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Paratuberculosis

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HISTORICAL INTRODUCTION

The clinical symptoms and pathoanatomical picture of paratuberculosis were first described during the 19th century. The name Johne's disease comes from the work of H. A. Johne and L. Frothingham, who, in 1895, demonstrated a connection between cattle enteritis and the presence of acid-fast microorganisms in sections of the intestinal mucosa. In 1906, Bang distinguished between tuberculous and nontuberculous enteritis and proposed that the latter be called pseudotuberculous enteritis. The identification of the etiological agent is attributed to F. W. Twort, who, in 1912, succeeded in cultivating and characterizing a mycobacterium, which in 1914 was shown to produce experimental enteritis. After the full characterization of *Mycobacterium paratuberculosis* as a distinct species within the genus *Mycobacterium*, the disease was renamed paratuberculosis (40, 115).

It was only towards the middle of this century, however, that the prevalence and wide geographic distribution of paratuberculosis were realized, and its effect on the livestock farming economy became apparent. The yearly losses due to the disease were estimated to exceed $1.5 billion in the United States (107). Moreover, since Johne's disease is one of the most widespread bacterial diseases of domestic animals, its impact on the world economy is enormous (10, 40, 146, 160).

The aim of the present review is to briefly summarize the most relevant early findings and to concentrate on the molecular biological aspects of more recent works. A book recently edited by R. J. Chiodini (39) contains the complete set of references pertinent to the history of Johne's disease.

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CLINICAL STAGES OF JOHNE'S DISEASE AND HOST RANGE

Paratuberculosis affects domestic and wild animals (in particular, ruminants) on five continents. Although the veterinary world has focused on cattle, sheep, and goats, many wildlife ruminants (deer, antelopes, mountain goats, bisons, camels, llamas, and others) are also affected by paratuberculosis. In addition, the etiological agent, *M. paratuberculosis*, multiplies in horses and mules, which become asymptomatic shedders. Laboratory animals (mice, rats, rabbits, hamsters, gerbils, and guinea pigs) and birds (pigeons and poultry) replicate experimentally injected *M. paratuberculosis* (40, 71, 86, 88, 89, 141, 151). A closely related species isolated from the wood pigeon (*M. avium* subsp. *silvaticum*) was reported to cause chronic enteritis in deer, goats, and pigeons (128, 205). The relationship between the agent of Johne's disease and similar organisms isolated from humans and birds is discussed in a following section. Here we focus on the disease in ruminants, which are the usual hosts.

Although it is commonly agreed that mycobacteria invade the intestinal tract when animals feed on contaminated pastures, there are grounds to believe that infection of newborn animals is the main cause of disease. Because of the incomplete development of their immune systems, neonates are particularly susceptible to the etiological agent. Intrauterine infection seems to be restricted to fetuses of females with advanced forms of the disease. In fact, this kind of transmission was claimed to occur in about 25% of the calves born to critically ill cows (222). Calves can also be infected by ingesting colostrum and milk from paratuberculous cows (40, 196). *M. paratuberculosis* crosses the intestinal mucosa, presumably by penetrating the M cells of the dome epithelium that covers ileal Peyer's patches. Mycobacteria are then phagocytosed by subepithelial and intraepithelial macrophages, wherein they multiply intracellularly. This results in the appearance of a

TABLE 1. Clinical stages of paratuberculosis

Stage[a]	Mycobacterial shedding	Immune reactivity		Clinical symptoms
		Humoral	Cellular	
I	Undetectable	Low	High	Absent
II	Medium	Medium	Medium	Absent
III	High	High	Low	Present[b]

[a] I, asymptomatic, nonexcretory; II, asymptomatic, excretory; III, symptomatic, excretory.

[b] Impairment of lactation, of weight increase, and of reproduction; emaciation and diarrhea; anorexia, cachexia, and death.

granuloma at the site of entry and in the involvement of nearby lymph nodes (153, 233). The basic intestinal reaction in cattle with paratuberculosis is chronic catarrhal inflammation, along with hyperplasia and intense histiocyte infiltration of the lamina propria (143).

Infection may spread due to the repeated ingestion of organisms or to the gradual replication of bacteria ingested at an earlier time. Larger regions of the intestinal tract and other body systems become involved as the disease advances with time. The diffuse granulomatous reaction results in a protein-losing enteropathy, and hence, hypoproteinemia and edema due to decreased osmotic pressure, and in progressive thickening of the gut (161–163). At later stages, the congested epithelial cells of the mucosa are sloughed into the intestinal lumen, and large numbers of bacilli are discharged. Bacterial concentration in the stools of infected animals may exceed 10^8 cells per g of feces, and the daily number excreted can be higher than 10^{12} mycobacteria per animal (40). This massive number of organisms accounts for the rapid spread of the infection, from even a single infected animal, to the whole herd.

The disease progresses through three clinical stages (Table 1). In stage I (subclinical with undetectable excretion), the infectious process develops without appreciable shedding of bacteria. This is followed by a subclinical excretory phase (stage II), during which the concentration of mycobacteria in the intestinal mucosa and lumen progressively increases. The terminal phase (stage III, clinical and excretory) is characterized by an intractable chronic diarrhea and the symptoms of a generalized infectious process. Emaciation, decreased milk production, diffuse edema, anemia, and infertility are the dominant late signs. Abortion and alopecia are rare events, even in the advanced stage of the disease. Animals die in a cachectic state.

Information on disease development was gathered by experimental infection trials. After oral administration of *M. paratuberculosis* cells to calves, the majority of animals developed a subclinical form of disease, although they shed organisms (stage II). Clinical symptoms appeared after prolonged periods in only a minority of cattle (125). Intravenous injection of the etiological agent produced similar results, though the experimental route differed from the natural one (192).

There is evidence that animals with the asymptomatic form of the disease outnumber those with the symptomatic form (22). A Pennsylvania slaughterhouse survey of 14,440 cull dairy cattle reported a prevalence of 7.2% of the animals to be infected with *M. paratuberculosis* on the basis of the recovery of the organism from tissues and/or manure samples. It is estimated that for every animal with clinical disease, 9 other animals in the herd are culture positive and another 10 to 15 animals are infected but undetectable by current diagnostic methods (222). As much as 60% of certain herds were reported to be asymptomatically infected (40, 198).

MICROBIOLOGICAL ASPECTS

The etiological agent of Johne's disease, *M. paratuberculosis*, is a gram-positive, acid-fast microorganism of 0.5 to 1.5 μm. On Watson-Reid agar plates, or the commonly used HEYM (Herrold's egg yolk medium) plates, it forms nonpigmented rough colonies. It is a very slowly growing, fastidious organism: to proliferate, many strains require the presence of mycobactin. Visible colony formation on solid media may require up to 4 months, irrespective of the presence of different supplements (40, 142).

While a dependence on metals for growth is common to all bacteria, the iron requirement of pathogenic mycobacteria is peculiar in that an organic source of this metal is needed for its uptake and utilization. The complexed iron is chelated by siderophores and undergoes transmembrane transport and cytoplasmic exchange. Two kinds of siderophores are known, mycobactins and exochelins: the former are the internal sequestering agents, and the latter are the external vectors. Exochelins are small protein molecules present in the extracellular fluid: high concentrations are released under conditions of iron deficiency. Liposoluble and hydrosoluble exochelins have been identified. In animals, the main storage form of ferric ion is ferritin, and the transport molecules for this metal are transferrin and lactoferrin. Mycobacterial exochelins remove Fe^{3+} from these animal chelators and transport it across the bacterial cell wall. An exchange of the chelated metal with intracellular mycobactin may then take place, thus yielding the iron storage form present in mycobacteria. Mycobactins are high-molecular-weight complex lipids containing a core to which Fe^{3+} is coordinately linked and alkyl side chains of different lengths. Being insoluble in water, these highly hydrophobic structures are confined to the cell membrane. Mycobactins are unique to mycobacteria and nocardiae: they have not been found in any other actinomycetes examined and are therefore of taxonomic importance. Moreover, only mycobacteria contain both mycobactins and exochelins. The absence of mycobactins from most strains of *M. paratuberculosis* and some *M. avium* isolates is intriguing. These strains multiply freely in parasitized hosts, while requiring an exogenous supplement of mycobactin for in vitro proliferation (5, 6, 145). Mycobactin dependence is no longer considered pathognomonic for identification of *M. paratuberculosis* since mycobactin-dependent wood pigeon mycobacteria and *M. avium* strains have been identified (128, 202).

No one test can identify a single mycobacterial species, and only after multiple tests are performed for specific markers (growth factors, growth inhibitors, specific enzymes, lipid and sugar components, protein profiles, restriction analysis of DNA, and host range pathogenicity) can a microorganism isolated from an animal source be classified as *M. paratuberculosis* (105, 106). Thus, different chromatographic methods have been used to identify specific lipid and sugar markers for diagnostic and taxonomic purposes (19, 58, 150, 199).

