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Analysis of Culture Filtrate and Cell Wall-Associated Antigens of *Mycobacterium paratuberculosis* with Monoclonal Antibodies

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Proteins secreted by *Mycobacterium* species have been suggested as major immune targets in the early phase of infection. In this study, we sought to identify specific antigens in culture filtrates and in soluble cell extracts of *Mycobacterium paratuberculosis*. The release of antigens into the culture medium during growth of the bacilli and the distribution of specific epitopes within the *Mycobacterium* species were investigated by immunoblot analysis with monoclonal antibodies (MAbs) raised against *M. paratuberculosis* antigens. MAb B6A interacted with a cellular antigen with an apparent molecular mass of 34.5 kDa in lysates of *M. paratuberculosis*. MAb B6A did not interact with lysates from any other mycobacterial species, suggesting recognition of an *M. paratuberculosis* species-specific epitope. MAb FL1-A1 reacted with an antigen of 44.3 kDa in *M. paratuberculosis* and a 9-kDa antigen in *Mycobacterium kansasii*. MAb PII-B1 reacted with concanavalin A (ConA)-binding cellular and filtrate molecules of *M. paratuberculosis* and with lysates of *Mycobacterium kansasii* and *Mycobacterium avium* 18. The affinity-purified glycosylated antigens migrated as a diffuse band of between 35 and 45.6 kDa and reacted strongly with ovine and bovine paratuberculosis serum and polyclonal serum against *M. tuberculosis* lipoarabinomannan antigens. These glycoconjugates were the earliest antigens detected in culture filtrates of *M. paratuberculosis*. Deglycosylation of the ConA-binding molecules with α-mannosidase enzyme abolished the reaction with MAb PII-B1 and with bovine but not ovine paratuberculosis serum, suggesting selective immunogenicity in the different animal species.

Mycobacterium paratuberculosis is the causative agent of Johne's disease or paratuberculosis, a chronic granulomatous enteritis of ruminants worldwide (11, 33). Although it has been recognized for over 80 years, Johne's disease remains an economically important disease of cattle, sheep, and goats. Animals with advanced disease shed large numbers of *M. paratuberculosis* in feces, but only some of these animals show clinical symptoms of paratuberculosis (11). A combination of early diagnosis of Johne's disease and elimination of animals in the asymptomatic preshedding stage remains the most effective strategy in limiting spread of the disease. *M. paratuberculosis* may also be a human pathogen. The bacilli has been isolated from intestinal tissues of human patients with Crohn's disease, a chronic inflammatory enteritis of unknown etiology with pathology similar to that of Johne's disease (9, 46). It is suggested that the clinical symptoms of Johne's disease, and possibly Crohn's disease, result from hypersensitivity-type responses (4, 33) and autoimmunity-induced responses against cellular antigens of *M. paratuberculosis* (30, 38). Therefore, it is of interest to identify and characterize specific antigens of *M. paratuberculosis* and to examine the role of these molecules as modifiers of cellular and humoral immunity in infected hosts.

Diagnostic methods for paratuberculosis rely on the ability of the test to detect infected animals and to discriminate between *M. paratuberculosis* and related species of mycobacteria (9, 13, 21, 27, 29, 42, 48). Animals in the early stages of infection do not shed bacilli in feces; hence, diagnosis of Johne's disease by direct bacterial culture or by use of DNA techniques may have limited application (31). The specificity and reliability of serodiagnostic tests are determined by the preparations of mycobacterial antigens employed (11, 13). *Mycobacterium* species express cellular components containing common or broadly cross-reactive antigenic determinants. Thus, serum antibodies from animals with paratuberculosis reacted extensively with antigen preparations from different mycobacterial species (11, 12, 25, 48). The identification of well-characterized and species-specific components of *M. paratuberculosis* would provide the means to improve the specificity and sensitivity of immunodiagnostic assays for Johne's disease. De Kesel et al. (17, 18) and Gilot et al. (26) identified a novel 34-kDa protein and characterized a peptide bearing a species-specific epitope of *M. paratuberculosis*. Serodiagnosis with this peptide permitted diagnosis of Johne's disease in infected animals at all stages of the disease (48). Recent studies by White et al. (49) showed that there were differences in the amounts and distribution of intracellular and extracellular antigens among different isolates of *M. paratuberculosis*. It remains essential to examine the best combination of well-characterized, species-specific antigens and epitopes of *M. paratuberculosis* for immunodiagnosis of Johne's disease caused by all isolates of this pathogen, for improved detection of *M. paratuberculosis*-specific antibodies and epitopes in Crohn's disease sera, and to examine the role of the bacilli in the pathogenesis of Johne's and Crohn's diseases.

Accordingly, we generated monoclonal antibodies (MAbs) against *M. paratuberculosis* antigens and used these MAbs to study the distribution of specific epitopes in culture filtrates and cellular extracts of *M. paratuberculosis* and among different *Mycobacterium* species. In this study, we have identified antigens expressing *M. paratuberculosis*-specific epitopes, broadly cross-reactive epitopes, and epitopes expressed in a limited number of the mycobacterial species examined. We report that *M. paratuberculosis* releases substantial amounts of concanavalin A (ConA)-binding glycosylated molecules into the medium during growth.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. paratuberculosis* ATCC 19698 (type strain) and *Mycobacterium kansasii* ATCC 35775 were obtained from the American Type Culture Collection (Rockville, Md.). *Mycobacterium smegmatis* mc^2155 was kindly supplied by W. R. Jacobs, Jr. (Albert Einstein College, Bronx, N.Y.). *Mycobacterium phlei* 425 and *Mycobacterium aurum* CIPT 1210005 (type strain) were from the microbiology culture collection (Department of Microbiology, University of Guelph, Guelph, Ontario, Canada). *Mycobacterium fortuitum* was a clinical isolate originally isolated by S. Rosendal (University of Guelph). *M. paratuberculosis* and *M. kansasii* were grown in Middlebrook 7H9 broth containing 0.5% (vol/vol) glycerol, 0.025% Triton WR1339, chloramphenicol, penicillin, and 10% (vol/vol) OADC enrichment (Difco Laboratories, Detroit, Mich.). Culture medium for *M. paratuberculosis* was also supplemented with Mycobactin J (2 μg/ml), and the cells were harvested at between 12 and 16 weeks of culture. *M. phlei*, *M. fortuitum*, *M. smegmatis*, and *M. aurum* were grown in Middlebrook 7H9 broth supplemented with 0.5% (vol/vol) nutrient broth powder (Difco) and either 0.5 or 0.05% (vol/vol) Tween 80 and harvested at an optical density at 600 nm of approximately 0.8. All cultures were incubated at 37°C without agitation and harvested by centrifugation (8,000 × *g* for 10 min). *Mycobacterium avium* 18 (formerly *M. avium paratuberculosis* 18) (10) antigens were obtained from Allied Monitor (Fayette, Mo.). *Mycobacterium tuberculosis*, lysates, and culture filtrates were a gift from Julian Davies, University of British Columbia. Verification of the *M. paratuberculosis* strain used in this study was performed as described previously (14) with PCR primers specific for the 5′ and 3′ ends of *M. paratuberculosis*-specific insertion sequence IS*900* (31).

