occurring in pairs, and at 8 to 9 weeks following inoculation broth cultures contained a pure culture of a strongly acid-fast *Mycobacterium* species (Fig. 3). Subcultures of these cultures readily grew in 3 to 4 weeks in 7H9 broth medium and in 6 to 8 weeks on HEYM and 7H10 agar media. Transformation into classical bacillary forms of mycobacteria was readily repeated with third-passage white colonies but not with earlier translucent or opaque colonies. Although they were reproducible, at least three flasks of broth needed to be heavily inoculated with white colonies to ensure that at least one culture would transform.

Culture and transformation. The transformation process suggested that early growth represented spheroplasts, although colonies developing on agar were similar to those of classical bacteria. In addition, growth and transformation occurred on nonhypertonic media. The poor and slow growth of these isolates on HEYM prompted our investigation of the utilization of a hypertonic medium for propagation of organisms for further study. Attempts to propagate cells from early white colonies on hypertonic HEYM, 7H10, Kirchner agar, butter agar, 7H9 broth, Kirchner broth, isolation and holding medium, and modified Medill-O'Kane media were not successful. In fact, hypertonic HEYM and Middlebrook 7H9 failed to support growth, though transformation occurred in both normal HEYM and 7H9. Other media did not support growth of either bacillary or spheroplast cultures. A suitable liquid medium for rapid propagation of spheroplasts without transformation could not be found.

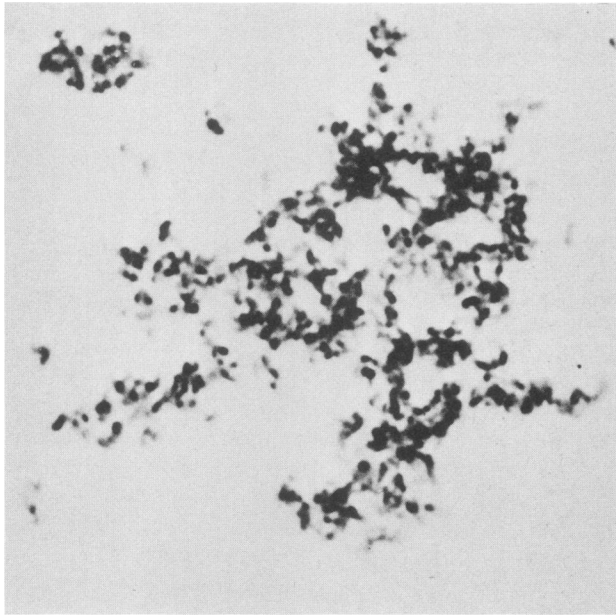


FIG. 3. The transformation process was completed by the emergence of pure cultures of acid-fast bacilli which developed by continued incubation of coccobacilli. (Ziehl-Neelsen; magnification, $\times 1,200$.)

Electron microscopic observations. Bacillary forms of strains Ben and Dominic revealed a well-defined cell wall measuring 6.0 to 13.0 nm thick (Fig. 4). Mesosomes or cytoplasmic invaginations and dense layered membranes were present within many cells (Fig. 4 and 5). There were multiple electron-translucent areas probably representing oil-red O inclusions as seen at the light level in some mycobacteria (27). Cytoplasm contained low-density areas with fine granular material or threads consistent with nuclear regions. Multiple small round to oval granules of various electron densities were the most prominent feature of the cytoplasm. There were occasional electron-dense areas resembling those of volutin granules. Many cross sections contained a poorly defined internal structure, but the cell wall was readily identified (Fig. 5).

Electron microscopic examination of opaque and white colonies of strains Ben and Dominic revealed microorganisms bound by a unit membrane but devoid of cell wall material (Fig. 6). The plasma membrane was poorly defined in most cells. Spherical forms of variable sizes were most prominent, but ovoid, teardrop, bean-shaped, and other forms were also observed. Many forms showed budding at single or multiple sites along the plasma membrane (Fig. 7), representing globular elements known as microspherules (32). A few cells appeared to be undergoing fission, suggesting active replication. Internal structures were limited to closely packed small granules of various electron densities and spheroidal low-density areas containing fine granular material. Mesosomes, electron-translucent areas, and volutin granules were not observed. The organisms were identified as spheroplasts since there was no chemical or immunologic evidence that cell wall material was totally lacking.

