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Lymphocyte Proliferation in Response to Immunodominant Antigens of *Brucella abortus* 2308 and RB51 in Strain 2308-Infected Cattle

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Lymphocyte proliferation in response to proteins from the *Brucella abortus* strain 2308 (S2308) and the lipopolysaccharide (LPS) O-antigen-deficient mutant of S2308, strain RB51 (SRB51), was measured in S2308-infected cattle following abortion. Supramammary and superficial cervical lymph node lymphocytes from infected cattle proliferated most when incubated with 27- to 18-kDa proteins of S2308 or SRB51. Proteins of SRB51, which contained no LPS O antigens, induced lymphocyte proliferation similar to that induced by S2308 proteins, which contained LPS O antigens. These results indicate that 27- to 18-kDa proteins, but not LPS O antigens, of S2308 and SRB51 are immunodominant in S2308-infected cattle as assessed by lymphocyte proliferation assays.

Brucella abortus is an intracellular bacterium that causes abortions in cattle (5) and undulant fever, endocarditis, arthritis, and osteomyelitis in humans (16). Like protective immunity to other intracellular bacteria, protective immunity to *B. abortus* results mainly from activated macrophages and lymphocytes and other cell-mediated immune responses (7). Studies evaluating cellular immune responses in cattle have focused on identifying *B. abortus* antigens that might replace the attenuated *B. abortus* strain 19 (S19) vaccine that is currently used to immunize cattle. Such studies have relied on measuring proliferation by lymphocytes in response to isolated *B. abortus* proteins in cattle that have been immunized or hyperimmunized with the attenuated *B. abortus* S19 vaccine (1–3, 11, 15). However, these types of analyses do not definitively identify antigens that cattle respond to during disease and abortions that results from infection with virulent strains of *B. abortus*. An analysis of the antigens that cattle respond to during clinical infections but not after vaccination with S19 would more likely identify antigens that are critical for immunity to brucellosis. Furthermore, proteins isolated from *B. abortus* S19 are contaminated with lipopolysaccharide (LPS) O antigens (1, 10, 14), and cattle produce antibodies to these antigens when vaccinated with S19 (6, 13). Therefore, previous reports of lymphocyte proliferation in S19-vaccinated cattle in response to isolated S19 proteins may have instead resulted from proliferation in response to LPS O antigens that contaminated the proteins.

The objectives of the current study were to determine which proteins of *B. abortus* are recognized as immunodominant in cattle with brucellosis and whether the response to the proteins results from contamination with LPS O antigens. These objectives were accomplished by infecting cattle with virulent *B. abortus* strain 2308 (S2308) and measuring lymph node (LN) cell proliferation in response to proteins isolated from both S2308 and its rough mutant, strain RB51 (SRB51), which lacks the LPS O antigens (9, 10).

Isolation of S2308 and SRB51 proteins. Cultures of *B. abortus* S2308 and SRB51 were grown, harvested, killed by gamma irradiation, and then stored at −70°C as described previously (12). The bacteria were thawed, and whole-cell lysates from S2308 or SRB51 were separated into 106- to 18-kDa proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1) as described previously (12). The S2308 and SRB51 proteins were eluted from the gel into 22 fractions with a Blotelutor B35 (Biometra, Göttingen, Germany). Each protein fraction was then filter sterilized and added to RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing L-glutamine, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10% fetal bovine serum (HyClone Laboratories, Logan, Utah), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5×10^{-5} M 2-mercaptoethanol. This supplemented medium is referred to here as RPMI. A 50-μl aliquot of each of the 22 protein fractions (undiluted or at a 1:2 or 1:4 dilution) of S2308 or SRB51 was added to two separate wells of a 96-well flat-bottom microtiter plate, and the plates were stored at −70°C. Each added fraction was also tested for the presence of LPS O antigens by a dot enzyme-linked immunosorbent assay with a monoclonal antibody to S2308 LPS O antigens (8). This analysis revealed that O antigens were present in S2308 fractions (fractions 1 to 16) that contained 106- to 18-kDa proteins (Fig. 1). These results are similar to those reported for other studies that have shown that 94- to 20-kDa proteins from S2308 (10) and most of the proteins from other smooth strains of *B. abortus* are contaminated with LPS O antigens, which is revealed when the proteins are separated by gel electrophoresis (1, 12, 14). No LPS O antigens were detected in the 22 protein fractions of SRB51 (Fig. 1). This result agrees with results of previous reports that have shown SRB51 does not contain O antigens (9, 10).