Preparation of culture filtrate and soluble cellular extracts. For preparation of culture filtrate antigens, the bacilli were harvested by centrifugation (8,000 × *g* for 20 min). The supernatants were filtered successively through 0.45- and 0.22-μm-pore-size Millipore (Bedford, Mass.) filters and lyophilized. For preparation of soluble cell extracts, the bacilli were washed twice in sterile saline containing 0.05% Tween 20. The cell pellet was suspended in sterile 10 mM Tris-HCl (pH 7.5) at 1 g (wet weight) per ml, heat killed (100°C, 5 min), and sonicated for 15 min at 4°C (40-s bursts at maximum setting). Acid-washed glass beads (106-μm diameter) were added to the sonicate, and the suspension was vortexed at top speed for 5 min and centrifuged at 15,000 × *g* for 20 min. The supernatant containing soluble cellular extracts (the S1 fraction) was filtered through a 0.45-μm-pore-size filter and stored at −20°C.

ConA lectin chromatography. A 2-ml sample of concentrated culture filtrates or 1 ml (5 mg/ml) of the S1 fraction was mixed with 1 ml of ConA-agarose beads in lectin buffer (10 mM Tris-HCl, 150 mM NaCl, 2 mM each $CaCl_2$ and $MgCl_2$, 0.05% Tween X-100 [pH 7.5]) and incubated for 1 h at 25°C. The eluates were collected and run four to five times through the ConA lectin matrix. The final eluate contained the ConA-nonbinding fraction. The ConA lectin beads were washed with the equivalent of 20 bed volumes of the lectin buffer to remove all nonbinding molecules. The lectin-binding antigens were eluted with 0.4 M α-D-mannopyranoside in lectin buffer, dialyzed against several changes of sterile saline, and lyophilized.

Preparation of crude filtrate oligosaccharides. The oligosaccharides were extracted as described by Sugden et al. (43). A 100-ml filtrate from 8- or 12-week cultures of *M. paratuberculosis* was lyophilized and suspended in 10 ml of 90% phenol in water (6:4), and the slurry was stirred for 60 min at 25°C and centrifuged at 30,000 × *g* (30 min at 4°C). The aqueous fraction was collected and dialyzed in running water for 72 h to remove the phenol, lyophilized, and suspended in 5 ml of water. Four volumes of ethanol-ether (2:1) chilled on dry ice were added to the crude oligosaccharide extract, and the mixture was stored at −20°C for 48 h and centrifuged at 15,000 × *g* for 15 min. The pellet was rinsed in chilled ethanol-ether, dialyzed in running water for 48 h, and lyophilized. The pellet contained the pooled culture filtrate oligosaccharides. The oligosaccharides were suspended in ConA-binding buffer and subjected to ConA lectin affinity chromatography.

Deglycosylation of ConA-binding antigens. ConA-binding glycoconjugate molecules from culture filtrates and soluble cell extracts of *M. paratuberculosis* were treated with the exoglycosidase enzymes α-mannosidase (EC 3.2.1.24), β-mannosidase (EC 3.2.1.25), and α-glucosidase (EC 3.2.1.20). The antigens (1 mg) were suspended in 50 mM citrate buffer containing 1 mM zinc acetate at the appropriate pH optimum for each enzyme and boiled for 10 min before the addition of the enzymes (5 to 10 mU of enzyme per reaction). Enzymatic deglycosylations were performed at 25°C for 18 h. Digestion reactions were terminated by the addition of an equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiling for 10 min.

MAbs and polyclonal antibodies. BALB/c mice were injected subcutaneously with 100 μg of *M. paratuberculosis* soluble cell extracts in Freund's incomplete adjuvant, followed by four booster injections of the antigen in sterile saline at 3-week intervals. The protocol for generation and selection of hybridomas was as described previously (28). Rabbits were injected intramuscularly with 500 μg of *M. paratuberculosis* soluble cell extract in Freund's incomplete adjuvant at 3-week intervals and bled after 3 months. Rabbit anti-*M. tuberculosis* lipoarabinomannan (LAM) polyclonal serum was a gift from R. Stokes (University of British Columbia). Sera from cows and sheep with paratuberculosis were provided by E. A. Sugden (Animal Diseases Research Institute, Nepean, Ontario, Canada) and D. Butler (Ontario Veterinary College, University of Guelph, Ontario, Canada). Specific MAbs for mycobacterial 71-kDa (HAT1) and 65kDa (HAT2) heat shock proteins and the 38-kDa PhoS-(HAT5) proteins were provided by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases and by I. Rosenkrands (2), Statens Seruminstitut, Copenhagen, Denmark, respectively.

