Antigens of *Brucella abortus* S19 Immunodominant for Bovine Lymphocytes as Identified by One- and Two-Dimensional Cellular Immunoblotting

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Cellular immune responses are essential for protection against intracellular bacteria such as brucellae. Therefore, identification of *Brucella abortus* antigens that activate primed bovine lymphocytes is fundamental for discerning the breadth of cellular response in bovine brucellosis. Potentially antigenic components of *B. abortus* S19 were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by nitrocellulose blotting. Specific one-dimensional blot segments induced proliferation of peripheral blood lymphocytes from all 25 of the vaccinated cattle tested and were defined as immunodominant. Individual proteins that stimulated lymphocyte proliferation were further characterized by two-dimensional cellular immunoblotting by two different approaches. Individual one-dimensional stimulatory blot segments were eluted, concentrated, and then subjected to two-dimensional cellular immunoblotting. Alternatively, entire two-dimensional gels containing all of the *B. abortus* components were blotted and nitrocellulose sections containing individual proteins were assayed for lymphocyte activation. Thirty-eight *Brucella* proteins that induced lymphocyte proliferation were resolved by both procedures. Phenotypic analysis of the proliferating cell population demonstrated the presence of CD4+, CD8+, and immunoglobulin M+ lymphocytes. Two immunogenic proteins, 12 and 31 kDa, identified by two-dimensional cellular immunoblotting, were subjected to partial N-terminal amino acid analysis. The 12-kDa protein was within the area of greatest lymphocyte proliferation, while the 31-kDa protein was chosen for comparison with a 31-kDa protein previously reported by others. A search of the National Biomedical Research Foundation protein data bank showed that the sequences were not homologous with other known proteins. Identification of *Brucella* proteins immunogenic for bovine lymphocytes provides an important step in distinguishing the various proteins involved in pathogenicity and/or disease resistance.

Immunity to intracellular bacteria, such as brucellae, is dependent on activation of appropriate host cellular responses (6, 13, 16). Identification of individual bacterial proteins that elicit lymphocyte proliferation is therefore important. Complex protein preparations have been used previously to investigate in vitro cellular stimulation (10, 35, 38); however, isolation and identification of individual stimulatory components in complex mixtures is difficult. Alternatively, genes that encode individual bacterial proteins can be cloned and subsequently assayed for immunogenic potential (9, 33). This strategy, however, may result in expenditure of considerable time and resources ultimately to identify proteins that are not recognized by lymphocytes from animals immunized to the intact bacterium. Previously, influenza hemagglutinin bound to nitrocellulose was used to obtain viral proteins stimulatory for T-cell clones (41). A similar nitrocellulose technique has been used to identify bacterial proteins recognized by freshly isolated peripheral blood mononuclear (PBM) cells from vaccinated cattle (4). These techniques facilitated identification of microbial components stimulatory for host cells without cloning genes or purifying individual proteins.

Brucellae are gram-negative facultative intracellular bacteria that cause disease in human beings (1) and contagious abortion in many animal species (40). In the United States, infection of cattle with *Brucella abortus* is still evident, even with current control measures utilizing *B. abortus* S19 vaccination. Disadvantages associated with the use of this live attenuated vaccine include its ability to cause disease in cattle (7) and human beings (42) and the difficulty in distinguishing vaccinated from infected animals (34). Because of these limitations, vaccine alternatives based on characterized bacterial antigens that stimulate immune responsiveness are essential. Brucellae, however, have a cell envelope that possesses unique characteristics that make the study of individual *Brucella* components difficult. Unlike other gram-negative bacteria, most *Brucella* species do not form spheroplasts when treated with Tris, EDTA, and lysozyme (23). Comparative studies of *Escherichia coli* and *Brucella* spp. have shown that nonionic detergents are less effective than ionic detergents in extracting proteins from brucellae (23). Therefore, most studies of *Brucella* proteins have used ionic detergents (11, 37, 38).

Identification of internal and cell envelope *Brucella* proteins that induce cellular responses has been arduous because of the detergents necessary for protein extraction. Evaluation of immunogenic *Brucella* proteins has been limited to complex protein preparations (2, 32, 39) or a small number of recombinant protein products (29, 31). In the present study, individual *B. abortus* proteins that induced proliferation of lymphocytes from previously immunized cattle were identified by immunoblotting, which permitted screening of a complex bacterial preparation for immunogenic antigens. By using heterogeneous lymphocyte populations, the greatest repertoire of lymphocytes specific for an array of *Brucella* antigens was determined in a large popu-

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lation of animals. Two of the immunogenic Brucella proteins of interest distinguished by two-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were subjected to partial N-terminal amino acid analysis. Particular Brucella proteins that are recognized by lymphocytes may be important in disease resistance and ultimately would be included as components in alternative vaccines.

