Comparison of the Proteosomes and Antigenicities of Secreted and Cellular Proteins Produced by Mycobacterium paratuberculosis

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Bovine paratuberculosis is diagnosed either by detecting the causative agent Mycobacterium paratuberculosis (also known as Mycobacterium avium subsp. paratuberculosis) or an immune response to the agent (6). Microbiological culture of the organism from feces is a widely used diagnostic test and is considered the reference assay against which other tests are compared. Culture of M. paratuberculosis on conventional solid bacteriological media is laborious and slow, requiring up to 16 weeks for assay completion (6). Detection of a cellular immune response by either skin testing or stimulation of leukocytes for gamma interferon release is useful for earlier diagnosis of infection, but these assays suffer from high variability and lower specificity (11, 16, 17, 20). Serologic assays, such as enzyme-linked immunosorbent assays (ELISAs), have low diagnostic sensitivity during early phases of the infection but are a useful tool for Johne’s disease control because of low-cost, high-throughput, standardized protocols and correlation with M. paratuberculosis fecal-shedding levels. A recent comparison of commercial ELISA kits for bovine paratuberculosis diagnosis illustrated that the assays performed comparably overall, with diagnostic sensitivity ranging from 27.9 to 44.5% for fecal culture-positive cattle (8). Interestingly, numerous individual cattle were strongly responsive in one assay and not another (8). Although exact details of the antigen preparation methods for commercially available ELISA kits are proprietary, either protoplasmic fractions, other cellular extracts, or lipoarabinomannan are likely the basis of most assays because of the higher antigen yields and ease of preparation (1, 4, 7, 9, 21, 34, 37, 46). Based on a literature review, proteins secreted from M. paratuberculosis into the extracellular environment have received little attention as serodiagnostic assay antigen candidates (21). In contrast, secreted proteins of Mycobacterium tuberculosis are the focus of considerable research as human medicine tries to improve diagnostic assays for tuberculosis (18, 22).

In previous work, we observed that culture conditions significantly influence protein expression profiles of M. paratuberculosis (39). The purpose of the present study was to compare the M. paratuberculosis protein antigens found in the cellular to extracellular compartments by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and ELISA. This work forms the basis of a search for secreted proteins that may improve serodiagnostic tests for bovine paratuberculosis.

MATERIALS AND METHODS

Bacterial strains. M. paratuberculosis JTC303 was propagated in Middlebrook 7H9 (7H9) broth (Becton Dickinson, Cockeysville, MD) and modified Watson-Reid (WR) broth supplemented with mycobactin J (Allied Monitor, Fayetteville, MO). In addition, 7H9 broth was supplemented with 10% oleic acid-albumin-dextrose-catalase (Becton Dickinson). WR broth (pH 6.0) was prepared without the addition of sodium pyruvate (44). Mycobacterium phlei (ATCC 11758) was cultivated in WR broth, and cellular protein extracts were used to absorb cross-reacting serum antibodies prior to immunoblotting (preabsorption). Cellular

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extracts and culture filtrates were harvested when growth had reached stationary
phase (38).

**Culture filtrates (CF).** Bacterial cells grown in WR broth were removed by centrifugation at 30,000 × g for 10 min, followed by filtration (0.2-μm pore size; Naïge Nunc, Rochester, NY). The filtrate was then concentrated roughly 100-fold by using Centricon Plus-80 (Millipore, Billerica, MA) and dialyzed in 10 mM phosphate-buffered saline (PBS; pH 6.8). The concentration of soluble CF proteins was determined by the BCA protein assay kit (Pierce, Rockford, IL).

**Cellular extracts (CE).** *M. philo* and *M. paratuberculosis* were grown in WR or 7H9 broth, harvested by centrifugation (30,000 × g for 10 min), and washed three times in 10 mM PBS (pH 6.8). The resultant cell pellets were then homogenized by using an overhead stirrer (Wheaton Instruments, Milville, NJ) for 3 min on ice to minimize clumping of cells. After the addition of a protease inhibitor cocktail (Complete, Mini, EDTA-free, 0.05% [vol/vol]; Roche Diagnostics GmbH, Germany), 10 ml of the homogenized cell pellets was mixed with 20 g of glass beads (0.10 to 0.11 mm; Glassperlen; B. Braun Biotech International GmbH, Germany). The mixture was vigorously agitated for 10 min, while cooling with liquid CO₂ using a high-speed agitator (model MSK; Braun Instruments, Allentown, PA). The homogenate was then centrifuged at 30,000 × g for 30 min, and the CE proteins were stored at −70°C. The concentration of CE proteins was also determined by using a BCA protein assay kit (Pierce, Rockford, IL).