Binding of MAbs to *M. paratuberculosis* filtrate and cellular antigens was also examined by the enzyme-linked immunosorbent assay (ELISA) procedure (28). The culture filtrates and *M. paratuberculosis* antigens were diluted in 10 mM phosphate-buffered saline (pH 7.4). All incubations were at 37°C for 2 h. The second antibody was goat anti-mouse (immunoglobulin G [IgG] or IgM) alkaline phosphatase (AP)-labeled antibody. The change in absorbance (optical density at 450 nm) of the AP substrate was read after 1 h of incubation at room temperature.

SDS-PAGE and Western blot (immunoblot) analysis. Samples (20 μg) of antigens were analyzed by PAGE in SDS–12% (wt/vol) polyacrylamide gels under reducing conditions. The gels were stained by Coomassie brilliant blue or silver staining methods (47). Two-dimensional gel electrophoresis was performed with the denaturing isoelectric-focusing slab gels (8 M urea, 4.5% acrylamide, 0.6 ml of ampholytes [0.2 ml at pH 5 to 7 and 0.4 ml at pH 3.5 to 10]) (37), followed by SDS-PAGE. For immunoblot analysis, antigens separated by one- and two-dimensional SDS-PAGE were electroblotted onto nitrocellulose membranes and blocked with 5% (wt/vol) skim milk. The blots were reacted with MAbs, polyclonal sera, or digoxigenin (DIG)-labeled ConA lectin. The blots were developed with AP-labeled anti-mouse IgG or IgM or AP-labeled rabbit anti-DIG antibody, followed by the AP substrate BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium) in AP buffer (0.1 M NaCl, 50 mM $MgCl_2$, 50 mM Tris-HCl [pH 9.6]). To test whether the antibodies were reacting with carbohydrate epitopes, the blots were treated with sodium metaperiodate and sodium borohydride prior to incubation with skim milk and antibody or lectin analysis (50). All incubations with antibodies were at 25°C for 2 to 3 h, while incubation was for 1 h for the lectin treatment. The blots were washed in 10 mM sodium phosphate (pH 7.4)-buffered saline containing 150 mM NaCl and 0.05% (vol/vol) Tween 20.

RESULTS

Fractionation of cellular and culture filtrate antigens of *M. paratuberculosis* by ConA lectin affinity chromatography. Mycobacteria express a variety of glycoconjugates, including oligosaccharides, glycolipids, and probably glycoproteins, which are found on the cell surface of the bacilli and in the external milieu (22, 24, 25, 32). Glycoconjugates such as LAM and glycolipids (8, 15, 19, 36), and antigens secreted by actively growing mycobacteria (1, 2, 7), play significant roles as modifiers of cellular and humoral immunity in infected hosts. ConA lectin affinity chromatography has been used to purify mycobacterial molecules containing α-D-mannose- and α-D-glucose-like residues (15, 16, 22).

In this study, we investigated the distribution of ConA-binding glycoconjugates and lectin-nonbinding molecules in 8-week-old culture filtrates and in soluble cell extracts of *M. paratuberculosis* ATCC 1969. The ConA-nonbinding fractions from culture filtrates were not routinely analyzed by SDS-PAGE because the high content of bovine serum albumin in the medium distorted the migration of other molecules in the sample.

Few antigens were stained by Coomassie brilliant blue in the ConA-binding soluble cellular extract (S1) fraction (Fig. 1A, lane 2) or in the phenol-extracted polysaccharide fraction (lane 5). In contrast, the ConA-nonbinding S1 and the ConA-binding filtrate fractions contained a large number of weakly staining bands (Fig. 1A, lanes 3 and 4, respectively), with the exception of a 69-kDa major band in the filtrate fraction (Fig. 1A, lane 4). The silver-stained gel showed a greater number of stained bands for each fraction (Fig. 1B). A number of bands at approximately 34, 44, and 55 kDa appeared to be unique or enriched in the ConA-nonbinding S1 fraction (Fig. 1B, lane 3), while only a 24-kDa silver-staining band was detected in the S1 fraction (Fig. 1B, lane 1). The ConA-binding filtrate fraction contained unique bands at 26, 27, 42, 47, 69, and above 100 kDa (Fig. 1B, lane 4), suggesting that these molecules may be preferentially secreted to the external environment during

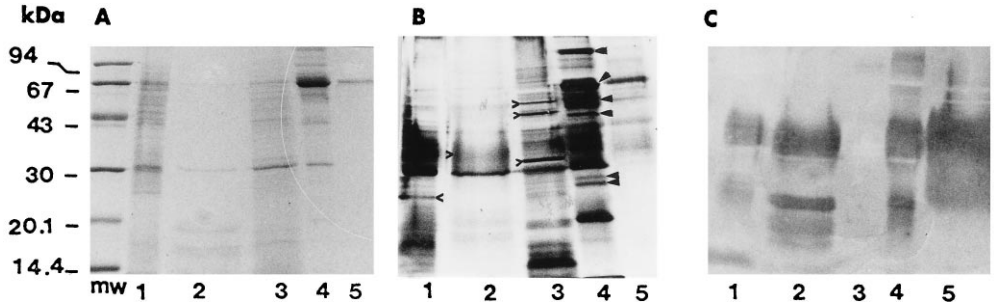

FIG. 1. ConA lectin-binding and nonbinding fractions of cellular and culture filtrate antigens from *M. paratuberculosis* ATCC 19698, analyzed by Coomassie blue staining (A), silver staining (B), and DIG-labeled ConA lectin blotting (C). Lanes: 1, soluble cell extracts (S1); 2, ConA-binding S1 fraction; 3, ConA-nonbinding S1 fraction; 4, ConA-binding culture filtrate fraction; 5, phenol-extracted culture filtrate polysaccharides; mw, molecular mass markers.

growth of *M. paratuberculosis*. A diffuse band which stained a light-brown color was clearly observed in the ConA-binding S1 fraction (Fig. 1B, lane 2), was also observed as a smear in the S1 fraction and in the ConA-binding filtrates (Fig. 1B, lanes 1 and 4), but was less prominent in the phenol-extracted oligosaccharide-containing fractions (Fig. 1B, lane 5).

