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► **To cite this version:**

M.-F. Thorel. Relationship between mycobacterium avium, M. paratuberculosis and mycobacteria associated with Crohn's disease. *Annales de Recherches Vétérinaires*, 1989, 20 (4), pp.417-429. hal-00901909

HAL Id: hal-00901909

<https://hal.science/hal-00901909>

Submitted on 11 May 2020

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Review article

Relationship between *Mycobacterium avium*, *M. paratuberculosis* and mycobacteria associated with Crohn's disease

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(received 31 August 1988, accepted 18 January 1989)

Summary — Recently some mycobactin-dependent mycobacteria were isolated from patients with Crohn's disease. These mycobacteria should be very similar to *M. paratuberculosis* which is closely related to *M. avium*. The author has investigated the relationship between *M. avium*, *M. paratuberculosis* and the mycobacteria associated with Crohn's disease.

***Mycobacterium avium* — *Mycobacterium paratuberculosis* — Crohn's disease — biochemistry — pathogenicity — immunology**

Résumé — Etude des relations existant entre *M. avium*, *M. paratuberculosis* et les mycobactéries associées à la maladie de Crohn. Récemment des mycobactéries mycobactine-dépendantes ont été isolées de lésions de patients souffrant de la maladie de Crohn. Ces mycobactéries seraient semblables à *M. paratuberculosis* et à *M. avium*. L'étude des caractères culturels et biochimiques, de la sensibilité aux antibiotiques, de la sérologie, du pouvoir pathogène, des propriétés immunologiques et des caractères génomiques ont permis à l'auteur d'analyser les relations existant entre *M. avium*, *M. paratuberculosis* et les mycobactéries associées à la maladie de Crohn.

***Mycobacterium avium* — *Mycobacterium paratuberculosis* — maladie de Crohn — biochimie — pathogénie — immunologie**

INTRODUCTION

Previous characterisation of Crohn's disease isolated mycobacteria has shown that they are mycobactin-dependent organisms with cultural characteristics similar to *M. paratuberculosis*. However, *M. paratuberculosis* is closely related to mycobacteria of the *Mycobacterium*

avium-intracellulare complex (MAI complex), and there is now no universally recognized criterion for their precise taxonomic classification.

MAI complex is a group of mycobacteria capable of causing a wide range of diseases in animals and man, primarily as opportunist pathogens. *M. avium-intracellulare* is a ubiquitous environ-

mental pathogen that was rarely responsible for disseminated disease before the epidemic of acquired immune deficiency syndrome (AIDS).

Mycobactin-dependence has been observed in some *M. avium* strains particularly on primary isolation, and mycobactin-dependent organisms similar to *M. paratuberculosis* have been isolated from wood pigeon, deer and hare (Rankin & McDiarmid, 1968; Matthews & Sargent, 1977; Matthews *et al.*, 1978).

According to McFadden *et al.* (1987c), the question of whether *M. paratuberculosis* is a specific pathogen or an environmental opportunist, like *M. avium*, is considered to be relevant to the etiological significance of the isolation of *M. paratuberculosis* from Crohn's disease.

Crohn's disease

Crohn's disease is fundamentally a granulomatous process, which most often involves the terminal ileum, but it may develop in any part of the gastrointestinal tract. Granulomatous colitis, or Crohn's disease of the large bowel is now recognized with increasing frequency, yet it is often difficult to distinguish it clinically from ulcerative colitis. Although regional enteritis has been extensively investigated, its etiology remains obscure and no specific therapy has been developed.

The presence of an infectious agent was postulated soon after the disease was distinguished from ileocaecal tuberculosis. A reexamination of Crohn's disease, in the perspective of expanded concepts of infectious disease, suggests that the infectious agent hypothesis may be the more fruitful.

The similarity between regional enteritis and ileocaecal tuberculosis is well known, yet other related diseases have received relatively little attention. Johne's disease, for example, is a mycobacterial disease of animals which causes chronic diarrhea and bowel changes similar to those noted in regional enteritis in man.

In 1923, Dalziel, in an early description of Crohn's disease, suggested that the cause might be related to Johne's disease, a mycobacterial enterocolitis that had then been recently described in cattle.