It is particularly difficult to distinguish among species closely related to *M. paratuberculosis*. Wood pigeon mycobacteria do not grow on egg medium and are inhibited by *para*-nitrobenzoate, whereas *M. paratuberculosis* and *M. avium* multiply in both instances. The test for production of alkaline phosphatase is negative for *M. paratuberculosis* but positive for both *M. avium* and wood pigeon mycobacteria. Wood pigeon mycobacteria are pathogenic for chickens but not for rabbits, *M. avium* is pathogenic for both, and *M. paratuberculosis* is pathogenic for neither (33, 58, 142, 204, 206–208). Restriction fragment length polymorphism (RFLP) analysis of mycobacterial DNA with nucleic acid probes is the most powerful diagnostic

procedure available to distinguish among genomes of these closely related species (119, 131, 156). Studies along these lines are described in the next section.

GENOMES OF *M. PARATUBERCULOSIS* AND RELATED MICROORGANISMS

Mycobacteria belong to the taxonomic group that includes the genera *Corynebacterium*, *Mycobacterium*, and *Nocardia* (CMN group) and the new genus *Rhodococcus* (7). The base composition of the genomes of different bacteria is an essential taxonomic parameter, which is currently measured by biophysical methods (thermal profile or buoyant density) and expressed as the percentage of guanine plus cytosine (G+C) in DNA. The base composition of CMN organisms varies from 48 to 73% G+C: it is 48 to 59% for corynebacteria, 63 to 73% for rhodococci, 62 to 70% for mycobacteria, and 64 to 69% for nocardiae (82). However, the etiological agent of human leprosy, *M. leprae*, does not fit into this scheme: it has a 55 to 58% G+C composition (45, 98, 104). The base composition of *M. paratuberculosis* DNA is 66 to 67% G+C (103, 130), and that of other pathogenic mycobacteria is as follows: 66 to 68% for *M. avium*, 65 to 69% for *M. intracellulare*, 64% for *M. kansasii*, and 63 to 66% for *M. bovis* and *M. tuberculosis* (44, 60, 102).

The sizes of mycobacterial genomes have been determined by renaturation kinetics and, more recently, by pulsed-field gel electrophoresis. The *M. paratuberculosis* genome has a molecular size of 4.4×10^6 to 4.7×10^6 bp. Thus, it is larger than the genomes of closely related mycobacteria: 3.5×10^6 bp for *M. avium*, 3.0×10^6 to 4.2×10^6 bp for *M. tuberculosis*, and 2.0×10^6 to 3.3×10^6 bp for *M. leprae* (3, 4, 44, 45, 102–104, 130).

The genetic relatedness of *M. paratuberculosis* with other mycobacterial species has been established by DNA-DNA hybridization (Table 2). Nearly 100% homology between most strains of *M. paratuberculosis* and *M. avium* has been reported (100, 103, 174, 231). The DNA of *M. paratuberculosis* was also found to be highly homologous to the DNA from several wood pigeon mycobacteria, organisms genetically related to some *M. avium* and *M. intracellulare* strains (100, 231). On the other hand, *M. paratuberculosis* DNA is less related to DNA of other mycobacteria (100, 103, 130, 174, 231).

Hybridization with the complete genome does not distinguish among very closely related organisms (for instance, *M. paratuberculosis* isolates, wood pigeon mycobacteria, and some *M. avium* serovars). A deeper insight into the genetic relatedness of *M. paratuberculosis* with other mycobacterial species was obtained by RFLP analysis. Nucleic acid probes proved to be the appropriate tools for such sophisticated discrimination. With this technique, labelled probes from the DNA of one bacterial strain are hybridized with the restriction fragments obtained by electrophoretic fractionation of endonuclease-digested chromosomes from other mycobacterial species (Southern blot). The degree of similarity of patterns between the reference and the unknown organisms provides a measure of the genetic relatedness of the compared species. These data can be used to evaluate DNA base substitution levels in closely related species and strains. Accurate estimation is obtained with probes prepared from single-copy DNA segments, which are known to undergo little rearrangement. From base substitution data, phylogenetic trees, such as that shown in Fig. 1, can be constructed (134).

Probes containing repetitive DNA elements have been used to measure genetic relatedness among isolates of the same species (52, 134). Thus, the insertion sequence IS*900* of *M. paratuberculosis* was found to produce similar patterns from several *M. paratuberculosis* isolates (Fig. 2), suggesting homogeneity of this mycobacterial species (134). IS*900*, a 1.45-kb insertion sequence present in 15 to 20 copies in the *M. paratuberculosis* genome, has several unique features: (i) it lacks terminal inverted repeats, (ii) it does not induce duplication of the insertion sequences at the target site, (iii) it possesses homologous target site sequences within the ge-

TABLE 2. Relatedness of *M. paratuberculosis* genome to other mycobacterial species

Mycobacterial species	% DNA-DNA hybridization[a]
M. avium D4	91
M. avium ATCC 25291	92–102 (95)
M. avium serotype 2	96–105
M. avium serotype 7	49
M. avium serotype 8	92
M. avium serotype 9	92
M. avium serotype 19	35
M. paratuberculosis 18 (*M. avium* st 2)	76–91
M. paratuberculosis 316 F	(100)
M. paratuberculosis Linda	92
M. paratuberculosis C286	90
M. paratuberculosis 21	90
Wood pigeon 64	(94)
Wood pigeon 36	(93)
Wood pigeon M21	99
Wood pigeon VI/72	98
M. intracellulare serotype 6	111
M. intracellulare serotype 9	65
M. intracellulare TMC 1406	53
M. scrofulaceum serotype 41	18–21
M. scrofulaceum serotype 42	11–18
M. scrofulaceum serotype 43	14–30
M. fortuitum	3–17
M. tuberculosis	52
M. phlei	2.7–19
M. kansasii	23
M. gordonae	27

[a] Data from references 100, 103, 174, and 231. Numbers without parentheses refer to hybridization with the ATCC 19698 strain and those within parentheses refer to hybridization with the 2E strain of *M. paratuberculosis*.

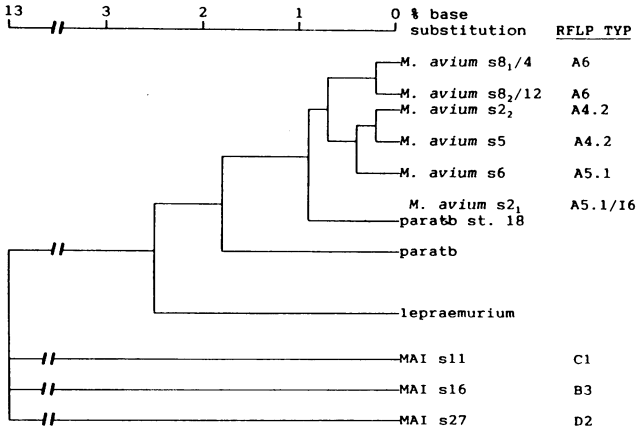

FIG. 1. Phylogenetic tree of a group of pathogenic mycobacteria. The dendogram was constructed with DNA base substitution data as calculated from RFLP analysis of the genomes of *M. paratuberculosis* (paratb), *M. avium*, some strains of the *M. avium-M. intracellulare* (MAI) group (different serovars), and *M. lepraemurium*. Reprinted from reference 133 with permission of the publisher.

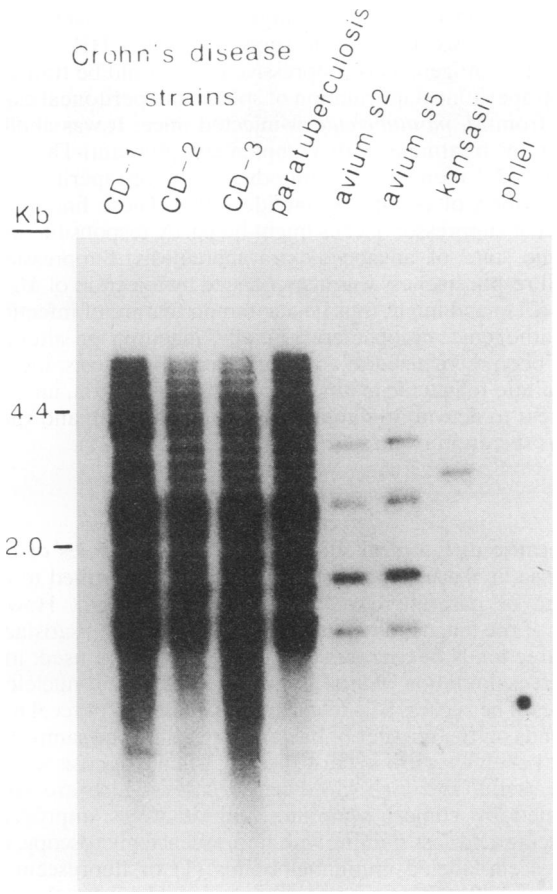

FIG. 2. RFLP analysis of mycobacterial genomes. *Pvu*II-digested DNA from *M. paratuberculosis* strains of human (Crohn's disease [CD]) and bovine (Johne's disease [paratuberculosis]) origin and other mycobacteria were probed with clone pMB22 containing the insertion sequence IS*900* of *M. paratuberculosis*. Reprinted from reference 133 with permission of the publisher.