To confirm the presence of carbohydrate residues on the *M. paratuberculosis* antigens, electrophoretic blots of the separated molecules were reacted with a glycan detection kit (Fig. 1C). The profiles of the lectin-binding molecules were similar in the S1, ConA-binding S1, and culture filtrate fractions (Fig. 1C, lanes 1, 2, and 4, respectively) and in the phenol-extracted filtrate oligosaccharides (Fig. 1C, lane 5). The diffuse bands reacting with the ConA lectin could not be resolved into separate bands on SDS-PAGE. The migration of these mycobacterial antigens was similar to that observed with capsular polysaccharides from gram-negative bacteria. The lectin did not bind to antigens in the ConA-nonbinding S1 fraction, thus confirming the absence of carbohydrates in these molecules (Fig. 1C, lane 3). The phenol-extracted oligosaccharides stained poorly with silver stain but reacted strongly with the lectin (Fig. 1B and C, lanes 5). The poor staining with silver suggests that the purified oligosaccharide molecules contain a high proportion of neutral sugars. The binding of ConA lectin to the diffuse antigens of *M. paratuberculosis* was not affected by proteinase K treatment of the samples but was completely abolished by treatment of the blots with sodium metaperiodate and sodium borohydride, implying that the diffuse bands of lectin-binding molecules from *M. paratuberculosis* cellular and extracellular fractions are primarily oligosaccharide in composition (data not shown).

Epitope specificity of MAbs against *M. paratuberculosis* antigens. The immunological cross-reactivities of 14 MAbs generated against *M. paratuberculosis* antigens were tested by ELISA and Western blotting with lysates from six other mycobacterial species and culture filtrates and ConA-binding and -nonbinding cellular antigens of *M. paratuberculosis* (Table 1). Of particular interest were MAbs that recognized *M. paratuberculosis*-specific epitopes and culture filtrate antigens. MAb B6A reacted with a major antigen of approximately 34.5 kDa and a minor band at 33 kDa in lysates of *M. paratuberculosis* (Fig. 2A, lane 1) and not in lysates of any other species, including the closely related *M. avium* 18 (lane 9). The epitope recognized by MAb B6A may be an *M. paratuberculosis* species-specific epitope. MAb FL1-A1 reacted with a protein with a molecular mass of 44.3 kDa in *M. paratuberculosis* and an antigen of approximately 9 kDa in lysates of *M. kansasii* (Fig. 2B, lanes 1 and 3, respectively). However, in heat-treated

TABLE 1. MAbs to *M. paratuberculosis* antigens

MAb	Isotype	Molecular mass(es) (kDa [10^3]) of *M. paratuberculosis* antigen(s)	Cross-reactivity of the MAbs[c]
Protein specific			
B6A	IgG1	34.5 and 33	*M. paratuberculosis*
FL1-A1	IgG1	47.5,[a] 44.3, 37.5,[a] 35	*M. paratuberculosis*, *M. kansasii*
D4B	IgG3	39.7	*M. tuberculosis*, *M. smegmatis*
FLII-B3	IgG3	38–45[a,b]	*M. kansasii*, *M. avium*
D1A	IgG1	69.6[a,b], 38[a,b]	*M. tuberculosis*, *M. smegmatis*, *M. fortuitum*, *M. phlei*, *M. aurum*
D2B	IgG2b	45	All 7 *Mycobacterium* species
D5B	IgG3	45–47	All 7 *Mycobacterium* species
A3A	IgG3	45–47	All 7 *Mycobacterium* species
B1A	IgG1	45–47	All 7 *Mycobacterium* species
FL1-AB	IgG1	36.7,[a] 33[a]	*M. tuberculosis*, *M. kansasii*, *M. fortuitum*, *M. aurum*, *M. avium*, *E. coli*
Carbohydrate specific			
PII-B1	IgM	35–46.6[a,b]	*M. tuberculosis*, *M. kansasii*, *M. avium*
PII-D5	IgG2b	35–46.6[a,b]	*M. tuberculosis*, *M. kansasii*, *M. avium*
PII-D1	IgG3	35–46.6[a,b]	*M. tuberculosis*, *M. kansasii*, *M. avium*
PII-C3	IgG3	35–46[a,b]	*M. kansasii*, *M. avium*

[a] ConA-binding antigen of *M. paratuberculosis.*
[b] Antigen in culture filtrates of *M. paratuberculosis.*
[c] MAbs were screened against cell lysates and culture filtrates from *M. tuberculosis*, *M. kansasii*, *M. smegmatis*, *M. fortuitum*, *M. aurum*, *M. phlei*, and *M. avium* 18.