Crohn *et al.* (1932) distinguished regional ileitis from intestinal tuberculosis, by failure to demonstrate the presence of tubercle bacilli. Subsequent attempts to culture mycobacteria from Crohn's disease tissue have been unsuccessful, though incubation was continued for only six weeks.

Golde and McGill (1968) suggest that regional enteritis is caused by an organism which may be related to the mycobacteria, but which cannot be cultured with existing techniques or identified with certainty in pathological sections.

Burnham *et al.* (1978) isolated a single mycobacterial strain *M. kansasii* from one of 27 patients with Crohn's disease, but only after eight months incubation. Cultures from 22 other patients with Crohn's disease, seven with ulcerative colitis and only one control subject, yielded pleomorphic organisms with the electron-microscopic appearances of cell-wall-deficient organisms. It seems possible that cell-wall-deficient mycobacteria are present in Crohn's disease lymph nodes and that they do not grow on ordinary culture media.

White *et al.* (1978) extended this work to look for antibody to this organism in the

sera of patients with inflammatory bowel disease, using an indirect fluorescent antibody technique which has been employed with success in tuberculosis. Nine of eleven patients with Crohn's disease, eight of ten with ulcerative colitis, but none of the 22 controls, were positive at 1/20 dilution of serum. The negative results in some patients may indicate an analogy with leprosy, in which antibodies are easily demonstrated in the lepromatous form, but not in the tuberculoid form.

Chiodini *et al.* (1984a) reported the isolation of an unclassified *Mycobacterium* species from a 15-yr-old female with Crohn's disease, and production of a chronic ileitis in a goat following oral inoculation. Later the isolation of a similar *Mycobacterium* species was reported from an additional patient.

Paratuberculosis

Paratuberculosis is a contagious and enzootic disease of ruminants, caused by the multiplication of a specific bacterium, *M. paratuberculosis* (Johne's bacillus), in the mucous membrane of the intestine.

First recognized in cattle, then in sheep, and later in goats, paratuberculosis is found more often among domestic and wild ruminants. The disease has also been reported in horses and pigs.

Under natural conditions, the disease in cattle spreads by ingestion of *M. paratuberculosis* from the contaminated environment. The disease persists in breeding stocks after the introduction of infected animals. A potential source of infection in calves, is milk contaminated with the faeces of diseased cattle.

Clinically, the bovine disease is characterized by chronic diarrhea and

emaciation. In sheep and goats, there may be simple intestinal catarrh, but if diarrhea develops, the animal usually dies within a few days or weeks.

Tuberculosis infection in wood-pigeon

The occurrence of tuberculous infections in the wood pigeon (*Columbus palumbus* L.), other than that associated with typical *Mycobacterium avium*, has been recorded (Christiansen *et al.*, 1946; McDiarmid, 1948-1962; Soltys & Wise, 1967). Previous authors have either failed to isolate the causal organism, or scanty growth has been obtained after prolonged cultivation on media usually employed for the isolation of *M. paratuberculosis*.

These last few years, we have had the opportunity to observe lesions in the livers and spleens of wood-pigeons that resembled the lesions of avian tuberculosis; large numbers of acid-fast bacilli were found on smears made from these lesions, but culturing of the bacteria was not successful when media currently used in tuberculosis bacteriology were employed.

The strains isolated from the wood-pigeons formed a relatively homogeneous group, which could be distinguished from *M. avium*, but were closely related to *M. paratuberculosis* (Thorel & Desmetre, 1982).

INFECTIOUS AGENTS

Cultural characteristics : Table I

Mycobacterium paratuberculosis grows slowly at 37 °C; Crohn's disease myco-

Table I. Cultural properties of mycobacteria ^a.