LN cell suspensions from S2308-infected cattle. Four pregnant polled Hereford heifers at the fifth to sixth month of pregnancy were infected with S2308 by placing 50 μl of 0.85% NaCl saline solution containing 5×10^6 CFU of S2308 into the right and left conjunctival sacs (total dose, 10^7 CFU). The supramammary and superficial cervical LNs were obtained from S2308-infected heifers at necropsy, which was performed 1 to 2 weeks after the animals aborted. Each LN was minced with scissors and processed to form a single-cell suspension as

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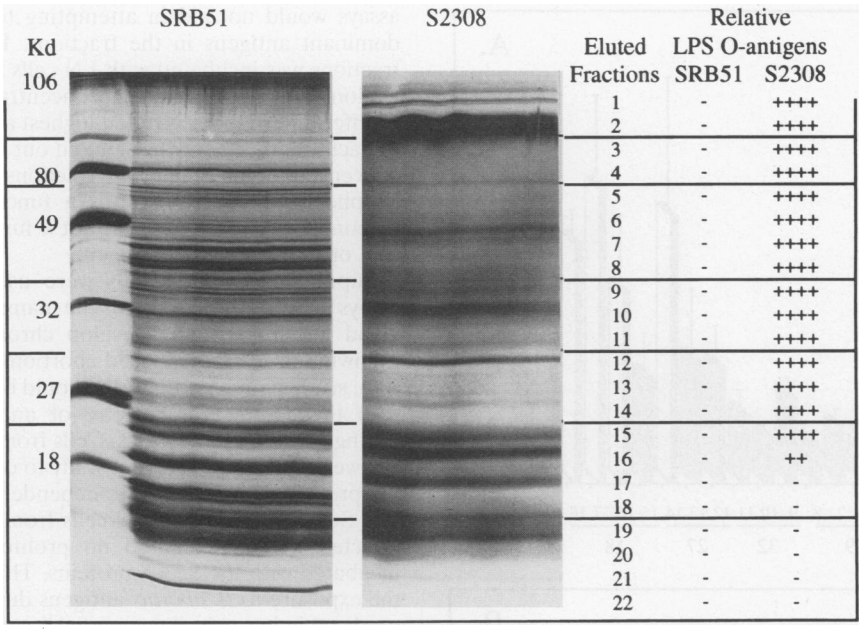

FIG. 1. Silver-stained gel electrophoresis of sodium dodecyl sulfate-extracted *B. abortus* SRB51 and S2308 proteins and LPS O-antigen content in eluted protein fractions. Positive (+) and negative (−) signs indicate the relative amount of LPS O antigens contained in the eluted protein fractions as measured by a dot enzyme-linked immunosorbent assay with a monoclonal antibody to S2308 LPS O antigen.

described previously (12). Cell viability in all LN samples was greater than 90% as assessed by cell staining with trypan blue.

LN cell proliferation in response to S2308 and SRB51 proteins. Plates containing the 22 isolated S2308 or SRB51 protein fractions were thawed, and a 50-μl aliquot of the LN cell suspensions (10^7 cells per ml) in RPMI was added to each fraction. All LN cell cultures were incubated for 5 or 7 days at 37°C in 5% CO_2 and mixed every two days during the incubations as previously described (12). After the 5- or 7-day incubation, cell cultures were pulsed for 18 h with 1.0 μCi of [^{3}H]thymidine per well. Cells were harvested onto glass filters and measured for radioactivity in counts per minute in a liquid scintillation counter. The count per minute from cells incubated alone (background) was subtracted from the count per minute of cells that were incubated with the protein fractions. Cell proliferation results were expressed as mean counts per minute minus background counts per minute ± the standard deviation incorporated by duplicate cultures. Stimulation indices (SI) were calculated by dividing the count per minute of LN cells incubated with proteins by the count per minute of LN cells incubated alone.