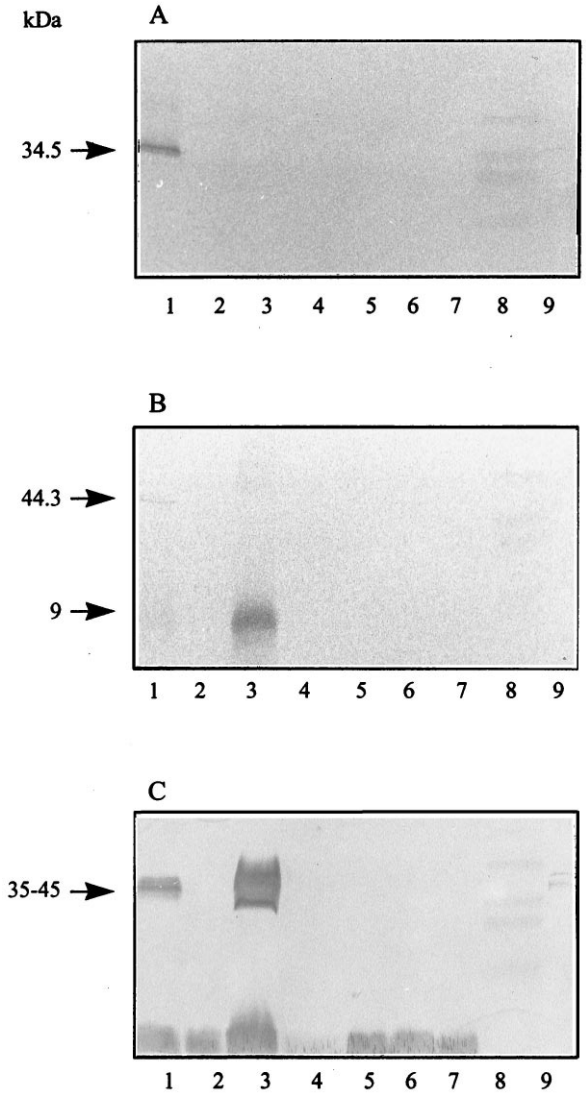

FIG. 2. Western blotting of lysates of different mycobacterium species with MAbs against *M. paratuberculosis* antigens. Reactivities of MAbs B6A (A), FL1-A1 (B), and PII-B1 (C) are shown. Lanes: 1, *M. paratuberculosis*; 2, *M. tuberculosis*; 3, *M. kansasii*; 4, *M. smegmatis*; 5, *M. phlei*; 6, *M. aurum*; 7, *M. fortuitum*; 8, molecular mass standards; 9, *M. avium* 18. Molecular mass values of *M. paratuberculosis* antigens reacting with the MAbs are indicated on the left.

(100°C for 20 to 30 min) cell lysates of *M. paratuberculosis*, the 44.3-kDa antigen was not detected and MAb FL1-A1 reacted with a doublet of antigens at 6 and 9 kDa (data not shown). Whether the 9-kDa immunoreactive peptides are proteolytic products or monomers of the 44.3-kDa antigen is to be determined. We are attempting to purify sufficient amounts of the *M. paratuberculosis* and *M. kansasii* 9-kDa immunoreactive peptides for further immunochemical analyses.

Four MAbs, including PII-B1, reacted with a diffuse band of protease-resistant antigens of between 35 and 45.6 kDa in *M. paratuberculosis* and *M. kansasii* and showed a weak reaction with three to four bands with similar molecular masses in *M. avium* 18 (Fig. 2C, lanes 1, 3, and 9, respectively). A weak reaction was also detected with the *M. tuberculosis* lysates (Fig. 2C, lane 2) but was not reproduced on photography. MAb PII-B1 also reacted with a fast-migrating band in cell lysates of all the *Mycobacterium* species (Fig. 2C). The reaction with the low-molecular-mass mycobacterial antigen was abolished by treatment of the lysates with lysozyme and was not detected in culture filtrate of *M. paratuberculosis* or *M. kansasii* (data not shown). These data implied that MAb PII-B1 is reacting with an antigenic determinant located on a peptidoglycan-associated oligosaccharide and which is exposed or unmasked in other mycobacteria upon lysis and dissociation of the cell wall during SDS-PAGE. The epitope appears to be expressed in a lysozyme-stable configuration in the high-molecular-weight polysaccharide complexes of *M. paratuberculosis*, *M. kansasii*, and *M. avium.*

Three MAbs (D1A, D4B, and FLII-B3) reacted with protease-sensitive antigenic determinants expressed by a limited number of the mycobacterial species, including *M. tuberculosis*, *M. kansasii*, *M. fortuitum*, and *M. avium* (immunoblots not shown) (Table 1). MAbs D2B, D5B, B1A, and A3A reacted with protein antigens with apparent molecular masses of 45 to 47 kDa in lysates and not culture filtrates of the *Mycobacterium* species examined (immunoblot data not shown) (Table 1). However, the intensities of staining were different for each MAb and within the mycobacterial species, suggesting that these MAbs were reacting with different epitopes on a highly immunogenic protein(s) that is common to all the species. Monoclonal reagents showing similar patterns of epitope heterogeneity within the *Mycobacterium* species have been reported (45).

Localization of antigenic determinants. Immunoblot analyses were performed to examine the distribution of antigenic determinants recognized by different MAbs in culture filtrates and soluble cell extracts of *M. paratuberculosis* (Fig. 3 and Table 1). MAb B6A reacted with a major band of approximately 34.5 kDa and a minor band at 33 kDa in the S1 fraction, and these antigens were also recovered in the ConA-nonbinding S1 fraction (Fig. 3A, lanes 1 and 3, respectively). The faster-migrating antibody-labeled band was invariably detected in the ConA-nonbinding S1 fraction, and it may be a degradation product or a subunit of the 34.5-kDa antigen which was enriched by lectin fractionation. MAb FL1-A1 reacted with four antigens of approximately 47.5, 44.3, 37.5, and 35 kDa upon nonreducing SDS-PAGE (Fig. 3B, lane 1). The 44.3- and 37.5-kDa antigens were also detected in the ConA-binding S1 fraction (lane 2), suggesting small amounts of glycan residues. MAbs FL1-A1 and B6A did not react with the unfractionated culture filtrate or the ConA-binding or -nonbinding filtrate fractions when tested by ELISA, suggesting that the nominal antigens were not shed into the medium during growth of *M. paratuberculosis* (data not shown).

MAb PII-B1 reacted with a diffuse band of between 35 and 45.6 kDa in the S1 fraction, the ConA-binding S1 fraction, the ConA-binding filtrate fraction, and the phenol-extracted filtrate polysaccharides (Fig. 3C, lanes 1, 2, 4, and 5) but not in the ConA-nonbinding S1 fraction (lane 3). The labeled antigens were similar in migration and molecular mass to the major lectin-binding antigen detected in Fig. 1C. ELISA analyses confirmed the presence in culture filtrates of antigens reacting with MAb PII-B1. These data provide evidence for specific release by *M. paratuberculosis* of polysaccharides molecules into the medium during growth of the bacilli. In addition to the polysaccharide (MAb PII-B1) antigens, *M. paratuberculosis* also secreted or released protease-sensitive ConA-binding antigens recognized by MAbs FLII-B3 (38 to 45 kDa), D1A (69.6 and 38 kDa), and FL1-AB (36.5 kDa) (Table 1).