	<i>M. paratuberculosis</i>	"Wood-pigeon" <i>mycobacteria</i>	<i>Crohn's disease</i> <i>mycobacteria</i> "Linda"	<i>M. avium</i>
Colony morphology	rough	rough	rough	smooth
Bacilli morphology (μ)	2	2	2	1
Growth rate (weeks)	4–8	3–6	4–6	2–3
Mycobactin dependency	+	variable	+	variable
Growth on Lowenstein– Jensen medium ^b	–	–	–	+

^a –, negative; +, positive; ^b growth was optimal at 37 °C.

bacteria also grows very slowly at 37 °C; the primary colonies developed on Herrold medium with mycobactin, after 5–16 weeks of incubation for *M. paratuberculosis*, and after 3–18 months of incubation for Crohn's disease mycobacteria. Subcultures grew in 4–6 weeks. The cells of primary colonies are mycobactin-dependent, acid fast, aerobic and rough (Figures 1–4). The colonies are 0.5–1 mm. Cells were arranged in clumps for both *M. paratuberculosis* and Crohn's disease mycobacteria (Thorel & Desmettre, 1982; Chiodini *et al.*, 1984b).

These organisms (*M. paratuberculosis*, Crohn's disease mycobacteria and wood-pigeon mycobacteria) failed to grow on Lowenstein–Jensen medium.

Biochemical characteristics : Table II

The strains form a thermostable catalase; nitrates are not produced (Virtanen's procedure). *M. paratuberculosis*, wood-

pigeon mycobacteria, like *M. avium*, do not produce niacin, but Crohn's disease mycobacteria weakly produces niacin.

The arylsulphatase test, negative at 3 days for the 4 strains, is positive at 14 days for Crohn's disease mycobacteria alone. The β glucosidase and the urease tests are negative. The Tween hydrolysis (10 days), negative for *M. avium* and Crohn's disease mycobacteria, is variable for both *M. paratuberculosis* and wood-pigeon mycobacteria.

The mycobacteria isolated from patients with Crohn's disease are very similar to *M. paratuberculosis* in both cultural and biochemical characteristics (Thorel & Desmettre, 1982; Chiodini *et al.*, 1984b; Chiodini, 1986).

Antimicrobial susceptibility : Table III

Chiodini *et al.* used 7H9 broth with mycobactin and appropriate antibiotic concentrations for Crohn's disease myco-