Supramammary LN cells from S2308-infected cattle exhibited the greatest proliferative response when incubated for 5 days with S2308 fractions (fractions 14 and 16 to 19) that contained 27- to 18-kDa or <18-kDa proteins (Fig. 2A) and with SRB51 fractions (fractions 15 to 20) that contained 18-kDa or <18-kDa proteins (Fig. 2B). Four of the same fractions of S2308 and SRB51 (fractions 16 to 19) produced an SI of greater than 10 when incubated for 5 days with the LN cells. However, differences occurred between the S2308 and SRB51 fractions in that fraction 14 of S2308 but not of SRB51 and fraction 20 of SRB51 but not of S2308 induced an SI of greater than 10. The remaining fractions of S2308 (fractions 1 to 13, 15, and 20 to 22) and SRB51 (fractions 1 to 15, 21, and 22) produced an SI of less than 10 (Fig. 2). Proliferative responses similar to those shown in Fig. 2 (5-day incubation) occurred when supramammary LN cells from S2308-infected cattle were incubated for 7 days with each of the same 22 protein fractions of S2308 or SRB51 (data not shown). Superficial cervical LN cells from three of the four S2308-infected cattle proliferated when incubated with the protein fractions of S2308 or SRB51 (data not shown). S2308 proteins of 27 to 18 kDa and SRB51 proteins of 18 kDa or smaller induced the greatest proliferation when incubated for 5 or 7 days with the superficial cervical LN cells (data not shown). All tested dilutions (1:2 and 1:4) of the 22 protein fractions revealed that S2308 proteins of 27 to 18 kDa and SRB51 proteins of 18 kDa or smaller induced the greatest proliferation when incubated for 5 or 7 days with either supramammary or superficial cervical LN cells (data not shown).

Results of this study showed that following abortions, S2308-infected cattle have LN cells that proliferate primarily in response to the 27- to 18-kDa proteins of S2308. Similar results also occurred when LN cells were incubated with proteins from SRB51, which contained no detectable LPS O antigens. Previous studies have shown that S2308 proteins are contaminated with LPS O antigens, which is revealed when the proteins are separated by gel electrophoresis (10, 12, 14). The same finding occurred in the current study; however, several results indicated that S2308 proteins but not S2308 LPS were responsible for inducing proliferation following incubation of proteins with the LN cells. For example, no relationship existed between cell proliferation and the presence or absence of LPS O antigens in the S2308 protein fractions. Instead, LN cells from S2308-infected cattle exhibited the greatest proliferation in response to S2308 protein fractions that either contained (fractions 14 and 16) or did not contain (fractions 17–19) LPS O antigens. This would be an unexpected result if the O antigens were the sole source of the stimulatory activity in the tested protein fractions. In addition, SRB51 proteins did not contain O antigens, and yet these proteins induced levels of LN cell proliferation similar to those induced by S2308 proteins. Collectively, these results suggest that the LN cell proliferation found in the current study arose from stimulation by S2308