Two-dimensional (isoelectric-focusing and SDS-polyacrylamide) gel electrophoresis followed by Western blot analysis of the S1 fraction of *M. paratuberculosis* was performed to determine the isoelectric point (pI) values of the antigens recog-

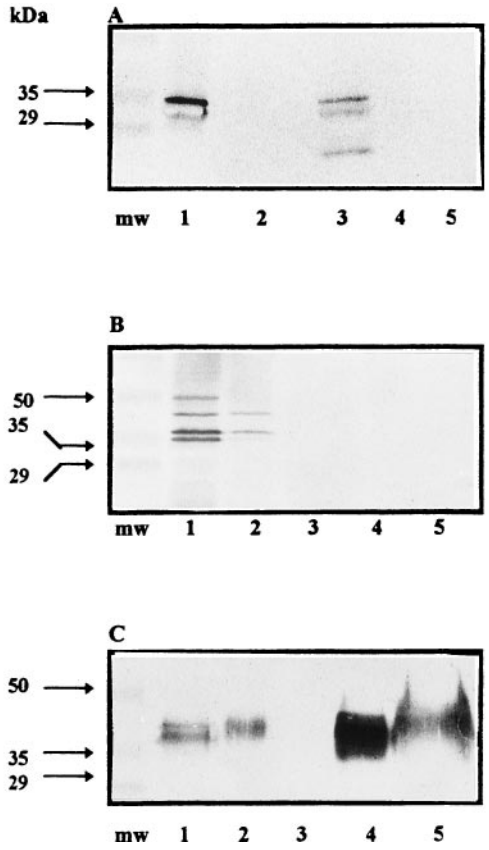

FIG. 3. Western blot analysis of the ConA lectin-fractionated antigens with MAbs against *M. paratuberculosis*. (A) MAb B6A; (B) MAb FL1-A1; (C) MAb PII-B1. Lanes: 1, soluble cell extracts (S1); 2, ConA-binding S1 fraction; 3, ConA-nonbinding S1 fraction; 4, ConA-binding culture filtrate fraction; 5, phenol-extracted culture filtrate polysaccharides; mw, molecular mass markers.

nized by the MAbs. The 44.3- and 37.5-kDa antigens reacting with MAb FL1-A1 had pI values of 8.9 and 6.85, respectively. The pI values for the 34.5- and 33-kDa antigens reacting with MAb B6A were 6.65 and 6.2, respectively. The antigens reacting with MAb PII-B1 appeared as a series of closely spaced vertical bands with pI values between 6.9 and 8.25 and migrated at molecular masses between 35 and 45 kDa on the two-dimensional gel. The appearance of these antigens as a train of vertical spots suggests heterogeneity in size and charge of the glycosylated antigens (data not shown).

Cross-referencing of the *M. paratuberculosis* ConA-binding antigens with those described in *M. tuberculosis* was attempted. ConA-binding cellular and culture filtrate antigens of *M. paratuberculosis* were purified by electroelution, and immunoblots of these antigens reacted with specific *M. tuberculosis* MAbs. MAb HAT1, specific for the 71- to 72-kDa protein of mycobacteria (the 71-kDa protein is a member of the DnaK family of heat shock proteins) (40, 51), reacted with the 69.6- and 38-kDa cellular and culture filtrate ConA-binding antigens also recognized by MAb D1A in *M. paratuberculosis*. The epitope recognized by MAb D1A was not expressed in *M. kansasii* or *M. avium* (immunoblots not shown) (Table 1). Andersen et al. (2) reported that the 70-kDa antigen was released gradually during growth of *M. tuberculosis*. The mode of release of the D1A antigens during growth of *M. paratuberculosis* was not examined. MAbs HAT2, HAT5, and HAT6 did not react with the ConA-binding cellular or filtrate components of *M. paratuberculosis* (data not shown).

We also attempted to determine the similarities between the 34.5-kDa cell-associated antigen recognized by MAb B6A in this study and a 34-kDa *M. paratuberculosis* antigen characterized by De Kesel et al. (18) and Gilot et al. (26). The novel 34-kDa antigen contains an *M. paratuberculosis*-specific epitope and epitopes expressed in other mycobacterial species (26). Comparisons of amino (N)-terminal primary sequences of proteins have been used to determine the similarities between target proteins and other known proteins. Thus, the B6A antigen was purified by preparative SDS-PAGE and by electroelution from the gel and subjected to amino acid sequence analysis. Unfortunately, the purified protein(s) was not amenable to N-terminal amino acid sequencing. In other studies, we have found that the 34.5-kDa antigen, as well as the antigens recognized by MAbs D5B, B1A, and PII-B1, remained in the detergent phase following Triton X-114 phase separation (5) of soluble cell extracts of *M. paratuberculosis* (data not shown). These data suggest that these are lipid-associated or integral (cell wall or cell membrane) proteins and concur with the cellular localization of these antigens (Fig. 2 and Table 1).