nome, and (iv) it has oriented insertion (85). The expression product of IS*900*, the p43 protein, is similar to the polypeptides encoded by transposable elements from streptomycetes (IS*116* and IS*110*) (70). IS*900* has recently been used as a transposing vector in *M. smegmatis* (70). Sequences related to IS*900* were also found in wood pigeon mycobacteria (IS*902*) and *M. avium* (IS*901*) (119, 120, 156). IS*900* and IS*902* have been used in taxonomic studies of *M. paratuberculosis* and *M. avium* isolates, respectively (27, 53, 90, 119, 120, 134, 221).

rRNA sequences have also been used to evaluate the genetic relatedness of different bacterial genera and species. Indeed, these sequences are highly conserved in evolution. The number of rRNA genes is low in mycobacteria compared with that of the reference organism *Escherichia coli* (which has seven copies of rRNA genes). Two copies of rRNA genes per chromosome were identified in saprophytic mycobacteria, and a single copy was seen in pathogenic mycobacteria (15, 114). RFLP analysis of the 5S rRNA gene region clearly indicates that the strain closest to *M. paratuberculosis* is *M. avium* serovar 2 (36). The development of sequence analysis has provided taxonomists with very high resolution power. A comparison of 16S rRNA sequences from a large number of mycobacteria has shown that the 16S rRNA of *M. paratuberculosis* is 99.9% homologous to *M. avium*, 98.1% homologous to *M. bovis*, more than 95% homologous to other mycobacteria, and 85 to 92% homologous to the related genera *Corynebacterium*, *Rhodococcus*, and *Nocardia* (188). This technique also revealed two clusters within the genus *Mycobacterium*: one constituted by the nonpathogenic rapid growers, and the other made up of the pathogenic slow growers (188). The latter group included *M. paratuberculosis*, *M. avium*, and *M. intracellulare*. *M. leprae* and *M. bovis* were the next closest species (57, 172, 184, 188, 213).

These studies indicate that the *M. avium* group is larger and more heterogeneous than the *M. paratuberculosis* group and that the two species are more closely related genetically than the other mycobacterial species.

MAJOR IMMUNOLOGICALLY ACTIVE COMPONENTS OF *M. PARATUBERCULOSIS* AND OTHER MYCOBACTERIA

Three groups of immunogenic mycobacterial glycolipids have been analyzed in detail. One group is composed of glycopeptidolipids that include polar mycoside C. These polymers contain monoglycosylated and acylated peptides linked to oligosaccharide residues endowed with serotype specificity. Glycopeptidolipid 1 of *M. paratuberculosis* was found to be strongly immunogenic and to cross-react with the glycopeptidolipid of *M. avium* serotype 2 (28–30).

Sophisticated structural studies have been recently carried out on lipoarabinomannan (LAM), a glycolipid anchored to the membrane and inserted in the mycobacterial cell wall. The latter is made up of two interconnected polymers: the arabinogalactanmycolate complex and peptidoglycan. In addition to arabinose (mostly in the furanose form) and mannose, LAM contains glycerol, polyolphosphates, and the diacyl-glycerol esters of hexadecanoate and methyloctadecanoate (7, 139). LAM is a dominant mycobacterial immunogen that strongly reacts with the sera from patients with leprosy and tuberculosis (18, 147) and with the sera from animals with paratuberculosis (137, 193–195). LAM-based immunoassays are very sensitive but lack species specificity. Mycobacteria excrete large quantities of hydrosoluble LAM complexes that exert various effects on the immune system: they stimulate B lymphocytes, induce suppressor T cells, and modulate lymphokine production (32, 67, 154, 182). The lateral arabinofuranose chains of this polymer seem to be mainly responsible for LAM's antigenicity (139).

The best-known members of the phenolic glycolipid group are the phenolic glycolipid 1 and 2 components of *M. leprae*, which accumulate in large quantities in the tissues of patients with lepromatous leprosy. They are highly immunogenic and species specific (99, 140). Some synthetic phenolic glycolipid 1 derivatives have been synthesized and used as reagents for serological tests in human leprosy (43, 126). These types of compounds have not been explored in *M. paratuberculosis*.

Mainly, two techniques are used to screen *M. paratuberculosis* antigens: two-dimensional immunoelectrophoresis on agarose gel, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Protein antigens are fractionated under natural conditions by the former technique and under denaturing conditions by the latter technique.

Rabbit hyperimmune sera to *M. paratuberculosis* identified 44 *M. paratuberculosis* antigens in a two-dimensional immunoelectrophoretic reference system (87). Most of these antigens cross-reacted with components from other mycobacterial species. Thus, of the 44 antigens indexed, 27 reacted with anti-*M. avium* serum and 24 reacted with anti-*M. bovis* BCG serum,

but only antigen 17 was specific for *M. paratuberculosis*. The antigen complex A36, identified by this technique, is described in detail in a later section.

When similar investigations were carried out with paratuberculous animal sera, the number of antigens identified was smaller than that revealed by hyperimmunized rabbit antisera. Of the 15 main components recognized by paratuberculous bovine sera, only antigens 2 and 5 were absent from *M. avium* and *M. bovis* strains (118). In another work, seven antigens were identified by sera from paratuberculous sheep. Two of them, antigens A (31 kDa) and D (400 kDa), were used as reagents in enzyme-linked immunosorbent assays (ELISAs), but the tests lacked species specificity (20, 193). The NH_2-terminal portion of antigen A is similar to that of antigen 85 of *M. bovis* BCG, a 31-kDa fibronectin-binding protein (224, 225), and the amino acid sequence of antigen D closely resembles that of bactoferritin (cytochrome b_1) from *E. coli* (21, 193). Another *M. paratuberculosis* component, antigen 16, which is involved in iron transport, is produced in large amounts when *M. paratuberculosis* is grown in the presence of a high concentration of iron, in mycobactin-free medium (54). Unfortunately, in the various publications cited, antigens are numbered according to different reference systems, which makes comparison difficult.

SDS-PAGE fractionation and Western blot (immunoblot) analysis afford higher resolution of mycobacterial proteins than agarose electrophoresis but yield denatured fractions. When *M. paratuberculosis* sonicates were fractionated by this procedure, 20 proteins within the 34- to 84-kDa region were recognized by paratuberculous bovine sera (9, 61), and fewer components of 30 to 43 kDa were recognized by sera of infected sheep (212).

IMMUNOLOGICAL ASPECTS

The immunological picture of Johne's disease mimics that of human leprosy: in both mycobacterioses, hyperreactive and anergic forms can be recognized. At the hyperreactive pole, which is characteristic of early stages of paratuberculosis, there are strong cell-mediated immune reactions that limit the proliferation of the etiological agent. This stage of Johne's disease resembles the tuberculoid form of human leprosy. At the opposite pole is anergy, which occurs in the terminal stage of paratuberculosis and resembles the lepromatous phase of human leprosy (11, 143).

Between the two poles there is an intermediate stage corresponding to the borderline form of human leprosy, which is characterized by a progressive weakening of cell-mediated immunity and increased blood levels of antimycobacterial immunoglobulin. The resemblance between the immunological spectra of leprosy and paratuberculosis is supported by numerous observations of low humoral response in subclinical cases of Johne's disease and weak cell-mediated reactions in advanced forms (11, 40, 59, 63, 125, 159). Indeed, in the early stages of paratuberculosis, there is a high proliferative response of lymphocytes to mycobacterial antigens and a positive response to cutaneous tests with johnin or with avian sensitin. These skin tests are based on delayed-type IV hypersensitivity reactions (DHR), which mimic those elicited by lepromin in leprosy and by tuberculin in tuberculosis (40, 83, 93).

On the other hand, lymphocytes from cattle with evident clinical symptoms of paratuberculosis yield weaker responses to mitogens than those from healthy animals (11, 40, 59). The occurrence in sera of paratuberculous cattle of a soluble factor suppressing mitogen-induced transformation of normal bovine lymphocytes has been reported (59). Moreover, intraperitoneal injection of mice with large amounts of inactivated *M. paratuberculosis* induced the suppression of DHR against an unrelated antigen. This suppressive effect could be transferred by intraperitoneal inoculation of spleen and peritoneal exudate cells from *M. paratuberculosis*-injected mice. It was abolished either by treatment with complement plus anti-Thy 1-2 or anti-Lyt 2-2 monoclonal antibodies or by intraperitoneal administration of cyclophosphamide (109). These findings suggest that suppressor T cells might be partly responsible for the anergic state of advanced paratuberculosis. Suppression of T-cell responsiveness was also induced by injection of *M. bovis* cells (211) and might thus be a common feature of infection by all pathogenic mycobacteria. Finally, macrophage alterations have been recognized in advanced paratuberculosis, including the failure to inactivate phagocytosed mycobacteria, unresponsiveness to activating signals (gamma interferon), and spontaneous secretion of interleukin-1 (12, 59, 117, 234).