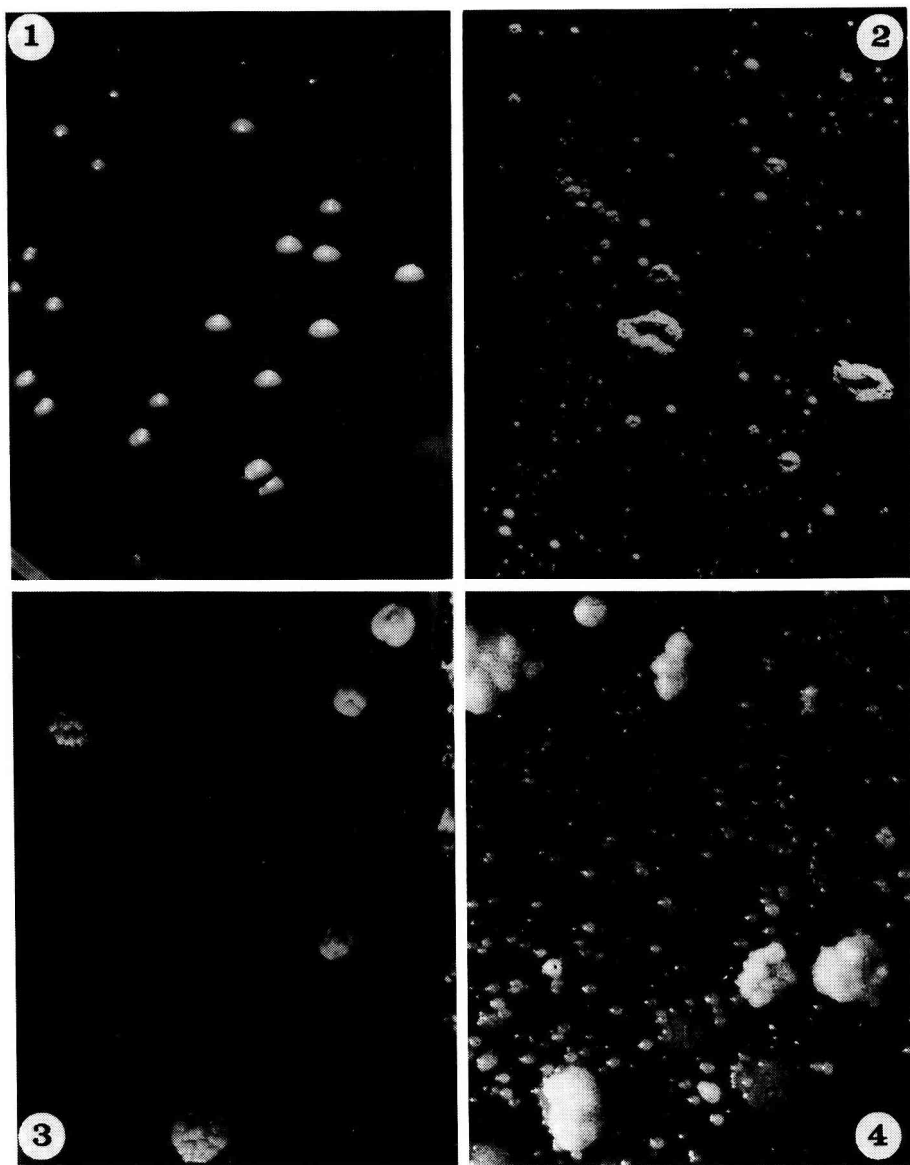


Fig. 1. Smooth colonies of *Mycobacterium avium* (x 2).

Fig. 2. Rough colonies of wood-pigeon mycobacteria (x 2).

Fig. 3. Rough colonies of *Mycobacterium paratuberculosis* (x 2).

Fig. 4. Smooth and rough colonies mycobacteria associated with Crohn's disease (x 2).

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Table II. Biochemical properties of mycobacteria ^a.

	<i>M. paratuberculosis</i>	"Wood-pigeon" <i>mycobacteria</i>	<i>Crohn's disease</i> <i>mycobacteria</i> "Linda"	<i>M. avium</i>
Niacin production	-	-	+ weak	-
Nitrate reductase	-	-	-	-
Catalase				
20 °C	+	+	+	+
68 °C	+	+	+	+
Tween 80 hydrolysis (10 days)	variable	variable	-	-
Arylsulfatase activity				
3 days	-	-	-	-
14 days	-	nd	+	-
β glucosidase	-	-	nd	-
Urease	-	-	-	-

^a -, negative; +, positive; nd, not done.

bacteria (Chiodini *et al.*, 1984c). We used 7H11 medium, with appropriate antibiotic concentrations, for *M. avium* and wood-pigeon mycobacteria, and the same medium, with mycobactin, for *M. paratuberculosis*.

These medium were inoculated with a 10⁻² dilution. *M. paratuberculosis* is resistant to most of antimicrobial agents like *M. avium*. Wood-pigeon mycobacteria is susceptible to rifampicin, ansamycin, cycloserin, streptomycin, capreomycin, kanamycin and paranitrobenzoate. Crohn's disease mycobacteria is susceptible to rifampicin, streptomycin, kanamycin, like wood-pigeon mycobacteria, and resistant to cycloserin, like *M. paratuberculosis* and *M. avium*.

Serology and thin layer chromatography analysis

One strain of Crohn's disease mycobacteria was autoagglutinated after four hours of incubation. Another, when it was suspended in 0.05% Tween 80, agglutinated with serotypes 42, 13 and 18, whereas, bacilli that were suspended in 7H9 broth, agglutinated with serotypes 4, 43 and 3. All the preparations were autoagglutinated by 24 h (Chiodini *et al.*, 1984b).

Wood-pigeon mycobacteria does not agglutinate in the presence of any of the sera defining the *M. avium-intracellulare* serovars, but wood-pigeon mycobacteria

Table III. Antimicrobial susceptibility in vitro of mycobacteria.