Immunochemical analysis of culture filtrate and cell-associated polysaccharides of *M. paratuberculosis*. Recently, Ortalo-Magne et al. (35) showed the release of capsular polysaccharides, LAM, and D-mannans into the media during growth of *M. tuberculosis*. Sugden et al. (43) purified LAM and lipid-free arabinomannan (AM) from *M. paratuberculosis* bacilli and reported that the AM was similar to the AM extracts from *M. tuberculosis*. Therefore, we examined the antigenic relationships between the polysaccharide antigens of *M. paratuberculosis* and *M. tuberculosis* LAM and the recognition of these antigens by MAb PII-B1 (Fig. 2C) and by serum obtained from animals with paratuberculosis (Fig. 4B and C). Polyclonal antiserum against *M. tuberculosis* LAM (Fig. 4A) reacted with a diffuse band of antigens in cell lysates, in ConA-binding filtrate fractions, and in the phenol-extracted culture filtrate polysaccharides of *M. paratuberculosis* (Fig. 4A, lanes 1 to 3) and with antigens of similar mobility and banding patterns in *M. avium*, *M. tuberculosis*, and *M. phlei* (lanes 4 to 6, respectively). However, bovine paratuberculosis serum did not react with *M. tuberculosis* antigens (Fig. 4B, lane 5), and the ovine paratuberculosis serum failed to react with extracts from *M. avium* and *M. tuberculosis* (Fig. 4C, lanes 4 and 5, respectively). In addition, MAb PII-B1 reacted weakly with cell extracts from *M. tuberculosis* and failed to react with *M. phlei* (Fig. 2C, lanes 2 and 5, respectively). The heterogeneity in antigenic cross-reactions of these polyclonal sera with lysates of the different *Mycobacterium* species implies that LAM-like molecules contain species-specific, subgroup-specific, and broadly cross-reactive epitopes. The immunogenicity of these antigens in Crohn's disease sera was not investigated.

Further studies were performed to examine the properties of the carbohydrate epitopes on the ConA-binding polysaccharides of *M. paratuberculosis*. Deglycosylation of the ConA-binding polysaccharide antigens with β-mannosidase (Fig. 5, lanes 3) or α-glucosidase (data not shown) enzymes had no effect on the binding of the MAbs, polyclonal antibodies, or ConA lectin to the glyconjugates. In contrast, α-mannosidase treatment was most effective in eliminating the binding of MAb PII-B1 (Fig. 5A, lane 2) and bovine paratuberculosis serum (Fig. 5B, lane 2) to the polysaccharides and significantly reduced ConA lectin binding to these antigens (Fig. 5D, lane 2). The ovine paratuberculosis serum reacted with an antigen band of higher mobility in the α-mannosidase-treated polysac-

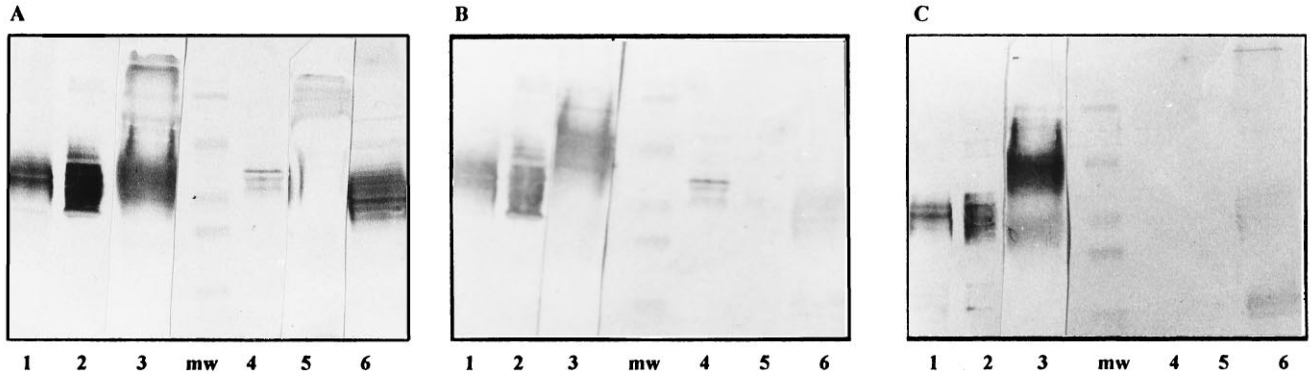

FIG. 4. Cross-reactivity of rabbit polyclonal serum against *M. tuberculosis* LAM (A) and sera from cow (B) and sheep (C) with paratuberculosis when reacted with *M. paratuberculosis* lysates and culture filtrate antigens and with lysates from different mycobacteria. Lanes: 1, *M. paratuberculosis* lysate; 2, ConA-binding culture filtrate fraction; 3, phenol-extracted culture filtrate fraction; mw, molecular mass standards; 4, *M. avium* 18; 5, *M. tuberculosis*; 6, *M. phlei.*

charides (Fig. 5C, lane 2). The alteration in mobility of the antigens suggests loss of α-linked mannose residues which were apparently not serologically active in the ovine host. The binding of the ovine paratuberculosis serum to the polysaccharide antigens was eliminated by oxidation of the antigens with sodium borohydride, indicating recognition of carbohydrate-containing epitopes by the antiserum (data not shown).

DISCUSSION

Accumulated evidence supports a role for secreted components in immunity against mycobacteria (1, 7). Many studies have also reported ConA-binding (glyco)proteins in culture filtrates of species of mycobacteria (15, 16) other than *M. paratuberculosis*. Furthermore, ConA-binding filtrate antigens of *M. tuberculosis* were immunoreactive in patients with tuberculosis (23, 24). Although extracellular secretion or release of proteins in *M. paratuberculosis* has been observed (26, 44, 49), there are few reports on ConA-binding cellular and culture filtrate antigens. In this study, at least 17 antigens in the 21- to 69-kDa range were observed in the ConA-binding filtrate fraction of *M. paratuberculosis*. The 26-, 27-, 42-, 47-, and 69-kDa antigens were unique or primarily enriched in the ConA-binding culture filtrate fraction, implying selective release or secretion into the environment. White et al. (49) also reported extracellular molecules with similar molecular masses (12- to 45-kDa range) in several *M. paratuberculosis* isolates, and filtrate antigens of 24, 68, and 28 to 43 kDa were recognized by bovine paratuberculosis serum in *M. paratuberculosis* and *M. avium* filtrates (49). Studies by Bech-Nielsen et al. (3) also reported an immunoreactive region in the 28- to 42-kDa range in extracellular and cellular fractions of *M. paratuberculosis* and *M. avium* which could not be resolved into individual bands by SDS-PAGE. Our studies show that the diffuse band of immunoreactive antigens consists of ConA-binding LAM-like polysaccharides of *M. paratuberculosis* that are released into the medium during growth of the bacilli. Furthermore, the serological activity of these molecules in mice (BALB/c) and cattle could be attributed to the α-linked mannose residues. In contrast, the α-linked mannan residues were apparently not immunogenic in sheep. Misaki et al. (34) reported that serological activity of rabbit polyclonal serum against *M. tuberculosis* cells was due entirely to the α-(1→5)-linked D-arabinofuranosyl residues, with no antibody activity against the α-linked mannose residues. Whether the serological activity and specificity of the bovine and ovine paratuberculosis serum against the mannan or possibly the arabinogalactan residues of the *M. paratuberculosis* polysaccharides are due to differences in the antigenicity of the carbohydrate residues in different animal species or to antigen preparations, source of antigen (*Mycobacterium* species and strain), and immunization protocols remains to be determined.