DIAGNOSTIC TOOLS

Because of the fecal shedding of large quantities of mycobacteria, it is crucial that infected cattle be identified to avoid spread of paratuberculosis throughout the herd. However, most of the diagnostic tools available are less than satisfactory.

Three kinds of coproanalytical procedures are used: microscopic examination of stool samples, culture, and nucleic acid probes. The success of microscopic examination of fecal matter depends on the number of bacteria present in the sample (113, 171). Positivity is low in early disease, when diagnosis would be most useful, and high in advanced cases, which are already revealed by clinical symptoms. Sensitivity is improved by replacing acid-fast staining with fluorescence microscopy, using fluorescein-labeled immunoglobulins (1) or fluorescent dyes (auramine, fluoresceine, and rhodamine). However, these procedures, which do not distinguish *M. paratuberculosis* from other mycobacteria, are of little practical use.

Culture, though more sensitive than microscopic examination of fecal samples, is a slow and exacting procedure because of the long generation time of *M. paratuberculosis*. Moreover, the decontamination step, which is required to remove the majority of organisms in the intestinal flora, may affect mycobacterial viability (64, 170). Biopsy materials and samples of milk have also been cultured for research purposes (197). Radiometric detection of *M. paratuberculosis* by culture of filter-concentrated fecal specimens was claimed to be more sensitive (>50% more positive cases) than conventional culture of sedimented bacteria on solid media (55). However, centrifugal concentration of mycobacteria from fecal samples seems to be a simpler and more sensitive coproanalytical procedure. The most advanced identification methods, based on nucleic acid probes, are described in a separate section.

Histopathological analysis of the terminal ileum and mesenteric lymph nodes has been carried out on biopsy material from paratuberculous cattle (13, 25, 116, 164). This approach, which allows rapid diagnosis, is mainly applied to examination of tissue obtained at necroscopy. The presence of mycobacterial clumps in granulomatous tissue is confirmatory (223).

Immunological procedures such as immunodiffusion, complement fixation, and immunoenzymatic (ELISA) tests are described in the serology section.

Cutaneous testing with tuberculin-like preparations (sensitins) is used as a diagnostic procedure in several countries (40, 66, 112, 124) but not in the United States. *M. avium* extracts (avian tuberculin) often replace *M. paratuberculosis* extracts (johnin), being more easily available. Although the two bacteria are genetically related (35), there are some

differences in their antigens (78). Sensitins are heterogeneous mixtures of substances inducing nonspecific inflammatory reactions and of antigens displaying extensive cross-reactions among members of the genus *Mycobacterium* (40, 78, 79). Their use for diagnostic purposes entails obvious difficulties due to the lack of species specificity. Thus, in countries where cutaneous testing for tuberculosis is compulsory (European Community), comparative cutaneous testing of positive cattle with both avian and bovine tuberculins is the rule. Cattle displaying high reactivity to bovine tuberculin and low reactivity to avian tuberculin are considered tuberculous and thus are submitted to compulsory slaughtering.

The cellular immune status in mycobacterial diseases can also be explored by some in vitro immunoassays. Since gamma interferon is considered a basic mediator of DHR, its synthesis in response to *M. paratuberculosis* preparations and components can be considered a measure of cellular immune reactivity against the mycobacteria. An enzyme immunoassay and a bioassay for gamma interferon have been comparatively analyzed in paratuberculous cattle. The former test had a higher sensitivity, being able to detect 71.8 to 93.3% of subclinical cases, a small percentage of which (16.7 to 33.3%) was detected by the latter test (17). In the diagnostic kit from IDDEX (Westbrook, Portland, Maine), the level of gamma interferon synthesized by bovine lymphocytes in response to mycobacterial antigens is measured by an ELISA, which, however, lacks species specificity since it relies on cross-reacting antigens. Lymphocyte proliferation tests for Johne's disease are based on labeled thymidine incorporation into the DNA of lymphocytes from paratuberculous cattle which are incubated in vitro with mycobacterial components. Assays of this sort have been used in research laboratories (24, 63, 69, 79, 96) and, less frequently, in diagnostic veterinary laboratory work.

A36 ANTIGEN COMPLEX OF *M. PARATUBERCULOSIS* AND TMA COMPLEXES FROM OTHER MYCOBACTERIA

As mentioned, two-dimensional immunoelectrophoresis is a powerful tool for antigen fractionation. When a sample of mycobacterial cytoplasm fractionated in one dimension migrates in a second dimension in agarose gel containing anti-mycobacterial immunoglobulins, a series of precipitation arcs of antigen-antibody complexes are formed. After staining, antigens are numbered according to reference systems such as that of Closs et al. for *M. bovis* (46): antigen 60 (A60) is the major and least mobile component of *M. bovis* BCG. All mycobacteria (and some organisms of the CMN group) yield immunoelectrophoretic patterns similar to that of *M. bovis*. The term thermostable macromolecular antigen (TMA) complexes has been proposed for the A60-like components of various mycobacterial species (A7 in *M. leprae* and A36 in *M. paratuberculosis*) (Table 3). The term TMA highlights the unique properties of these complexes: thermostability and high molecular weight ($>10^6$) (46, 47, 49, 87, 92). The pattern of A36 from *M. paratuberculosis* is shown in Fig. 3.

Chemical analysis of TMA complexes revealed the presence of polysaccharides, proteins, and lipids. The branched glycan core and lipid components have negligible antigenicity (23). Upon dissociation with detergents and fractionation by gel electrophoresis (SDS-PAGE), TMA complexes yield 30 major protein components of 20 to 70 kDa, mainly responsible for the antigenicity of these complexes. Proteins within the 25- to 45-kDa region seem to be immunodominant in mycobacterioses (50, 61, 79).

TABLE 3. TMA complexes of pathogenic mycobacteria

Microorganism	Mycobacteriosis	TMA	
		Name	Antigenicity[a]
M. bovis	Tuberculosis	A60	High
M. tuberculosis	Tuberculosis	A60	High
M. avium	Nontuberculous mycobacteriosis		High
M. leprae	Leprosy	A7	High
M. paratuberculosis	Johne's disease (Crohn's disease)	A36	High

[a] As determined by the level of immunoglobulins present in patients and infected animals.

TMA complexes elicit humoral and cellular immune reactions and are immunodominant in mycobacterioses, as shown by the observation that a large proportion of anti-mycobacterial immunoglobulin in the cerebrospinal fluid of tuberculous meningitis patients and in the blood of pulmonary tuberculosis cases is directed against A60 (14, 16, 31, 49, 50, 183). Sensitins, bacterial extracts used for cutaneous testing, contain TMA components that are partly responsible for the elicited DHR (79, 92). TMA-based immunoassays have thus been developed to explore the humoral and cellular immune reactions in infected hosts (48, 72). An A36-based serological test distinguished between healthy and paratuberculous cattle at all stages of the disease (61), and cutaneous testing with A36 in sensitized rabbits yielded DHR comparable to those elicited by johnin and avian tuberculin (79).

However, TMA-based immunoassays lack species specificity, owing to the pronounced cross-reactivity among complexes from different mycobacterial species (61, 79). The specificity lacking at the level of whole complexes was then sought at the level of their protein components. In practice, proteins used as reagents for immunoassay ought to possess B-lymphocyte-specific epitopes (serological assay) or T-cell-specific epitopes (cutaneous testing). To identify specific B-cell epitopes in A36 proteins, the reactivity of A36 antiserum, either preabsorbed or not absorbed with lysates of different mycobacteria, was compared by Western blot (Fig. 4). Five proteins of the *M.*

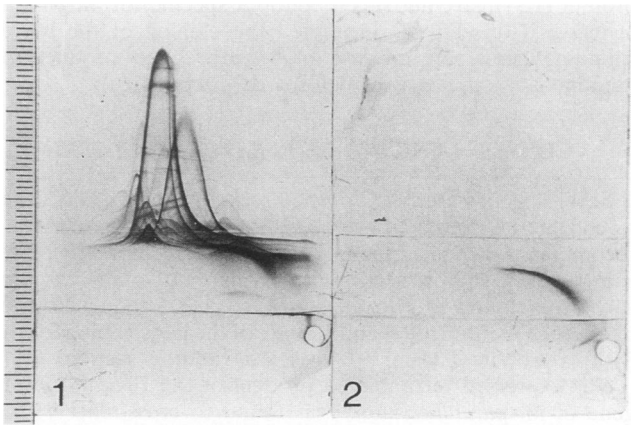

FIG. 3. Two-dimensional immunoelectrophoresis of *M. paratuberculosis* sonicate (1) and A36 complex (2). Migration: leftwards (first dimension) and upwards (second dimension). Rabbit antiserum directed against *M. paratuberculosis* sonicate was added to the second-dimension agarose gel. Reprinted from reference 61 with permission of the publisher.