Comparison of restriction endonuclease fragments of rDNA. Restriction of mycobacterial DNA and hybridization with *E. coli* 5S rDNA produced a single band per digest in autoradiograms, suggesting that, unlike other bacteria which have

multiple copies of the ribosomal genes, the mycobacteria may have a single copy within their genomic DNA.

Of 11 restriction enzymes examined, identical restriction fragments of the 5S rDNA gene were detected with the isolates from Crohn's disease and *M. paratuberculosis* (Table 1). The restriction patterns of the rDNA gene suggest that the isolates from Crohn's disease are evolutionarily closely related and should be considered members of *M. paratuberculosis*.

Examination of *M. avium-M. intracellulare* serovars 2 and 4 revealed homology with *M. paratuberculosis* and the Crohn's disease isolates when restricted with *Bgl*II, *Eco*RI, *Eco*RII, *Hinc*II, and *Pst*I, showing the long-known similarities of *M. paratuberculosis* and the *M. avium-M. intracellulare* complex (Table 1). Other restriction enzymes, i.e., *Ava*I, *Bam*HI, *Hind*III, *Pvu*II, *Sst*I, and *Xho*I, produced distinguishing rDNA fragments between the organisms examined. *M. kansasii*, distantly related taxonomically, shared identical fragments with other mycobacteria when restricted with *Bgl*II and *Hinc*II enzymes, which appear to produce fragments conserved within the genus. In addition, *M. kansasii*, *M. paratuberculosis*, and the Crohn's disease isolates shared restriction fragments when digested with *Ava*I. From these data it was concluded that spheroplasts could be compared and identified by appropriate choice of restriction endonucleases. Owing to the limited amount of available spheroplast DNA, only selected enzymes were used for analyses.

Digestions with *Eco*RI, *Bam*HI, and *Xho*I were selected as digests which would produce distinguishing rDNA fragments and allow identification of the spheroplasts (Table 1).

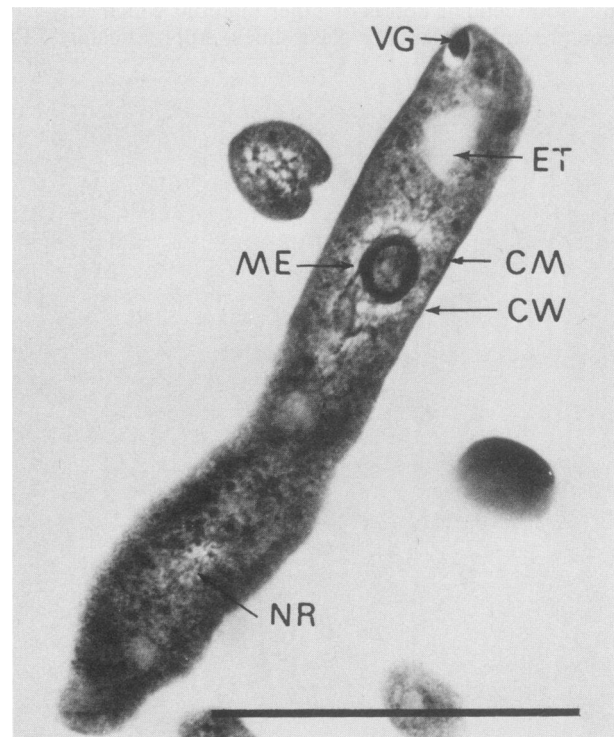


FIG. 4. Electron microscopic appearance of the bacillary state of *Mycobacterium* sp. Dominic after transformation from the spheroplast state. The organism has a well-defined cell membrane (CM), cell wall (CW), electron-translucent areas (ET), nuclear region (NR), volutin granules (VG), and mesosomes (ME). Bar = 1 μ m.