**Sera.** Sera were collected from healthy uninfected dairy cattle resident in herds at status level 4 in the U.S. Voluntary Bovine Johne’s Disease Herd Status Program (8). Sera from cattle known to be infected with *M. paratuberculosis* were collected from cows in eight different dairy herds. All were naturally infected, clinically normal, and fecal culture positive (8). One of the infected cattle, Dolly, was euthanized according to AALAC protocols to verify her disseminated *M. paratuberculosis* infection by histopathology and culture of tissues. Serum sufficient to complete the one- and two-dimensional electrophoresis (1-DE and 2-DE) immunoblot analyses was obtained from this cow (i.e., the positive control).

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seen when the serum was absorbed with particularly strong reactivity with the positive control serum (lane 3). Low molecular mass proteins (26 kDa) were more intensely stained using CF proteins (Fig. 2A and B, lane 3, versus panel B, lane 3).

Proteome and immunoblot analysis by 2-DE. Image analysis of 23-by-30-cm 2-DE gels resolved approximately 400 (CE) and 240 (CF) proteins (Fig. 3). The CE proteins were evenly distributed by mass and pI on the 2-DE gel. In contrast, most CF proteins were ≤37 kDa and had acidic pI values (pH 4 to pH 5.5). Several proteins in both CE and CF preparations appeared as strings of spots of similar masses but different pIs, suggesting the existence of proteins modified by phosphorylation, glycosylation, or acylation (Fig. 3). The 2-DE immunoblots of CE and CF preparations were done with or without M. phlei CE protein serum absorption (Fig. 4A). The total staining intensity of all proteins was determined and compared by image analysis software (Fig. 4B). The total immunoreactivity of serum from an M. paratuberculosis-infected cow was greater with CF than CE proteins, a finding in agreement with the 1-DE immunoblot results (Fig. 2). The number of immunoreactive spots after serum absorption was much lower for both CE and CF antigens, but the effect was greater for CE proteins than for CF proteins. Some CF proteins’ immunoblot staining intensity was not affected by serum absorption.

Antigenicity comparisons by 1-DE immunoblotting with multiple sera. The antigenicities of CE and CF proteins by 1-DE immunoblot analysis were determined by using six sera randomly chosen from the population of >400 sera from M. paratuberculosis-infected cattle. Using image analysis software, each band’s location was identified, and its net intensity was calculated. The total staining intensity of all bands was compared as the relative net intensity (Fig. 5). Although some sera had weak antibody responses to CE and CF, the signals to CF were in general much stronger.

Comparison of CF and CE by ELISA. Sera from 444 infected and 424 infection-free cattle were tested by ELISA with CE and CF proteins as the solid-phase antigen. The mean ELISA OD values for CE and CF antigens were significantly different (P < 0.0169 and P < 0.001) for infected and infection-free cattle, respectively (Fig. 6). Receiver-operator characteristic analysis showing the relationship between specificity and sensitivity revealed that the area under each curve was significantly greater for CF than for CE antigens (0.7716 and 0.6623, respectively; P < 0.05).

Variability of immunoblot patterns to CF proteins. Immunoblot analyses with sera from 31 M. paratuberculosis-infected cattle and 6 infection-free cattle were done after first absorbing sera with M. phlei cellular extract proteins. Among the sera from infected cattle, protein binding patterns were highly variable (Fig. 7A). Seventeen cattle showed strong serum antibody binding to CF proteins of approximately 10, 15, 23, 30, 35, and 50 kDa, but no single protein was reactive with the serum antibody from all 17 cattle. The protein binding patterns for sera from the other 14 M. paratuberculosis-infected cattle were the same as for the sera from uninfected cattle. Sera from uninfected cows, after absorption with M. phlei CE proteins, showed weak reactions to a 25-kDa band of M. paratuberculosis CF. This weak reaction also was seen after absorption of sera with M. paratuberculosis CE proteins (Fig. 7B).