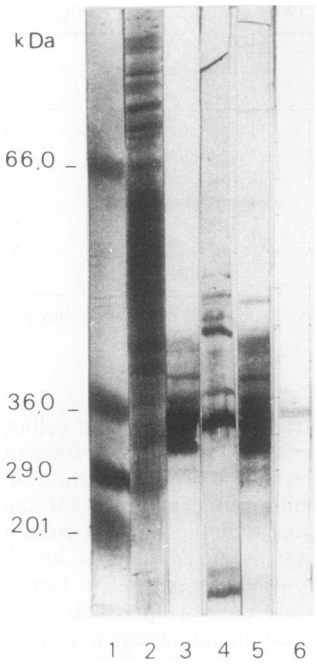

FIG. 4. Identification of immunodominant A36 proteins containing species-specific B-cell epitopes. Electrophoresed A36 proteins (SDS-PAGE), after blotting, were either stained with India ink (lane 2) or incubated with paratuberculous cattle sera (lanes 3 to 6) that were either preabsorbed (lane 6) or not (lanes 3 to 5) with a mixture of mycobacterial sonicates (*M. avium*, *M. bovis*, and *M. phlei*). Lanes 3 to 5 correspond, respectively, to stages I, II, and III. Bound primary immunoglobulins were revealed with peroxidase-labelled secondary immunoglobulin. Molecular mass markers are noted in lane 1.

TABLE 4. Antigenicity and specificity of some proteins of the A36 complex[a]

Protein (kDa)[b]	Antigenicity[c]				Specificity[d]		
	Rabbit serum[e]	Infected bovine serum[f]			*M. avium*	*M. bovis*	*M. phlei*
		I	II	III			
74	++	−	−	+	−	−	−
52	+	−	−	−	−	−	−
41	+	+	+	+	−	−	−
40	+++	+	+	+	−	−	−
37	++	++	−	++	−	−	−
35	+	++	++	++	−	−	+
34	+++	+++	+++	+++	+	+	+
31	++	+++	−	+++	−	+	+
29	+++	−	−	+	−	−	−
23	+++	−	+	−	−	+	+
22	+	−	++	−	−	−	+

[a] From reference 61 with permission of the publisher.
[b] A36 components were fractionated by acrylamide gel electrophoresis (SDS-PAGE) and identified by immunoblotting.
[c] The degree of antigenicity was evaluated from the immunoblot intensity as revealed by the corresponding sera: lack of (−) or low (+), medium (++), or high (+++) antigenicity.
[d] The presence (+) or absence (−) in A36 proteins of epitopes specific towards three mycobacteria were tested with an anti-A36 rabbit serum preabsorbed on lysates of the indicated bacteria.
[e] Recognition level of A36 proteins by an anti-A36 rabbit serum.
[f] Sera from cattle at different stages of Johne's disease: I, asymptomatic, nonexcretory; II, asymptomatic, excretory; III, symptomatic, excretory forms.

paratuberculosis A36 complex (all within the 20- to 35-kDa region) contain epitopes specific for *M. phlei*, three contain epitopes specific for *M. bovis*, and only one protein (P34) contains epitopes specific for *M. avium* (Table 4) (61). The parallel between this immunological specificity and DNA relatedness is obvious: the more genetically related two organisms are, the fewer the proteins endowed with specific epitopes. The identification of a 34-kDa immunodominant protein (P34) carrying B-cell epitopes specific for *M. paratuberculosis* (61) (Fig. 4) prompted the cloning of the corresponding gene and the use of its expression product for immunoassays in paratuberculosis (described later).

CLONED GENES OF *M. PARATUBERCULOSIS*

Several genes from *M. tuberculosis*, *M. leprae*, and other mycobacterial genomes have been cloned, primarily to prepare recombinant proteins in amounts sufficient for immunological assays (232). In this section, we focus on the few cases of successful cloning of *M. paratuberculosis* DNA sequences (Table 5). The identification and cloning of the gene coding for the 34-kDa protein (P34) are discussed in the next section.

The expressed recombinant product of the 70-kDa protein gene of *M. paratuberculosis* was found to cross-react with a monoclonal antibody directed against the 71-kDa component of *M. tuberculosis* (190). The latter antigen is one of the heat shock proteins present in all bacteria and which have similar compositions, hence, the strong cross-reactions with even remote species. Indeed, the genes of heat shock proteins from both prokaryotes and eukaryotes are, to a large extent, homologous: they contain sequences that are highly conserved in the evolution of all living beings (127). For instance, the 70-kDa protein of *M. paratuberculosis* shows a 58% relatedness with *E. coli* DNA K, a 50% relatedness with the heat shock protein of *Drosophila melanogaster*, and a 49% relatedness with that of *Homo sapiens*. Some stretches of the 70-kDa protein of *M. paratuberculosis* are more than 90% related to the 70-kDa protein of *M. leprae* and the 71-kDa protein of *M. tuberculosis*. The carboxyl termini seem to be the most specific portions of these molecules (190).

The 65-kDa protein of *M. bovis*, another heat shock protein, is present in the cytoplasm in relatively large amounts and is highly antigenic. Proteins of comparable size and function have been found in all mycobacteria. The gene coding for the 65-kDa heat shock protein of *M. bovis* has been entirely sequenced (200). With two primers complementary to each strand of this gene, segments of the genomes of five other mycobacterial species, including *M. paratuberculosis*, have been amplified by PCR and shown to bear strikingly similar sequences (91).

The gene product (p43) of the insertion sequence IS*900* has been produced in a recombinant strain of *E. coli*, using an exogenous promoter. An antiserum to p43 identified a 28-kDa processed product expressed at a level that is unusually high for classical transposases. If it were well recognized by bovine antisera, this recombinant protein could have a potential use in the diagnosis of Johne's disease (209).

Two promoters of unknown genes from *M. paratuberculosis* have been cloned. One induced the transcription of the promoterless *galK* gene in a promoter-probe plasmid (201). The cloned DNA segment contained the expected −35 and −10 promoter regions, and the Shine-Dalgarno ribosome binding sequence, preceding the ATG start codon of the *galK* gene. The other promoter sequence (P_{AN}), adjacent to the 3′ end of the IS*900* insertion element, was isolated from *M. paratuberculosis* and sequenced (158). The P_{AN} promoter was

TABLE 5. Proteins of *M. paratuberculosis* with known sequences

M. paratuberculosis protein (kDa)	Similar proteins (kDa) described in:			Characteristic	Reference(s)
	M. leprae	*M. bovis*	*M. tuberculosis*		
70	70	70	71	Heat shock	190, 232
65[a]	65	64	65	Heat shock	61, 91, 232
43	No	No	No	Transposase	85
34	No	No	No	Cell wall associated	62, 80

[a] Partly sequenced.

shown to control the expression of the reporter gene *lacZ* in *M. smegmatis* and *M. bovis*, hence, its possible use for the expression of heterologous genes in the BCG vaccine strain (158).

THE 34-kDa PROTEIN: IDENTIFICATION AND CLONING OF THE GENE

As mentioned, the 34-kDa protein (P34) of the A36 complex is immunodominant and contains B-cell species-specific epitopes (61) (Table 4). Three portions of the gene coding for P34 have been isolated from a λgt11 genomic library of *M. paratuberculosis*. The expression product of one of them (a362) was a 13.6-kDa polypeptide containing B-cell epitopes present in all tested *M. paratuberculosis* strains but not in other mycobacterial species (62). This recombinant polypeptide, which contained the carboxyl-terminal portion of P34, was used as a reagent for an ELISA for paratuberculosis. Infected cattle at all stages of the disease were correctly diagnosed (62, 80) (Fig. 5).