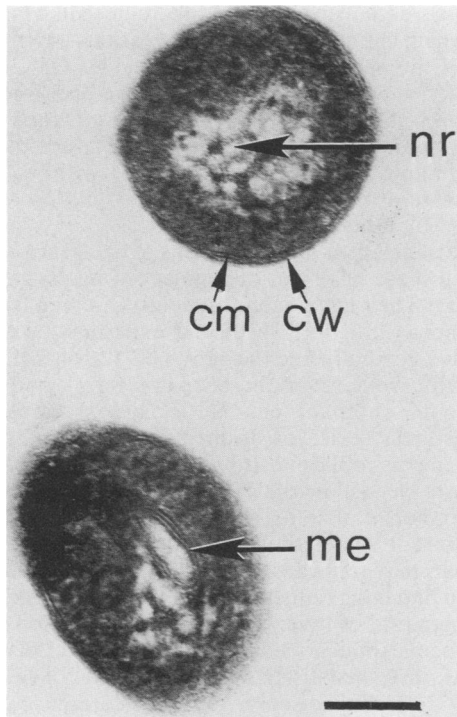


FIG. 5. Cross section of *Mycobacterium* sp. Dominic after transformation into the bacillary state. Multiple sections contained poorly defined internal structures, but the cell wall was readily defined. Abbreviations: cw, cell wall; cm, cell membrane; me, mesosomes; nr, nuclear region. Bar = 0.1 μ m.

DNA from bacillary and spheroplast forms of the Crohn's disease isolates were digested with *EcoRI*, *BamHI*, and *XhoI* and examined concurrently. Both spheroplast and bacillary forms of strains Dominic, Ben, and Leona produced 1.8-, 15.0-, and 8.3-kilobase fragments of rDNA following digestion with *EcoRI*, *BamHI*, and *XhoI*, respectively.

Serology. After 4 h of incubation, anti-Dominic serum agglutinated spheroplasts of strains Dominic and Ben and 7 of 10 spheroplasts isolated from other patients. Seroagglutination represented approximately 50% of soluble antigen; complete agglutination did not occur. Agglutination with bacillary forms of strains Dominic or Ben could not be determined owing to autoagglutination of cells within 4 h. There was no agglutination with *E. coli*, *P. multocida*, *P. aeruginosa*, *M. phlei*, or normal rabbit serum (preimmune). Spheroplasts were autoagglutinated by 24 h. Further testing was not possible owing to the limited quantities of spheroplasts.

DISCUSSION

Of major concern in the study of cell wall-deficient forms is the conclusive evidence that transformation to the parent form actually occurred, and that the observed transformation was not a contaminant or proliferation of a slow-growing organism within the spheroplast culture. In this report, we provide conclusive evidence of the relationship between spheroplasts and the *Mycobacterium* sp. by demonstration of identical restriction patterns of the rDNA genes.

Ultrastructural examination of early growth of the *Mycobacterium* sp. clearly identified the organisms as cell wall-