MATERIALS AND METHODS

Animals. Twenty-five Guernsey, Brown Swiss, Ayshires, and Holstein cows and steers (aged 1 to 8 years) were kept at the University of Wisconsin Department of Dairy Science, Madison. Twenty-two animals received one injection of B. abortus S19 vaccine (Coopers Animal Health, Inc., Kansas City, Kans.) at or before 6 months of age. Three cattle received two to four injections of the S19 vaccine. Cattle were bled between 1 and 8 years after final vaccination. Five nonvaccinated animals served as controls. All cattle were seronegative by the brucella card test (Hynson, Westcott and Dunning, Baltimore, Md.).

SDS extraction of B. abortus S19 proteins. Proteins from gamma-irradiated (137Cs) B. abortus S19 were prepared by a modification of a previously described method (37). Briefly, 30 mg (wet weight) of bacteria was suspended in 150 ml of extraction buffer containing 2% SDS, 10 mM of Tris, 0.7 M 2-mercaptoethanol, and 10% glycerol. The suspension was kept at 65°C for 4 h, and additional fresh buffer was added at 2 h. After 4 h, the suspension was subjected to lysozyme digestion overnight at 37°C (1 mg of lysozyme per 60 mg [wet weight] of bacteria). The suspension was boiled for 10 min and ultracentrifuged at 150,000 × g for 1 h at 4°C. The supernatant was aliquoted and frozen at −70°C until needed.

Protein determination. Protein concentrations were determined by using the bicinechonic acid protein determination assay (Pierce Chemical Co., Rockford, Ill.) in accordance with the manufacturer’s instructions.

Preparation of one-dimensional immunoblots. SDS-extracted B. abortus S19 proteins were prepared as described above and then separated by SDS-PAGE (14) using a 15% polyacrylamide gel. The gels were electroblotted onto nitrocellulose at 30 V overnight with an increase to 70 V for the final 2 h. Transfers were performed in 25 mM Tris–192 mM glycine–20% methanol. A portion of the blotted nitrocellulose was stained with 0.1% amido black (Sigma Chemical Co., St. Louis, Mo.) to visualize the transferred proteins. The unstained portion of the blot was cut into 32 sections (4 by 4 mm) corresponding to the entire length of the gel (35 μg of total protein per lane of gel) and placed into 96-well tissue culture plates (Costar, Cambridge, Mass.). One blot section was placed into each well. The plates containing the nitrocellulose sections were sterilized by gamma irradiation (137Cs). Freshly isolated bovine PBM cells were then added at 7 × 105 per well.

Production of two-dimensional immunoblots. Two-dimensional cellular immunoblotting was performed by two approaches. First, individual molecular weight regions that stimulated bovine PBM cells were eluted from the one-dimensional nitrocellulose blot. The protein-containing nitrocellulose strips were cut and placed into elution buffer containing 9.5 M urea (Bethesda Research Laboratories, Gaithersburg, Md.), 5% 2-mercaptoethanol, and 2% Nonidet P-40 (Sigma) and incubated for 3 days at room temperature and then at 37°C for 2 h. SDS was not added at this step because excessive SDS was determined to be inhibitory to lymphocytes. The Nonidet P-40 was used to extract the Brucella proteins from the nitrocellulose membrane. Because of the consistent protein patterns and the absence of proteins weighing less than 6 kDa, proteolysis of the separated Brucella components was considered minimal. Eluted proteins were concentrated (Speedvac concentrator, SVC 100H; Forma Scientific Inc., Marietta, Ohio) and individually subjected to two-dimensional gel electrophoresis (35 μl of protein per lane) by the method of O’Farrell (26). The first dimension was isoelectric focusing with a pH range of 3.0 to 9.0, and the second dimension was SDS–15% PAGE. The two-dimensional gels were electroblotted onto nitrocellulose and prepared for use in cell stimulation assays as described above. The second approach consisted of sectioning (4 by 4 mm) the entire two-dimensional nitrocellulose blot containing the SDS-extracted B. abortus S19 total-protein preparation (156 to 188 μg of protein per gel) and analyzing the nitrocellulose sections that induced PBM cell proliferation.