A portion of the cloned a362 DNA was then used as a probe to identify the 5′ end of the P34 gene on a Southern blot containing restriction fragments of the *M. paratuberculosis*

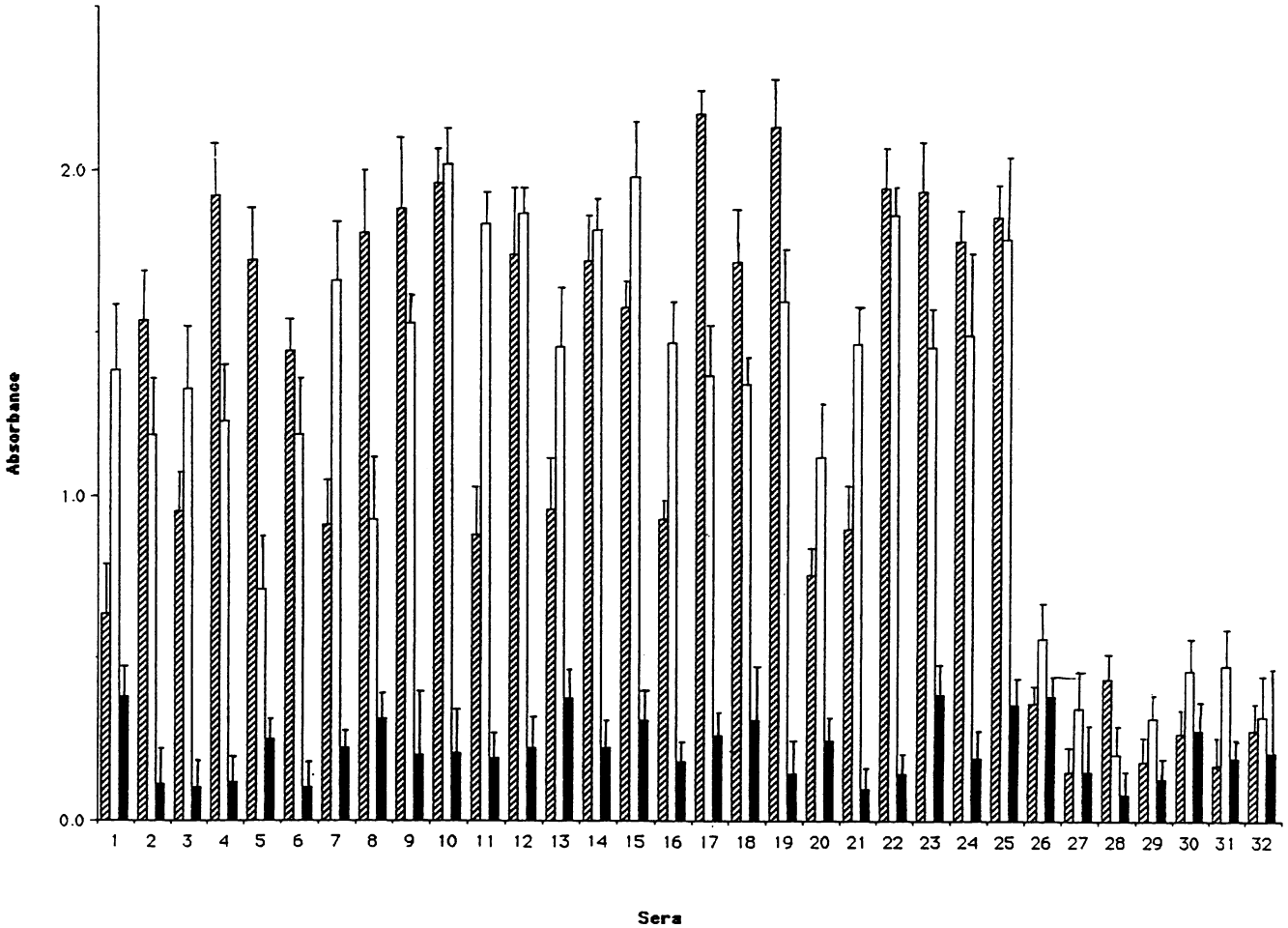

FIG. 5. Serological analysis of paratuberculous cattle. ELISAs were conducted on paratuberculous (samples 1 to 25) animals at stages I, II, and III and on healthy (samples 26 to 32) cattle. The antigens were the A36 complex (▨), a lysate of the recombinant *E. coli* MC1061 strain coding for the carboxy-terminal portion of the P34 protein (a362 polypeptide) (□), and a control of nonrecombinant *E. coli* MC1061 extract (■). Reprinted from reference 62 with permission of the publisher.

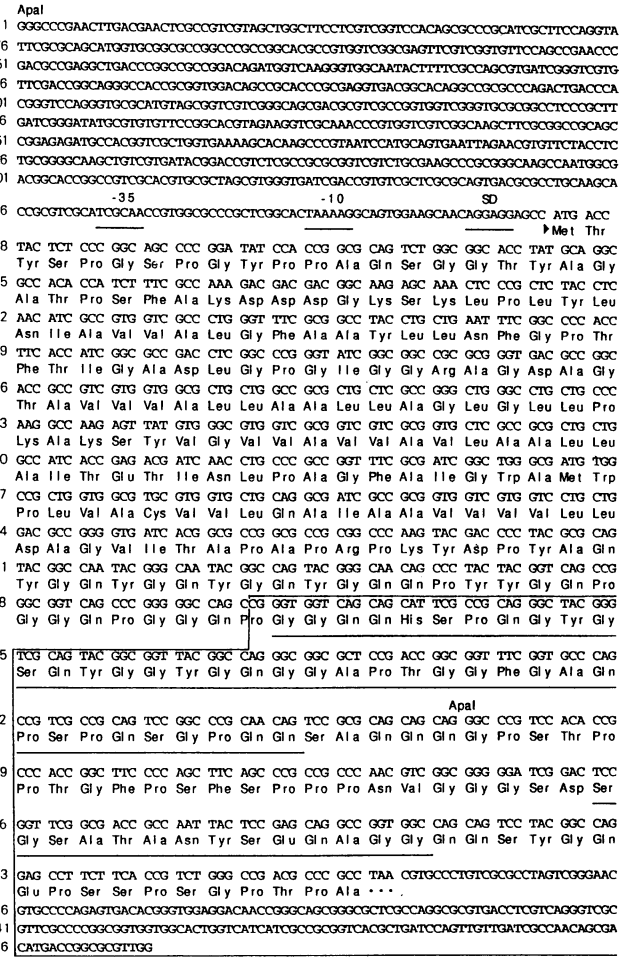

FIG. 6. Nucleotide sequence of the region containing the gene encoding the 34-kDa protein of *M. paratuberculosis*. The sequence of the gene, of its translation product, and of the putative promoter region (−10, −35) and the Shine-Dalgarno sequence (SD) are shown. The translation initiation codon is indicated by an arrow, and the stop codon is shown by dots. The amino acids confirmed by internal sequencing of the recombinant polypeptide are underlined. The sequence corresponding to the DNA fragment inserted in clone a362 is boxed. Reprinted from reference 80 with permission of the publisher.

genome. The DNA fragment of 1,500 bp thus detected was shown to contain the amino-terminal part of the P34 gene. The full sequence of the P34 gene is displayed in Fig. 6; a DNA bank search revealed its uniqueness. Putative regulatory sequences of the gene (start and stop codons, Shine-Dalgarno sequence, and promoter segments) have been identified. The high G+C content of the P34 gene (70%) agrees with that of the *M. paratuberculosis* genome (67%). P34 displayed a surprising hydrophilic profile: a strong hydrophobic portion corresponding to the NH_2 moiety of the molecule was followed by a highly hydrophilic part, the COOH moiety (80) (Fig. 7). The hydrophilic region is exposed at the outer cell surface, as shown by immune electron microscopy, while the hydrophobic portion is inserted in the envelope (62).

SEROLOGICAL ANALYSIS IN PARATUBERCULOSIS

In principle, proliferation of *M. paratuberculosis* in infected cattle induces the synthesis of specific immunoglobulins, serum

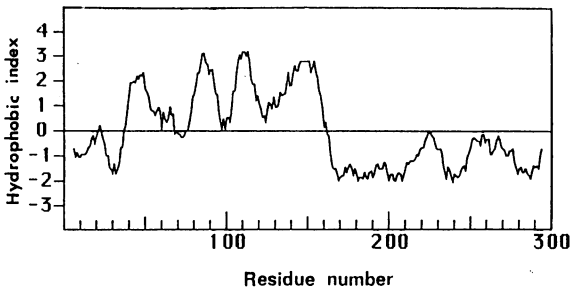

FIG. 7. Kyte-Doolittle hydrophobicity profile of the 34-kDa protein of *M. paratuberculosis*. This diagram, drawn with the DNA Strider computer program according to Kyte and Doolittle, identifies a strongly hydrophobic NH_2 moiety portion (amino acids 40 to 160) and a highly hydrophilic COOH terminal part (amino acids 161 to 298) exposed at the cell surface. Reprinted from reference 80 with permission of the publisher.

titer being proportional to the foreign antigen load. Thus, serological analysis would furnish both proof of infection (diagnosis) and information on disease evolution (prognosis). However, unless the antigen used for the test is specific for the chosen organism, diagnosis is impaired by cross-reactions with related bacteria of the CMN group. This is a serious obstacle because of the close genetic relatedness between *M. paratuberculosis* and other mycobacteria, particularly strains of the *M. avium* complex (30, 77, 135, 152, 165, 166). Moreover, the immunoglobulin titer is low in paratuberculous cattle at certain stages of the disease.