Antibiotic concentration ($\mu\text{g/ml}$)	<i>M. paratuberculosis</i>	"Wood-pigeon" mycobacteria	Crohn's disease mycobacteria "Linda"	<i>M. avium</i>
Rifampicin 1	1/5 ^b	4/5	s	r
Isoniazid				
1	r ^c	r	r	r
0.2	r	r	r	r
Ethionamide 10	r	r	2/3	r
Ethambutol 7.5	r	r	2/3	r
Ansamycin 1	3/5	s	nd	1/2
D cycloserin 30	r	3/5	r	r
Pyrazinamide 50	2/5	r	nd	r
Streptomycin 2	1/5	3/5	s	r
Capreomycin 10	1/5	3/5	1/3	r
Kanamycin 6	1/5	3/5	s	1/2
TCH5 ^a	r	r	r	r
PNB 500 ^a	2/5	4/5	nd	r

^a TCH, thiphen-2-carboxylic acid hydrazide; PNB, paranitrobenzoate; ^b n/n, number of susceptible strains/number of tested strains; ^c s, susceptible; r, resistant; nd, not done.

agglutinate in the presence of sera prepared from this strain. The results are similar with *M. paratuberculosis*; this suggests that they form at least one new serotype (Thorel & Desmetre, 1982).

According to Chiodini *et al.*, thin layer chromatography, performed by Brennan, failed to reveal any specific lipid pattern useful for identification from Crohn's disease isolated mycobacteria (Chiodini *et al.*, 1984b).

Mycobacterium paratuberculosis, however, contains a major immunoreactive

glycopeptidolipid, isolated and characterized by Camphausen *et al.*, 1985.

The glycolipid antigen belongs to the polar mycoside C glycopeptidolipid family present in other mycobacterial species. The polar glycopeptidolipid I of *M. paratuberculosis* conforms to the previously defined specifications of a species-specific antigen; the combination of sugars at the distal non-reducing end of the oligosaccharide has not been encountered before. This particular glycolipid does not correspond, in thin layer

chromatography mobility, to those previously encountered. The obvious antigen determinant appears to be characteristic of *M. paratuberculosis*. The glycolipid can be readily recognized in isolates of *M. paratuberculosis* by thin layer chromatography, and its presence may be used as a characteristic marker of the infectious agent. However, the polar glycopeptidolipid was highly reactive against sera, from only one animal of nine, with overt clinical paratuberculosis.

These oligosaccharide groups are responsible for the specific serological properties of *Mycobacterium avium-intracellulare-scrofulaceum* complex (Tsang *et al.*, 1983).

A typical thin layer chromatogram of the mycolic acid methyl esters from *M. paratuberculosis* and wood-pigeon mycobacteria formed the mycolic acid pattern characteristics of *M. avium*. The mycolic acid pattern characteristics of *M. avium* consist of : mycolate type I, α mycolates; type IV, ketomycolates; and type VI,

dicarboxylic mycolates (Daffe *et al.*, 1983; Minnikin *et al.*, 1984).

Pathogenicity : Table IV

The pathogenicity of *M. avium*, *M. paratuberculosis* and wood pigeon mycobacteria, was investigated in chickens, rabbits, guinea pigs, mice and calves (Berg Jorgensen and Clausen, 1976; Thorel *et al.*, 1984; Collins *et al.*, 1985). The pathogenicity of Crohn's disease mycobacteria was investigated in chickens, rabbits, guinea pigs, mice and goats (Chiodini *et al.*, 1984a, b).

Mycobacterium avium, *M. paratuberculosis* and wood-pigeon mycobacteria are pathogenic for mice. The resistance of guinea-pigs to *M. avium* is confirmed. Virulent strains of *M. avium* are usually lethal for rabbits within 50 days. Typical *M. avium* strains are pathogenic for chickens and produce an acute

Table IV. Pathogenicity of mycobacteria ^a.

	<i>M. paratuberculosis</i>	"Wood-pigeon" <i>mycobacteria</i>	<i>Crohn's disease</i> <i>mycobacteria</i> "Linda"	<i>M. avium</i>
Mice	+	+	+	±
Guinea pigs	-	-	-	-
Rabbits	-	variable	-	+
Chickens	-	+	-	+
Goats	+	nd	+	nd
Calves	+	+	nd	+

^a -, negative; ±, weakly positive; +, positive; nd, not done.

generalized infection in calves which usually results in death within 2—3 months.

Wood-pigeon mycobacteria has the pathogenic characteristics of *M. avium* for chickens, and of *M. paratuberculosis* for calves. Wood-pigeon mycobacteria, compared to *M. avium*, show a lower virulence for rabbits.