DISCUSSION

The proteome of M. paratuberculosis strain JTC303 CF was different from that of the CE. Specifically, CF proteins generally had lower pIs (4.4 to 5.5) and smaller masses (most <30 kDa) than CE proteins. A similar protein pattern was shown in CF prepared from M. paratuberculosis strain ATCC 19698 cultured in Sauton media, suggesting that this difference is not caused by the strains of organism or culture medium (30). Rosseels et al. also showed by SDS-PAGE that M. paratuberculosis...
culosis CF was markedly different from *M. tuberculosis* H37Rv CF, where major protein bands were visible at up to 100 kDa. Other mycobacteria also have been shown to have proteins with more acidic pI values than CE proteins (5, 19, 24). In work with *Escherichia coli*, *Helicobacter pylori*, and *M. tuberculosis*, researchers have suggested there is a correlation between pI and subcellular protein localization, especially for cytoplasmic and integral membrane proteins (33, 41). Cytoplasmic proteins have pIs in the range of 5 to 6, whereas integral membrane proteins have pIs of 8.5 to 9. The data presented by Pleissner et al. also revealed a distinct bimodal protein pI pattern centered at around 5 and 9 for *M. tuberculosis* proteins (29). We suspect that the correlation of a protein’s pI with subcellular localization extends to secreted proteins. Moreover, the narrow range of pIs we found in *M. paratuberculosis* CF contrasted with CE supports the idea that these proteins are actively secreted and not simply released by autolysis of the cells as has been previously proposed (43).

The low pI value of CF proteins may point to their role in macrophage phagolysosome fusion. The low pH within lysosomes, i.e., pH 4.5 to 5.0, favors activation of a hydrolytic enzyme that degrades a wide range of macromolecule and microbes (13). In addition to being optimal for enzyme activation, this low pH would make many secreted mycobacterial proteins insoluble, with solubility being lowest when the pH approaches the pI of the protein. This fact may explain why pathogenic mycobacteria avoid fusion of the phagosome in which they reside in cells with lysosomes (31).
and why they have mechanisms that modulate the pH inside the phagosome (36).

The immunoblot and ELISA results presented here showed that serum antibodies from naturally M. paratuberculosis-infected cattle react more strongly and to more proteins in CF than in CE. The obvious explanation for this difference is accessibility. Secreted proteins are more available for interaction with antigen-presenting cells, and T and B lymphocytes, which lead to antibody production. The accessibility of the immune system to most CE proteins, except for those on the mycobacterial cell surface, is likely to be limited.

The diversity of binding patterns of bovine serum antibodies to CF proteins observed in the present study is consistent with that reported for other mycobacterial infections in cattle, deer, and humans (23, 42). Beck et al. found at least four serum antibody response profiles when testing human tuberculosis patient sera against M. tuberculosis antigens (2, 3). Serum antibody responses are primarily affected by the stage of infection. Little or no detectable antibody is produced until the Th1 to Th2 immune response shift occurs (28). The same pattern is seen in paratuberculosis: the early cell-mediated immune response contains the infection for most of the natural history of the M. paratuberculosis infection. The organism appears to be in a dormant state during this stage of infection. When, for unknown reasons, the animal’s immune system loses control of the pathogen, or the pathogen begins active replication, the organism disseminates, and the host response shifts to a Th2 type immune reaction. The hallmark of this infection stage is production of detectable serum antibodies. A positive relationship has been shown between the level of serum antibody production and the number of M. paratuberculosis isolated from fecal samples (8, 27).

A second possible explanation for the diversity in antibody-antigen binding patterns is host response variability to the infection. Paratuberculosis has diverse histopathological forms paralleling those of leprosy, ranging from lepromatous (multibacillary) to tuberculoid-like (paucibacillary) with many intermediate lesion categories (15). All forms are thought to represent the advanced stages of disease resulting from infection early in life since adult animals are generally resistant to infection (28). Tanaka et al. showed that cytokine gene expression profiles differ in lepromatous versus tuberculoid forms of paratuberculosis (40). Lepromatous lesions are associated with strong Th2-type cytokine expression and a strong antibody response.

Hormonal, physiological, nutritional, and pharmaceutical influences on the host immune response, both direct and indirect, may also contribute to serum antibody binding differences seen during paratuberculosis in cattle. During the few weeks prior to and after parturition, serum levels of antibody to M. paratuberculosis drop precipitously as antibody is shunted from serum into the colostrum (35). Feola et al. demonstrated the effects of bovine growth hormone on M. paratuberculosis
growth rates both inside and outside bovine macrophages (12). Levamisol-treated cattle showed enhanced cell-mediated immune responses to \textit{M. paratuberculosis} (26). Other parasitic and corticosteroids also may have immunomodulatory effects that could influence the character of serum antibody production to this pathogen (32).