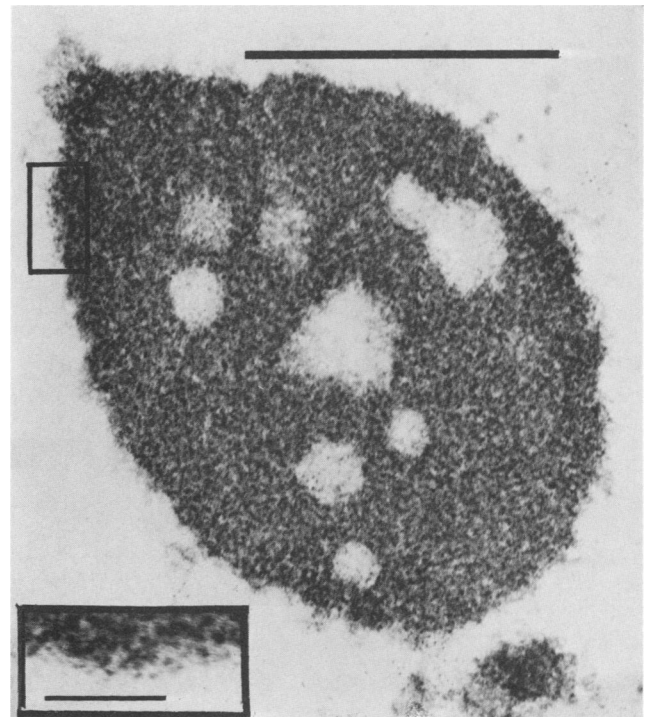


FIG. 6. Electron microscopic appearance of spheroplasts of *Mycobacterium* sp. Dominic. The organism is devoid of cell wall material, and internal structures are limited to small granules of various electron densities. A cell membrane (inset) was poorly defined in most cells. Bars = 0.5 and (inset) 0.1 μ m.

deficient forms or spheroplasts. Characteristics were consistent with other mycobacterial spheroplasts (21, 22, 31, 32), and there was no structural resemblance to the parent bacillary forms. Although the spheroplasts were actively growing, as suggested by the presence of microspherules, daughter cells, mycelial forms, and elementary bodies, indicating reversion to the bacillary state (31, 32), were not present. The lack of chemical and immunologic evidence for the complete absence of cell wall components precludes their characterization as protoplasts. Based on available information, primary isolation of *Mycobacterium* sp. Ben,

TABLE 1. Restriction endonuclease polymorphism of 5S rDNA between several *Mycobacterium* spp.^a

Enzyme	CD isolates	M ptb	MAI 2	MAI 4	M kan
<i>AvaI</i>	1.8	1.8	2.2/1.8	8.0	1.8
<i>BamHI</i>	15.0	15.0	9.5	9.5	6.5/5.3
<i>BglII</i>	7.8	7.8	7.8	7.8	7.8
<i>EcoRI</i>	1.8	1.8	1.8	2.8	1.9/1.7
<i>EcoRII</i>	1.3	1.3	1.3	1.3	3.3/2.5
<i>HindIII</i>	17.5	17.5	9.0	9.0	4.9
<i>HincII</i>	1.7	1.7	1.7	1.7	1.7
<i>PstI</i>	6.2	6.2	6.2	6.2	17.5/5.7
<i>PvuII</i>	12.0	12.0	9.5	7.0	14.0
<i>SstI</i>	5.9	5.9	5.6	12.0	5.6
<i>XhoI</i>	8.3	8.3	6.6	12.0	19.0

^a Values are sizes of rDNA fragments in kilobases; values separated by a slash indicate double bands in autoradiograms. Abbreviations: CD isolates, isolates from patients with Crohn's disease; M ptb, *M. paratuberculosis*; MAI 2, *M. avium-M. intracellulare* serovar 2; MAI 4, *M. avium-M. intracellulare* serovar 4; M kan, *M. kansasii*.