Mycobacterium paratuberculosis strains are pathogenic for calves, but are not normally pathogenic for chickens and rabbits.

According to Collins *et al.* (1985), it seems unlikely that small doses of lethal *M. avium* or *M. intracellulare* strains could produce clinical Johne's disease.

On the other hand, they suggest that the wood-pigeon isolates may constitute a distinct group with the pathogenic capability of *M. avium* in chickens, and of *M. paratuberculosis* in calves (Collins *et al.*, 1985).

Lesions did not occur in rabbits, chickens or guinea pigs, following intraperitoneal, intravenous, or subcutaneous injection of 5 mg of viable bacilli of Crohn's disease mycobacteria. No effect was observed in mice inoculated subcutaneously or in foot pads. Mice inoculated intravenously, developed non caseating granulomas of the liver, spleen, and mesenteric lymph nodes (Chiodini *et al.*, 1984a).

A goat, inoculated with Crohn's disease mycobacteria strain Linda, had a demonstrable delayed hypersensitivity to Johnin at three weeks after inoculation, which progressively increased until the time of autopsy. A response to tuberculin did not develop.

Humoral immunoglobulins of the IgM class were detected only during the first and second week post-inoculation. An IgG response did not occur.

Clinical disease did not develop during the course of this study. At autopsy 28 days post-inoculation, \approx 20 cm of the terminal ileum was thickened, and contained numerous transverse corrugations and focal hyperemia. The regional mesenteric lymph nodes were enlarged.

Histologically, the Peyer's patches of the terminal ileum contained multiple non caseating tuberculoid granulomas with giant cells. The adjacent mucosa was thickened with lymphocytes, macrophages and occasional giant cells. Regional lymph nodes had multiple tuberculoid granulomas within the cortical regions. Acid-fast bacilli were not demonstrable in any gastrointestinal section.

A *Mycobacterium* strain, with characteristics identical to those of the inoculum, was reisolated from the mesenteric lymph nodes. Organisms were not recovered from intestine.

Crohn's disease mycobacteria are pathogen, and are capable of producing a granulomatous disease of the intestine in the goat (Chiodini *et al.*, 1984a).

Immunological properties

ELISA studies, in an infected goat, showed an immunologic response to the *Mycobacterium* species, but there was also considerable cross-reactivity to *M. paratuberculosis* (Chiodini *et al.*, 1984a).

In view of these findings, a preliminary study to search for antibodies to this organism, and to *M. paratuberculosis*, was initiated using Crohn's disease, ulcerative colitis, and healthy, normal and non inflammatory bowel disease patient control sera. Other antigens used included *M. tuberculosis* and *M. kansasii* (Thayer *et al.*, 1984).

There was a good correlation between the antibody responses to *M. paratuberculosis* and the *mycobacterium* species, but not between *M. tuberculosis* and the *mycobacterium* species.

With the *paratuberculosis* antigen, the results showed a statistically significant difference between mean values for Crohn's disease patients and controls.

In the positive patients with *M. paratuberculosis*, 43% were also positive with *M. kansasii*, and all of the positives with *M. kansasii*, were also positive with *M. paratuberculosis*.

Sera of patients with Crohn's disease, appear to recognize an antigen present in *M. paratuberculosis* and *M. kansasii* those and probably also present in the unclassified *mycobacterium* species.

Genomic characteristics

Mc Fadden *et al.* (1987b) determined the total DNA base sequence homology between the Crohn's disease isolated unclassified *Mycobacterium* and the *mycobacterial* species *M. paratuberculosis*, *M. avium* complex serovars 2 and 5, *M. kansasii* and *M. phlei*, by measurement of DNA reassociation kinetics. This study was capable of distinguishing between the Crohn's disease isolate and *M. kansasii* and *M. phlei*, but the DNA homology found between the Crohn's disease isolate and *M. paratuberculosis*, *M. avium* 2 and *M. avium* 5, was in each case > 90% and was indistinguishable from that obtained with homologous DNA. This suggests that the Crohn's disease isolate and *M. paratuberculosis* are members of the MAI complex.