Culturing of bovine PBM cells. PBM cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed AS Diagnostics, Oslo, Norway). Cells were washed three times with sterile phosphate-buffered saline (PBS) containing 100 IU of penicillin and 100 μg of streptomycin per ml and suspended in RPMI 1640 (Sigma) supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 25 mM HEPES (N-2-hydroxyethylpiperezine-N′-2-ethanesulfonic acid), 5 × 10−5 M 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (Sigma). The cells, 7 × 106 per well, were cultured in 96-well tissue culture plates containing nitrocellulose blot 3 days at 37°C and then exposed to [3H]thymidine (1 μCi per well) for 8 h. The cells were harvested onto glass fiber filters and lysed, and radioactivity was counted in a liquid scintillation counter. Blot segments obtained from the one-dimensional gels described above were considered to have a 1X protein concentration. To examine the effect of the protein concentration in a blot segment on lymphocyte proliferation, one-dimensional gels containing 0.1X and 4X concentrations of Brucella total protein were produced. Results were considered significant when the mean counts per minute were 3 standard deviations above the background. Results for each animal were the means of duplicate or triplicate cultures in at least eight different experiments.

Flow cytomtery. PBM cells (106) in 0.1 ml of PBS containing 1% bovine serum albumin (Sigma), 0.2% sodium azide (NaN3), and 0.05 ml of a monoclonal antibody were incubated on ice for 30 min. The cell suspension was washed twice with PBS with NaN3 prior to suspension in 50 μl of anti-mouse immunoglobulin (lg)–dichlorotriazinylaminofluorescein (lg-DTAF; H & L, Jackson Labs, Avondale, Pa.). The cells were incubated in the dark for 30 min on ice, washed twice, suspended in PBS containing 1% paraformaldehyde, and stored in the dark at 4°C until analyzed by flow cytometry (Becton Dickinson, Mountain View, Calif.). The monoclonal antibodies used were 16-1E10 (anti-CD2 [27]), IL-A12 (anti-CD4 [3]), SBU-T8 (anti-CD8 [17]), and 33 (anti-bovine IgM [25]).

Partial amino acid sequencing of proteins. Proteins were separated by two-dimensional SDS-PAGE and electroblotted onto Immobilon (Millipore Corp., Bedford, Mass.) in accordance with the manufacturer’s instructions. The blotted proteins (31 and 12 KDa, 10 pmol of each) were stained with Coomassie blue (Bio-Rad, Richmond, Calif.) and sequenced by Gary Hathaway, University of California, Riverside, using an Applied Biosystems model A vapor phase protein sequencer. Obtained sequences were compared with known sequences in the National Biomedical Research
FIG. 1. Significant cellular proliferative responses ($P \leq 0.05$) of once- and hypervaccinated cattle to 32 nitrocellulose blot segments of \textit{B. abortus} S19 proteins. [$^3$H]thymidine incorporation responses are means of 22 once-vaccinated animals and 3 hypervaccinated animals that are 3 standard deviations above the background (nitrocellulose without antigen) minus the background. When the hypervaccinated response predominated, the vaccinated animals were within 3,000 cpm of the hypervaccinated animals. A Coomassie blue-stained SDS–15% PAGE gel of \textit{B. abortus} S19 aligned to the stimulatory blot segments and molecular masses are shown below. kd, kilodaltons.

Foundation protein data bank by using the University of Wisconsin Genetics Computer Group program FIND and allowing two mismatches.

Statistics. Significant lymphocyte proliferation was determined by the Student $t$ test.

RESULTS

Proliferation of bovine PBM cells in response to one-dimensional immunoblotted \textit{B. abortus} S19 proteins. \textit{B. abortus} S19 proteins were separated by one-dimensional SDS-PAGE and then assayed for induction of bovine PBM cell proliferation. Significant ($P \leq 0.05$) mean proliferative responses that were 3 standard deviations above the background (nitrocellulose without antigen) are shown in Fig. 1. The proliferative responses of PBM cells from 25 \textit{B. abortus} S19-vaccinated cattle to the one-dimensional blotted proteins were assayed multiple times. Three of the cattle received two to four injections of the S19 vaccine (hyper-vaccinated), whereas the remaining 22 cattle were vaccinated once. Cells from hyper- and once-vaccinated cattle proliferated in response to similar blot segments. However, individual animals varied in the degree of proliferation in response to any one blot segment. Blot segments containing antigens that stimulated cell proliferation in all of the cattle tested were defined as immunodominant. These proteins were present between 116 and 97, 87 and 43, 38 and 30, 25 and 23, and 21 and 6 kDa. When the hypervaccinated response predominated, the vaccinated animals were within 3,000 cpm of the hypervaccinated animals. Cells from non-vaccinated cattle did not respond in vitro to protein blots, whole gamma-irradiated \textit{B. abortus} S19, or nitrocellulose without antigen, as previously observed (4). Immunoblotting experiments in which different protein concentrations (0.1$x$ and 4$x$) were loaded on the gels determined that other stimulatory antigens were not evident in other blot segments (data not shown).