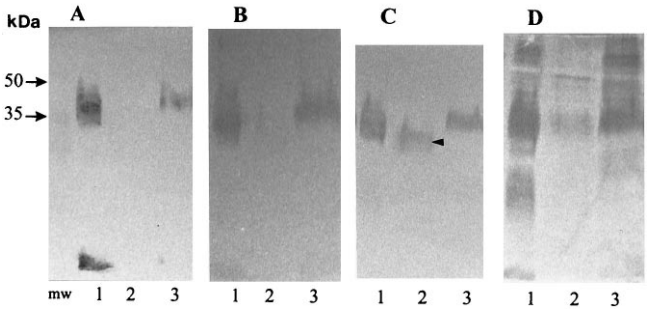

FIG. 5. Effect of enzymatic deglycosylation of ConA-binding S1 molecules from *M. paratuberculosis* on binding of MAb PII-B1 (A), sera from cow (B) and sheep (C) with paratuberculosis, and DIG-labeled ConA lectin (D). Lanes: 1, no enzyme control; 2, α-mannosidase; 3, β-mannosidase. (C) The arrowhead indicates an antigen band with higher mobility in the α-mannosidase-treated sample.

This is the first report of culture filtrate polysaccharides in *M. paratuberculosis*. Recent biochemical and structural studies have indicated that *M. tuberculosis* bacilli contain a cell surface capsular material containing proteins and polysaccharide components which are released into the environment during growth (35). Many studies have documented the role in pathogenesis of mycobacterial cell wall-associated polysaccharide complexes (19, 36, 39, 41). The release of polysaccharide complexes into the environment by *M. paratuberculosis* may be significant in the formation of granulomata and other hypersensitivity-type responses manifested in paratuberculosis and Crohn's disease.

Only a relatively small number of antigens, including the hsp60K (14) and hsp65K (21) stress proteins, LAM (43), antigens A and bacterioferritin (6, 44), and the 34-kDa antigen (17, 18, 26), have been fully characterized for *M. paratuberculosis*. The extremely low growth rate of *M. paratuberculosis* precludes large-scale isolation of antigens for use in diagnostic and immunochemical studies of the bacilli. The aim of this study was to identify and characterize specific epitopes and antigenic determinants of *M. paratuberculosis* and to eventually examine the immunological properties of specific antigens in

paratuberculosis. Using MAbs raised against *M. paratuberculosis* antigens, we have identified four groups of epitopes or antigenic determinants: an *M. paratuberculosis* species-specific epitope (B6A), an *M. paratuberculosis-M. kansasii* complex-specific epitope (FL1-A1), epitopes expressed in a limited number of mycobacteria (PII-B1, PII-D5, PII-D1, PII-C3, FLII-B3, D1A, and D4B), and broadly cross-reactive epitopes (FL1-AB, D2B, D5B, B1A, and A3A) (Table 1). Using these MAbs, we demonstrated that *M. paratuberculosis* bacilli released polysaccharide (PII-B1) antigens and protease-sensitive glycoconjugates reacting with MAbs FLII-B3 (38 to 45 kDa), D1A (69.9 and 38 kDa), and FL1-AB (36.5 kDa) into the environment.

There were similarities between some of the antigens identified in this study and previously reported *M. paratuberculosis* or mycobacterial antigens. For example, MAb D1A recognized the *M. paratuberculosis* homolog of the mycobacterial 71-kDa heat shock protein (40, 51). The 69.6-kDa (MAb D1A) antigen and a cross-reacting 38-kDa antigen were released during growth of *M. paratuberculosis*, and both antigens contained ConA-binding moieties. The role of heat shock proteins in autoimmunity has been suggested for both Crohn's disease and paratuberculosis (30). Since the 69.9-kDa antigen was a major filtrate antigen, it would be interesting to study its role in the immunopathology of Crohn's and Johne's diseases. White et al. (49) reported a 42-kDa antigen in lysates of *M. paratuberculosis* strains which was not detected in lysates of *M. avium* or *M. bovis* BCG strains. Although other *Mycobacterium* species were not included in that study, the authors suggested that the 42-kDa antigen may be specific for *M. paratuberculosis* species. There were not sufficient data at this time to allow a valid comparison of the 42-kDa *M. paratuberculosis*-specific antigen and the 44.3-kDa antigen recognized by MAb FL1-A1 in this study. Despite the similarities in molecular mass between the antigen recognized by MAb B6A and the previously described 34-kDa *M. paratuberculosis* antigen (17, 18, 26), other data suggest that we may have identified a new species-specific antigen. Unlike the 34-kDa antigen (26), the B6A (34.5-kDa) antigen was not detected in culture filtrates of the bacilli, implying that it is a nonsecreted antigen. Furthermore, the apparent hydrophobic properties of the protein are concordant with a cellular localization of this antigen. In this study, we did not identify MAbs to previously described 18-, 24-, or 38-kDa *M. paratuberculosis*-specific antigens (20). We are currently screening expression libraries of *M. paratuberculosis* with the MAbs to identify recombinant plasmids expressing selected antigens and to subsequently study the genes encoding the *M. paratuberculosis* proteins. Of particular interest are antigens attributed with species-specific epitopes and the extracellular or secreted antigens.

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