The determination of total sequence homology, by measurement of DNA reassociation kinetics has been shown to

present a good correlation with *mycobacterial* species classification (Gross and Wayne, 1970; Baess, 1979). However, this method is not sensitive enough to distinguish between closely related species.

Restriction endonuclease analysis of chromosomal DNA, has also been used to differentiate among *mycobacteria* (Collins and De Lisle, 1986).

Of 11 restriction enzyme patterns examined, identical restriction fragments of the 5S ribosomal DNA gene were detected with the isolates from Crohn's disease and *M. paratuberculosis* (Chiodini *et al.*, 1986). The restriction patterns of the ribosomal DNA gene suggest that the isolates from Crohn's disease are evolutionarily closely related and should be considered members of *M. paratuberculosis*.

This method requires preparation of high molecular weight DNA, which when digested, yields a large number of DNA fragments that are difficult to differentiate and interpret.

For this reason, Mc Fadden *et al.* (1987a) have developed a technique for distinguishing between these closely related *mycobacterial* species; the cloning of the genome of the unclassified *mycobacteria*. DNA, extracted from an unclassified Crohn's disease isolated *Mycobacterium* strain, was cloned. Cloned DNA probes were used to identify restriction fragment length polymorphisms in the DNA samples under study, to determine the relationship between this organism and other *mycobacteria*.

They showed that the three fastidious *mycobacteria*, isolated separately from samples of Crohn's disease tissues from three individuals, were indistinguishable, both from each other and from the type strain of *M. paratuberculosis*. Also Mc Fadden *et al.* (1987a) demonstrated

several restriction fragment length polymorphisms, distinguishing between the type strain of *M. paratuberculosis* and *M. avium* 2, and in each case, that the Crohn's disease isolated organisms gave the *M. paratuberculosis* pattern, and that *M. avium* serovar 5 gave the *M. avium* serovar 2 pattern. This suggests that they belong to two separately evolving groups.

Recently, Mc Fadden *et al.* (1987c), showed that the restriction fragment length polymorphisms, specific for *M. paratuberculosis*, were found in strains of *M. paratuberculosis* isolated in the USA and in France, and also in strains isolated from Crohn's disease patients, but not in any *M. avium* complex strains examined so far. This strongly suggests that this strain of *M. paratuberculosis* is genetically isolated from the *M. avium* complex strains examined. If this were not the case, then it would be expected that the markers would also be found in some *M. avium* complex strains.

The question of whether *M. paratuberculosis* should be considered as the same species, as organisms of the *M. avium* complex, must await a more thorough examination of the whole complex and further strains of *M. paratuberculosis*.

CONCLUSIONS

Our current bacteriologic methods may not be optimum for primary isolation of Crohn's disease mycobacteria; the use of mycobactin specifically prepared from this organism will improve our isolation capabilities.

The mycobacteria isolated from patients with Crohn's disease are very similar to *M. paratuberculosis*, in both

cultural and biochemical characteristics. Biochemically, differences occur only in arylsulfatase (14 days) and niacin reactions. Like *M. paratuberculosis*, these organisms are mycobactin dependent. This organism is a pathogen and is capable of producing a granulomatous disease of the intestine in goat, like wood-pigeon mycobacteria in calf. Crohn's disease mycobacteria and *M. paratuberculosis* also have many antigenic similarities. These organisms may be the causative agents in some cases of Crohn's disease, as is *M. paratuberculosis* in Johne's disease in ruminants. Further studies are required to determine if these organisms represent a new species of *Mycobacterium*, or a biovariant or subspecies of *M. paratuberculosis*.

According to Collins and de Lisle (1986), use of restriction endonuclease analysis may help clarify the relationship between *M. paratuberculosis* and *paratuberculosis-like* organisms, such as those isolated from wood-pigeons and man.

Recently, McClure *et al.* (1987) described the clinical, pathological, serological and cultural features of *paratuberculosis* in a colony of stump-tail macaques (*Macaca arctoides*). These findings extend the natural host range of *M. paratuberculosis*, to include non-human primates, and add support to current suggestions that *M. paratuberculosis* may be pathogenic for humans.

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