Proliferation of bovine PBM cells in response to two-dimensional immunoblotted proteins. Silver staining of the two-dimensional SDS-PAGE containing the SDS-extracted \textit{Brucella} protein preparation revealed hundreds of individual proteins (Fig. 2). Individual proteins present in molecular size regions of Fig. 1 that were recognized by lymphocytes from vaccinated animals were identified by two approaches. In the first approach, one-dimensional bands that stimulated bovine PBM cells were eluted from the nitrocellulose with a 9.5 M urea solution. Eluted proteins were concentrated, subjected to two-dimensional gel electrophoresis, and blotted onto nitrocellulose. The nitrocellulose containing the blotted proteins was cut into sections (4 by 4 mm), placed
into 96-well plates, and assayed for in vitro cellular proliferation. In the second approach, a two-dimensional SDS-PAGE gel containing all of the protein components of \textit{B. abortus} was electroblotted onto nitrocellulose and the entire blot was cut into sections (4 by 4 mm). The immunogenic proteins are identified with circles in Fig. 2. Ten different cultures were assayed multiple times by both approaches to identify proteins that consistently stimulated lymphocyte proliferation. The first approach allowed concentration of proteins for cellular assays and partial N-terminal amino acid sequencing, whereas the second approach confirmed the identities and locations of the cellular stimulatory proteins in the two-dimensional gels.

**Flow cytometry.** Flow cytometric analysis using monoclonal antibodies to CD2, CD4, CD8, and IgM was conducted on proliferating cells 6 or 10 days following stimulation with proteins isolated by one- or two-dimensional SDS-PAGE. Selected cultures received a second in vitro antigen stimulation on day 7 and were assayed for surface phenotype on day 10. Cells that proliferated in response to the individual proteins were approximately 23\% \pm 3\% CD4\(^+\), 12\% \pm 2\% CD8\(^+\), and 35\% \pm 1\% IgM\(^+\) after 6 and 10 days of stimulation. The distribution of lymphocyte phenotypes was similar regardless of the protein blot tested.

**Sequencing of two-dimensional cellular stimulatory proteins.** The N-terminal amino acids of two of the stimulatory \textit{B. abortus} proteins distinguished at the two-dimensional level (identified by arrows in Fig. 2) were partially sequenced (Table 1). The 12-kDa protein was present in the area of greatest lymphocyte proliferation and was of sufficient quantity for sequencing. The 12-kDa protein of greatest concentration in Fig. 2 was not possible to sequence accurately because of contamination by lysozyme used for bacterial digestion. The 31-kDa protein was chosen for comparison with a 31-kDa immunogenic protein previously reported by others (33). The 12- and 31-kDa sequences were not similar to other known protein sequences revealed in the National Biomedical Research Foundation protein data bank by the FIND program.

**DISCUSSION**

In the present study, by using one- and two-dimensional SDS-PAGE followed by immunoblotting, we identified individual \textit{B. abortus} 19 components that stimulated proliferation of freshly isolated bovine lymphocytes. Specific nitrocellulose blot segments from one-dimensional SDS-PAGE induced proliferation of PBM cells from 25 cattle and were termed immunodominant proteins. These proteins were present between 116 and 97, 87 and 43, 38 and 30, 25 and 23, and 21 and 6 kDa. Similar blot segments were immunodominant for PBM cells from once- and hypervaccinated animals.