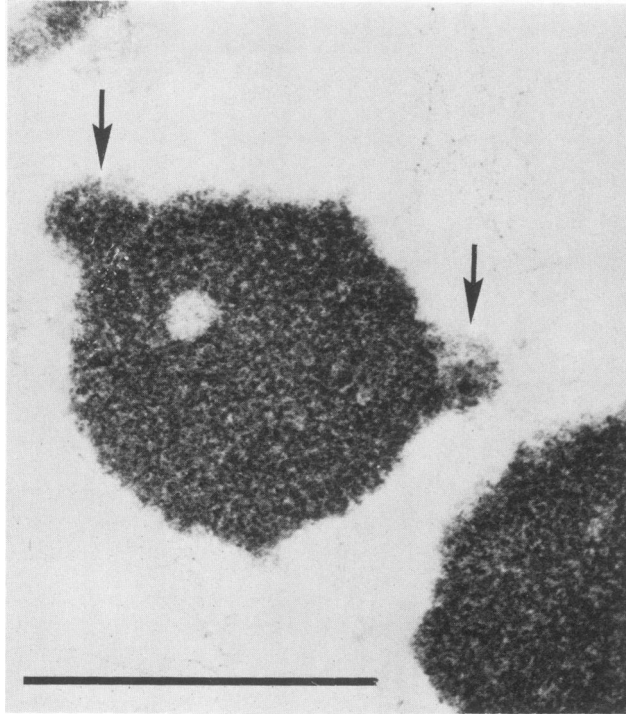


FIG. 7. Spheroplasts of *Mycobacterium* sp. Dominic grown on HEYM. Microspherules (arrows) indicating physiologic and metabolic activity were present in most cells. Bar = 0.5 μ m.

Dominic, and Leona appeared to be in spheroplast form. The poor internal morphology of the spheroplasts, their variability in size, and their poorly defined plasma membrane prevent their reliable detection in tissue sections by light or electron microscopy.

Identification of a spheroplast is generally dependent on its transformation to the bacillary state. Mycobacterial spheroplasts have characteristics completely different from those of the parent forms. Cell walls limit permeability into the cell cytoplasm, and the absence of this exclusion mechanism at the wall level alters biochemical reactivity (22). Thus, a complete cell wall is necessary for identification and determination of origin of the spheroplasts. The difficulties encountered in inducing transformation and our failure to date to transform the 12 unidentified spheroplasts reported herein are not unusual. Successive transfers, for 10 months, were required for transformation of *M. tuberculosis* spheroplasts into bacillary forms (18). Heavy variant growth is necessary for transformation to occur. In experimentally induced spheroplasts, reversion rates to the bacillary form were found to be as low as 0.1% (21, 31, 32). Reversion rates for naturally occurring spheroplasts are not known but are likely to approach the lower limits of those of experimental forms. Transformation of spheroplasts from four patients into classical mycobacteria and the demonstration of identical restriction fragments of the rDNA genes provided evidence that these spheroplasts were mycobacterial in origin.

Burnham and co-workers (4) recently proposed cell wall-deficient mycobacteria as etiologic agents of Crohn's disease. They reported isolation of a strain of *M. kansasii* from a lymph node of one patient with Crohn's disease and recovery of pleomorphic cell wall-deficient organisms from 22 of 27 Crohn's disease patients and 7 of 11 ulcerative colitis patients but in only 1 of 11 controls (4). *M. kansasii* is an

opportunistic organism causing a tuberculosislike pulmonary disease, which has been isolated from various environmental sources and human and animal lymph nodes (23, 36). Their cell wall-deficient organisms could not be transformed and thus may have represented one of the many L-form bacteria commonly found in patients with inflammatory bowel disease (3). Immunologic studies conducted with *M. kansasii* antigens were at first encouraging (4, 34) but could not be reproduced by other investigators (9, 35).

The role of cell wall-deficient forms of bacteria as etiologic agents of disease has been the subject of investigations for many years. They have been frequently isolated from clinical specimens (2, 3, 11, 17–20), but experimental challenge studies have generally been negative (8, 12, 14, 24). Disease often results from revertant bacillary forms and not the spheroplasts themselves. Nevertheless, pathogenic spheroplasts may exist, as do their morphologically similar relatives, the mycoplasma. It has been shown that bacterial spheroplasts persist in the body for longer periods, have poor chemotactic activity, and resist phagocytosis to a greater extent than their parent forms (24). Stable spheroplasts may replicate slowly over a long period of time, thereby maintaining infection at a subclinical or chronic level. A high rate of reversion induced by unknown factors may produce a sudden manifestation of clinical disease.

To date, intestinal tissues have been obtained from 26 patients with Crohn's disease, 13 with ulcerative colitis, and 13 with other intestinal disorders. Mycobacterial spheroplasts and their respective unclassified *Mycobacterium* spp. have been isolated from 4 patients with Crohn's disease, and related unidentified spheroplasts have been isolated from an additional 12 Crohn's disease patients (61%) but not from patients with ulcerative colitis or other bowel diseases. These findings add support for an etiologic role of mycobacteria in some cases of Crohn's disease.

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