Complement fixation (CF) using crude mycobacterial preparations was widely used in the past. In spite of its lack of species specificity and poor sensitivity, CF is still the reference test for cattle export (51, 77, 136, 165, 227). The crude carbohydrate antigen used in the CF test in Canada differs from the antigen used in the United States (65, 223). A commercial CF assay (Parafix; Iffa-Mérieux, Lyon, France) is available in Europe. The agarose gel immunodiffusion (AGID) test has drawbacks similar to those of the CF test. The reagent of a commercial AGID immunoassay (Rapid Johne's Test; Immucell Corp., Portland, Maine) is whole-cell cytoplasm of *M. paratuberculosis* strain 18, recently identified as *M. avium* serotype 2. AGID is essentially used to confirm the diagnosis of paratuberculosis in an animal with suspected clinical symptoms, since this test becomes positive in cattle at advanced stages of the disease (178–181). An AGID test based on the D antigen is currently used in Canada (137). The performance of another immunoassay, the indirect immunofluorescence test, is comparable to that of CF (1, 74–76).

The sensitivity of the enzyme immunoassays is very high because of the amplification of the measured signal. In these tests, antimycobacterial immunoglobulins are captured by a selected antigen, and the antibody-antigen complex is revealed by a second antibody coupled to an enzyme that catalyzes a reaction which is measured spectrophotometrically. Some ELISAs incorporating crude mycobacterial extracts or partly purified antigens have been developed (2, 56, 108, 152, 229). To avoid the large number of false-positive results observed, a preabsorption step with *M. phlei* was introduced to remove some cross-reacting antibodies (8, 148, 149, 152, 230). Two such tests are commercially available: Allied ELISA (Allied Monitor Inc., Fayette, Mo.) and CSL-ELISA (Johne's absorbed EIA; Commonwealth Serum Laboratories, Melbourne, Australia [also distributed by Idexx Corp., Portland, Maine]). The former assay is based on a partly purified antigen prepa-

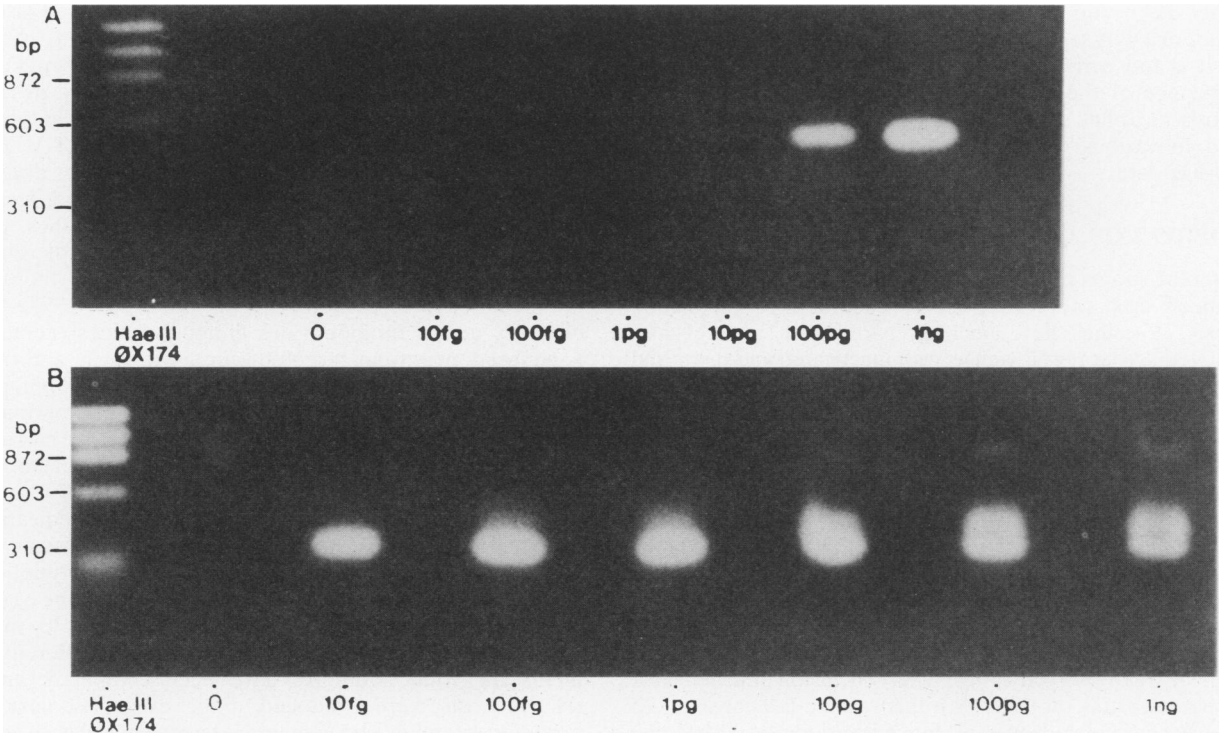

FIG. 8. Identification of amplified *M. paratuberculosis* DNA. Primary (A) and secondary nested (B) PCR-amplified *M. paratuberculosis* DNA samples, which were obtained with primers specific for the insertion sequence IS*900*, were electrophoresed and stained with ethidium bromide. The second amplification step leads to a 10^4-fold increase in sensitivity. Reprinted from reference 133 with permission of the publisher.

ration of *M. paratuberculosis* strain 18, whereas the latter uses whole cytoplasm of strain VRI 316:102-2. Recently, a LAM-based assay (LAM-ELISA) has been submitted to field trials. This assay lacks species specificity due to cross-reactivity among LAM preparations from different mycobacterial and corynebacterial species (137, 138). The dot immunobinding assay uses whole cytoplasm or partly purified antigens of *M. paratuberculosis* ATCC 19698 as reagents and yields only qualitative results (infected or uninfected cattle) (210, 228).

The levels of sensitivity of the CF test (the standard CF test used in the United States), the AGID test (Rapid Johne's test), Allied ELISA, and CSL-ELISA were claimed to be 38.4, 26.6, 58.8, and 43.4%, respectively, for all subclinical cases of paratuberculosis (stages I and II) and: 14.5, 4.3, 47.8, and 24.6% for stage I and 54.6, 40.7, 65.7, and 56.5% for stage II of the disease. Specificity values were 99, 100, 95, and 99%, respectively (185, 186).

An ELISA based on the previously described A36 antigen was able to correctly detect infected cattle at all stages of the disease (61, 62) (Fig. 5). After identification of species-specific B-cell epitopes in the 34-kDa protein (P34) component of A36, the carboxy-terminal portion of this protein (a362), which contains only species-specific epitopes, was used as the reagent for a serological assay (62). The a362 ELISA was negative with tuberculous cattle and positive with paratuberculous cattle (Fig. 5) (62, 217).

NUCLEIC ACID PROBES

Preparation of a nucleic acid probe requires the identification of a species-specific DNA segment, which is then cloned and possibly sequenced. The probe, either labeled DNA or RNA complementary to the identified genomic segment, is then hybridized with DNA released by lysis of unknown isolates. To circumvent the paucity of the infectious agent in biological samples, released DNA is amplified by PCR prior to probe hybridization. The PCR amplification of *M. paratuberculosis* DNA using IS*900* sequences (134) is illustrated in Fig. 8. One hundred picograms of DNA could be detected after a first step of amplification, whereas a secondary nested amplification step led to a 10^4-fold increase in sensitivity.

To date, only two species-specific DNA fragments have been identified in the *M. paratuberculosis* genome. Probes have been developed from them for the identification of *M. paratuberculosis* in stool samples (155, 157, 167, 218). One probe corresponds to a part of the IS*900* insertion sequence, which is present in multiple copies in the *M. paratuberculosis* genome. Labeled IS*900* probe was claimed to identify the DNA from 10^2 cells, after amplification by PCR. An IS*900*-based diagnostic kit, with nonisotopically labeled reagents, is now commercially available (Idexx Corp.). In studies performed in a paratuberculosis control program, this test had an 89% specificity but only a 13% sensitivity (214).

The other probe (F57) was prepared in our laboratory by cloning DNA segments from *M. paratuberculosis* in a transcription vector (167). Recombinant clone F57 proved to be specific for the Johne's disease agent. It failed to hybridize with the genomes of all mycobacteria tested, including *M. avium*, and it recognized all of the tested *M. paratuberculosis* strains isolated from paratuberculous animals and humans with Crohn's disease. Sequencing of the F57 insert yielded a 620-bp segment with a G+C content of 58.9%. Comparison with EMBL and UGEN banks revealed that the F57 sequence is unlike any other known gene, including IS*900* (167). F57 is now being used as a diagnostic tool for both paratuberculous cattle and patients with Crohn's disease.

Other diagnostic probes with non-species-specific *M. paratuberculosis* DNA segments have also been described (101, 213, 214). It is too early to draw definitive conclusions as to the performance of these genetically engineered probes for the diagnosis of Johne's disease. Large clinical trials being conducted in various countries are expected to furnish more conclusive data.

PROPHYLAXIS AND THERAPY OF JOHNE'S DISEASE

Different strains of *M. paratuberculosis* and its components have been used to prevent the spread of infection among livestock. Vaccines have been prepared with virulent and attenuated live strains as well as with inactivated and disrupted organisms (40, 175).