Because a number of proteins were observed in each blot, two-dimensional SDS-PAGE techniques were employed to identify immunodominant proteins. First, elution of stimulatory proteins from nitrocellulose blots permitted sufficient protein concentration for partial amino acid sequencing. Previous studies using cellular immunoblotting techniques did not include subsequent sequencing of immunogenic proteins (4, 12, 15, 20, 28). Our first approach also controlled for variability in the two-dimensional SDS-PAGE positions of proteins by assaying blot regions individually. Others have eluted proteins from two-dimensional gels, pooled the fractions from different gels, and assayed for lymphocyte proliferation capabilities (12). Elution of proteins from the two-dimensional gels does not account for various protein mobilities in different gels. Also, protein components may be lost as a result of electroelution. The second approach used to identify immunodominant proteins used the entire two-dimensional gels containing all of the \textit{B. abortus} components and then blotting onto nitrocellulose. The nitrocellulose was cut into sections and assayed for lymphocyte proliferation. This approach confirmed the locations of proteins from the two-dimensional gels that stimulated lymphocyte proliferation. However, this technique was labor-intensive and did not allow concentration of proteins for amino acid sequence analysis. Others have identified \textit{Leishmania} proteins by using a similar technique (20). Previously, we have reported the presence of \textit{Brucella} lipopolysaccharide (LPS) throughout electroblotted one-dimensional SDS-PAGE protein preparations (4). In the present study, phenotypic analysis of proliferating cells demonstrated preferential stimulation of CD4\(^+\) and IgM\(^+\) cells. Since \textit{Brucella} LPS does not bind polymyxin B (22), the contribution of \textit{Brucella} LPS to cellular stimulation could not be addressed. However, we have previously demonstrated that lymphocytes proliferated only in response to selected blotted \textit{Brucella} proteins (4). Specifically, lymphocytes primed to one set of blotted \textit{Brucella} proteins proliferated in response to proteins of the same but not different gel regions upon secondary in vitro lymphocyte proliferation. These findings indirectly support the absence of a mitogenic role for \textit{Brucella} LPS. Although \textit{B. abortus} components are mitogenic for human lymphocytes (36), there is no evidence to support a similar role for \textit{Brucella} LPS when bovine lymphocytes are used.

A number of \textit{Brucella} proteins have been isolated and identified by molecular weight (5) and have had specific functions assigned (8, 11, 30). In the present study, the partially sequenced 31- to 32-kDa protein that stimulates lymphocyte proliferation does not contain sequence homology to a previously reported 31-kDa \textit{Brucella} protein (18) that has been used to immunize mice against \textit{Brucella} infection (29, 33). Whether the stimulatory proteins identified in this study are similar to other reported \textit{Brucella} proteins comparable in molecular weight remains unknown (5, 11, 37). Comparison of the amino acid sequences of the 12- and 31-kDa proteins in this study with other known sequences in the National Biomedical Research Foundation protein data bank allowing two mismatches indicated that the proteins have not been previously sequenced.

The present findings identified an array of bacterial antigens that specifically stimulate freshly isolated lymphocytes from vaccinated animals. The role that these identified proteins might have in protection against disease or in pathogenesis remains to be established. However, demonstration that specific proteins are recognized by antigen-primed cells from immune protected animals provides a first indication of the relevance of selected \textit{Brucella} proteins. Others have used T lymphocytes to identify proteins of \textit{Mycobacterium leprae} unique to vaccine strains and patho-

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**TABLE 1.** Characteristics of two partially sequenced N termini of \textit{B. abortus} S19 cellular stimulatory proteins

<table>
<thead>
<tr>
<th>Molecular mass (kDa)</th>
<th>Relative pl(^a)</th>
<th>Partial amino acid sequence(^b)</th>
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<tr>
<td>31-32</td>
<td>6.2</td>
<td>XNKTIXVGMHPKDF</td>
</tr>
<tr>
<td>12</td>
<td>5.5</td>
<td>ADLAEIVEDLSA</td>
</tr>
</tbody>
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\(^a\) Isoelectric point as determined under experimental conditions.  
\(^b\) X, amino acid unknown.

**REFERENCES**

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genic mycobacteria that are not recognized by antibodies (24). This approach has been instrumental in evaluating the relative importance of the T-lymphocyte repertoire in M. leprae-immune individuals.

Additional experiments using selected protein concentrations to stimulate lymphocyte proliferation suggested that no additional lymphocyte-proliferative components were detected in the B. abortus preparation. The techniques employed in the present study allowed isolation and analysis of Brucella proteins despite the use of detergents in the extraction procedure. However, we cannot exclude the possibility that certain proteins identified by one-dimensional immunoblotting were overlooked when lymphocyte reactivity was assessed by two-dimensional blotting. With freshly isolated lymphocytes, the maximal Brucella-specific cellular repertoire is available to recognize particular antigens in a complex mixture of bacterial components. Alternatively, only a few antigens can be selected by using cloned lymphocyte lines, thus limiting the number of bacterial antigens for future evaluation. Use of cloned lymphocytes places the future availability to recognize certain proteins identified by antibodies clonal lymphocyte and not the Toxoplasma gondii, as demonstrated by one- and two-dimensional gel immunoblotting for the B. abortus strain 19 antigen. This approach has been instrumental in evaluating the functional reactivity of the T-lymphocyte clones. J. Exp. Med. 171:831-841.