Our study has several important implications for the serodiagnosis of bovine paratuberculosis. First, serologic tests may be improved by the use of \textit{M. paratuberculosis} proteins derived from CF instead of CE. Second, to increase the serodiagnostic sensitivity for \textit{M. paratuberculosis} infection, multiple antigens are likely required. Finally, depending on the stage of infection, not all fecal-culture-positive cows may have detectable serum antibodies to any \textit{M. paratuberculosis} protein.

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**REFERENCES**

1. Bech-Nielsen, S., J. B. Jorgensen, P. Ahrens, and N. C. Feld. 1992. Diagnostic accuracy of a \textit{Mycobacterium phlei}-absorbed serum enzyme-linked immunosorbent assay for diagnosis of bovine paratuberculosis in dairy cows. J. Clin. Microbiol. 30:613–618.

2. Beck, S. T., O. M. Leite, R. S. Arruda, and A. W. Ferreira. 2006. Use of Western blot/ELISA to improve the serological diagnosis of human tuberculosis. Braz. J. Infect. Dis. 10:93–104.

3. Bech-Nielsen, S., J. B. Jorgensen, P. Ahrens, and N. C. Feld. 1992. Comparative proteome analysis of culture supernatant proteins from virulent \textit{M. bovis} BCG strain 1173P2 and attenuated \textit{M. bovis} BCG strain 1173O. Environ. Microbiol. 69:5344–5349.

4. Bech-Nielsen, S., J. B. Jorgensen, P. Ahrens, and N. C. Feld. 2006. Immuno-modulatory effects of lipopolysaccharide from \textit{M. paratuberculosis} to acid environments as a function of culture medium. Appl. Environ. Microbiol. 72:5549–5553.
39. Sung, N., K. Takayama, and M. T. Collins. 2004. Possible association of GroES and antigen 85 proteins with heat resistance of Mycobacterium paratuberculosis. Appl. Environ. Microbiol. 70:1688–1697.

40. Tanaka, S., M. Sato, T. Onitsuka, H. Kamata, and Y. Yokomizo. 2005. Inflammatory cytokine gene expression in different types of granulomatous lesions during asymptomatic stages of bovine paratuberculosis. Vet. Pathol. 42:579–588.

41. VanBogelen, R. A., E. E. Schiller, J. D. Thomas, and F. C. Neidhardt. 1999. Diagnosis of cellular states of microbial organisms using proteomics. Electrophoresis 20:2149–2159.

42. Waters, W. R., M. V. Palmer, J. P. Bannantine, D. L. Whipple, R. Greenwald, J. Esfandiari, P. Andersen, J. McNair, J. M. Pollock, and K. P. Lyashchenko. 2004. Antigen recognition by serum antibodies in white-tailed deer (Odocoileus virginianus) experimentally infected with Mycobacterium bovis. Clin. Diagn. Lab. Immunol. 11:849–855.

43. Wayne, L. G., and G. A. Diaz. 1967. Autolysis and secondary growth of Mycobacterium tuberculosis in submerged culture. J. Bacteriol. 93:1374–1381.

44. Whittington, R. J., A. F. Hope, D. J. Marshall, C. A. Taragel, and I. Marsh. 2000. Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis: IS901 restriction fragment length polymorphism and IS1311 polymorphism analyses of isolates from animals and a human in Australia. J. Clin. Microbiol. 38:3240–3248.

45. Xiong, Y., M. J. Chalmers, F. P. Gao, T. A. Cross, and A. G. Marshall. 2005. Identification of Mycobacterium tuberculosis H37Rv integral membrane proteins by one-dimensional gel electrophoresis and liquid chromatography electrospray ionization tandem mass spectrometry. J. Proteome Res. 4:855–861.

46. Yokomizo, Y., R. S. Merkal, and P. A. Lyle. 1983. Enzyme-linked immunosorbent assay for detection of bovine immunoglobulin G1 antibody to a protoplasmic antigen of Mycobacterium paratuberculosis. Am. J. Vet. Res. 44:2205–2207.