The first vaccine for paratuberculosis was devised by Vallée and Rinjard in 1926. It consisted of a live virulent *M. paratuberculosis* strain suspended in liquid paraffin and olive oil to which pumice powder was added. This vaccine, which was inoculated subcutaneously, found wide application in France, apparently with success (140, 192). An attenuated strain of *M. paratuberculosis*, obtained after prolonged propagation in mycobactin-free medium, was used for vaccination in field trials conducted in France and England. When calves from infected herds were vaccinated within the first month of life, a protective effect was observed (192). The conclusion reached in a retrospective study on trials with this vaccine (all calves of 23 herds were vaccinated over prolonged periods) was that disease could be eradicated after an average period of 4 years (226). A commercial vaccine made of live attenuated *M. paratuberculosis* 316F suspended in an oil-based adjuvant (Neoparasec; Rhone-Mérieux) has been used extensively in France in field trials. After vaccination by subcutaneous injection, the animals produced long-lasting antimycobacterial antibodies and hence had positive reactions in the serological assays currently used for diagnostic purposes and export requirements (95).

The protective effects of vaccine preparations containing inactivated mycobacteria have been compared. A vaccine prepared with a virulent *M. paratuberculosis* strain afforded higher protection than that made with an attenuated strain (adapted to grow in mycobactin-free medium) (121, 123). The prophylactic practice in the United States is to restrict the injection of inactivated vaccines to calves less than 1 month old. Vaccinated cattle older than 1 year cannot be distinguished from infected cattle by means of the lymphocyte proliferation assay (96).

In vaccinated herds, there was a decrease in the number of both infected animals and those with advanced stages of the disease. A reduction in the percentage of shedders and in the quantity of excreted mycobacteria was also observed (40). However, the efficacy of vaccination should be viewed in light of environmental factors. Isolation of newborn animals immediately after birth and replacement of suckling with artificial nursing are probably as efficient prophylactic interventions as vaccination itself (122).

In some cases, vaccination produces cutaneous lesions (inflammation, swelling, and nodules) at the injection site (40). Moreover, inoculation of whole mycobacteria and crude extracts induces delayed hypersensitivity towards the sensitins from related species and positivity in serologic diagnostic tests (40, 95, 187). The use of purified proteins able to induce both cellular immune reactivity and protection from disease would be a more rational prophylactic approach. Ideally, different proteins should be used for vaccination and for diagnostic tests.

Treatment for paratuberculosis is rarely indicated or undertaken; however, it may be considered for animals of exceptional genetic value or companion animals (191). Drugs against *M. paratuberculosis* have been tested in vitro and in vivo. In short, most antimycobacterial agents, including antimetabolites and antibiotics, inhibit the in vitro growth of *M. paratuberculosis*. Antimetabolites include antituberculous drugs such as cycloserine, ethambutol, ethionamide, isoniazid, *para*-aminosalicylic acid, thiocarlide and thiosemicarbazones, phenazines, pyrizinamide, and antileprosy drugs such as dapsone and other sulfones. There are several reports on the use of antimetabolites in paratuberculosis (168, 169, 177). Among clinically active antibiotics are aminoglycosides (gentamicin, kanamycin, neomycin, streptomycin, tobramycin, and others), capreomycin, rifabutin, rifampin, and viomycin. Riminophenazine inhibited the development of the disease in experimentally infected mice and sheep (73). These antibiotics are more active on *M. tuberculosis* than on other pathogenic mycobacteria such as *M. leprae* and the *M. avium* complex (37, 38, 40).

Treatment of paratuberculosis requires daily medication for extended periods and results in palliation of the disease rather than a definitive cure (191). Clofazimine is the antibiotic most widely used for treatment of cattle (97, 144). In some cases, the addition of anti-inflammatory drugs appeared to improve symptomatology (141). In a recent work (97), different treatments for Johne's disease have been compared, and the relative costs were evaluated. Four drugs (isoniazid, clofazamine, rifampin, and gentamicin) were chosen as being both effective in vitro and in vivo and safe. It should be noted that none of the drugs used was approved for use in food animals in the United States and that these treatments did not eliminate infection, as shown by consistently positive fecal cultures and biopsies during therapeutic periods.

To conclude, in spite of the promising results of in vitro susceptibility tests, chemotherapy of paratuberculosis has been disappointing. The discrepancy between the in vitro and in vivo results is due to the inaccessibility of mycobacteria, which multiply within phagocytes and other cells of the intestinal mucosa and Peyer's patches. Antimycobacterial drugs penetrate such intracellular targets with difficulty.

CROHN'S DISEASE: RELATIONSHIP WITH PARATUBERCULOSIS

Although there had been earlier reports of chronic inflammatory bowel diseases, it was only in 1932 that B. Crohn provided the first description of the chronical granulomatous ileitis now known as Crohn's disease. Almost three decades later, a complete picture of the syndrome was established. It has been shown that every organ of the digestive tract may be involved and that, in some cases, there are alterations of the locomotor system and of the skin. It is still difficult to differentiate clinically Crohn's disease from such chronic gut infections as ulcerative colitis and intestinal tuberculosis (the inflammatory bowel diseases group). The basic clinical symptoms common to all of these disorders are enteralgia, diarrhea, and vomiting. Prolonged constipation may cause intestinal obstruction, and surgery is required to remove the obstructed ileum. Thus, the clinical symptoms of Crohn's disease mimic somewhat those of Johne's disease (34).

Histopathological analysis of biopsy material frequently shows diffuse granulomas and lymph node alterations resembling those of bovine paratuberculosis (176).

Acid-fast bacteria were rarely found in granulomas from Crohn's patients, and even the more sensitive immunohistochemical methods yielded negative results in most cases (110,

215). However, prolonged culture of biopsy specimens yielded three types of colonies: (i) acid-fast *M. paratuberculosis*; (ii) non-acid-fast, protoplast-like, wall-deficient organisms that eventually converted into acid-fast cells; and (iii) other mycobacterial species (26, 33, 34, 41, 81, 129, 203). More recently, PCR was used to amplify the DNA of organisms in biopsy material from patients with Crohn's disease: identification was made by probe hybridization. The probe was a portion of the *M. paratuberculosis* insertion sequence IS*900* (85, 94, 132, 155, 157, 219). By this approach, *M. paratuberculosis* was identified in biopsy specimens from 65% of Crohn's disease patients compared with 4.3% of ulcerative colitis cases, and 12.5% of healthy persons (173).

The problem of etiology was also approached by exploring the humoral immune status of Crohn's disease patients. Serological assays based on antigenic preparations of lipids (111) and glycopeptidolipids (42) and on antigen complex A60 (133) yielded negative results. While in these studies immunoglobulin G (IgG) was essentially analyzed, other studies disclosed significantly higher titers of antimycobacterial IgA in Crohn's disease patients than in ulcerative colitis cases and healthy controls (68, 84, 189, 220). In a recent study with a species-specific ELISA (using the cited a362 recombinant polypeptide), significant differences in titers between sera from Crohn's disease patients and control sera were observed for both IgG and IgA. Higher titers of anti-a362 IgA than IgG were observed in 36% of Crohn's disease patients (216).

Chemotherapeutic approaches to Crohn's disease have been unsuccessful. Antimetabolites (dapsone, ethambutol, isoniazid, and sulfasalazine) and antibiotics (rifampin, streptomycin, and viomycin) proved to be almost ineffective. Some improvement was obtained only when chemotherapy was given in combination with certain anti-inflammatory steroidal drugs (cortisone, prednisolone, and methotrexate). The failure of the chemotherapeutic approach can be ascribed to the intracellular location of mycobacteria and to the peculiar features of the digestive apparatus (intestinal tuberculosis is far less susceptible to therapy than the pulmonary and pleural forms of the disease) (34).

CONCLUSIONS AND PERSPECTIVES

The etiological agent of Johne's disease is one of the lesser known pathogenic bacteria. The slow rate of growth and the complex requirements for axenic proliferation are undoubtedly at the origin of our limited knowledge of the topic.

Composition, properties, and the metabolism of different polymers (glycolipids, polysaccharides, and proteins) of *M. paratuberculosis* are to be further defined. Investigations of this kind are expected to identify components of medical value (specific antigens and immunomodulators) and to contribute to the development of numerical taxonomy.

Gene cloning and sequencing will presumably reveal similarities and differences of components from *M. paratuberculosis* and other mycobacteria. In turn, the purification of different antigens would promote the development of more specific and more sensitive assays for the diagnosis, prognosis, and prophylaxis of Johne's disease.

The development of new diagnostic tests (probes) and immunoassays (ELISA) will help to identify infected animals and thus prevent propagation of paratuberculosis. The IS*900* probe provided by McFadden and coworkers (134) and the two specific tests developed in our laboratory, the F57 probe and the a362 ELISA, can be considered new effective diagnostic tools for experimental and clinical studies.

It is only through the convergence of different approaches that the control of Johne's disease (which is perhaps the most diffused infectious disease of domestic animals) can be envisaged and its eradication successfully planned.

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