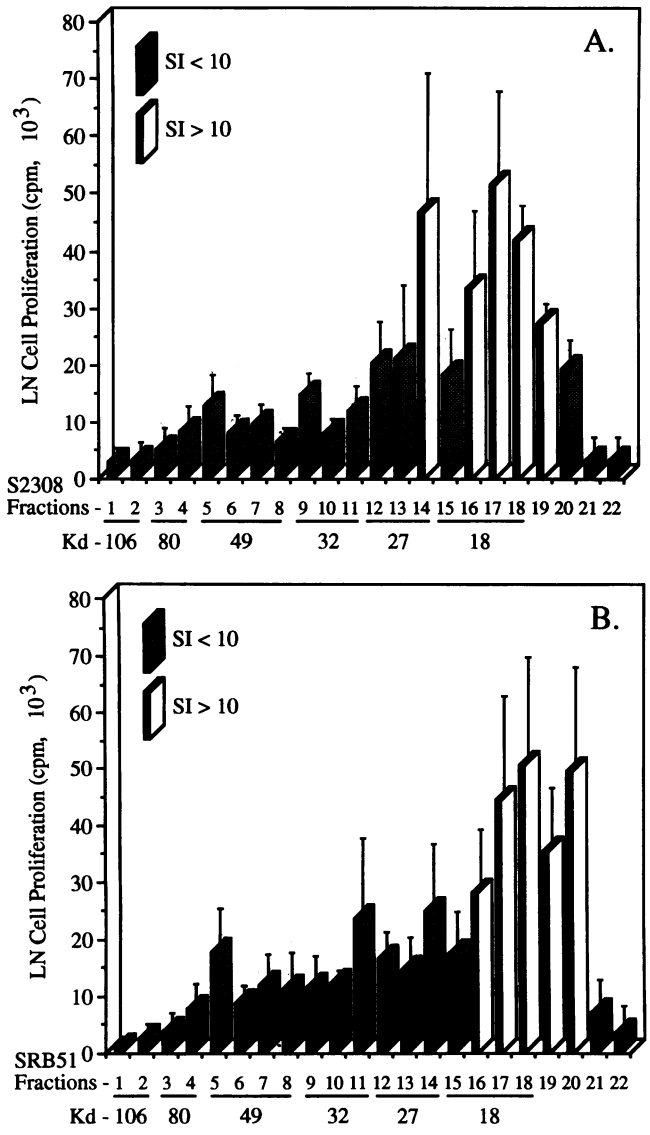

FIG. 2. Proliferation of supramammary LN cells from *B. abortus* S2308-infected cattle in response to S2308 (A) and SRB51 (B) proteins. LN cells were obtained from S2308-infected cattle following abortion. A total of 5×10^5 cells per well were incubated for 5 days with each of the 22 protein fractions of S2308 or SRB51 and then pulsed for 18 h with [^{3}H]thymidine. Results are presented as mean counts per minute ± the standard deviation for four S2308-infected cattle. Protein fractions giving a mean SI of more or less than 10 are indicated. The mean proliferative responses ± the standard deviation for LN cells incubated alone were 2,695 ± 898 (A) and 3,141 ± 1,076 (B).

proteins rather than by the LPS O antigens. A previous study has also shown that blood lymphocytes from S19-vaccinated cattle exhibit proliferative responses to several recombinant S2308 fusion proteins which contain no S2308 LPS O antigens (17).

In our study, each of the 22 fractions of S2308 and SRB51 did not contain a single protein but instead contained groups of proteins with similar molecular masses, and certainly only some proteins in each fraction stimulated lymphocyte proliferation. Therefore, adjusting each fraction to the same protein concentration when performing the lymphocyte proliferation assays would not aid in attempting to identify the immunodominant antigens in the fractions. Instead, each of the 22 fractions was incubated with LN cells at an undiluted concentration and at 1:2 and 1:4 concentrations. The lymphocyte proliferative responses to the highest and lowest concentration of each fraction were the same in our study. Thus, the antigen concentration in the tested fractions was high enough that lymphocyte proliferation was a function of the number of responding lymphocytes and not a function of the concentration of the stimulating antigen.

Supramammary LN cells were used in the proliferation assays because this LN drains the mammary gland and both the gland and LN usually develop chronic localized infections following *B. abortus*-induced abortions in cattle (4, 5). Therefore, supramammary LN cells should be chronically stimulated with the complete repertoire of antigens that are present during *B. abortus* infections. Cells from the superficial cervical LN were used in the current study to determine if responses in the proliferation assays were dependent on the location of the LN. Superficial cervical LN cells from one of the four S2308-infected heifers exhibited no proliferative responses when incubated with the S2308 proteins. This was probably because the exposure to *B. abortus* antigens during abortions would be predictably lower in the superficial cervical than in the supramammary LN.

Previous studies have not measured proliferation in response to S2308 proteins by lymphocytes obtained from S2308-infected cattle. However, studies by Brooks-Worrell and Splitter have shown that peripheral blood lymphocytes from S19-vaccinated cattle proliferate in response to 116- to 6-kDa proteins of S19 or S2308 (2, 3). In addition, the greatest proliferation appears to occur in response to S19 proteins of 66 to 43 and 21 to 6 kDa (3). These results differ from those reported in the current article because S2308 proteins of 27 to 18 kDa, or less than 18 kDa, induced the greatest proliferation when incubated with LN lymphocytes from S2308-infected cattle. However, a critical comparison of our results with those reported by Brooks-Worrell and Splitter is precluded by the fact that we used LN lymphocytes from S2308-infected cattle that had aborted whereas Brooks-Worrell and Splitter used blood lymphocytes from cattle that had been immunized or hyperimmunized with the attenuated S19 vaccine. Perhaps lymphocytes recognize different *B. abortus* antigens on the basis of the source of the lymphocytes (LN versus blood) and the immune status of the animal (S2308 infected versus S19 vaccinated). Thus, these factors might explain the differences in the results from our study with those reported by Brooks-Worrell and Splitter (2, 3).

We previously showed that LN cells in S19-vaccinated cattle proliferate mainly in response to 49- to 27-kDa proteins of S2308 (12). This differs from the major proliferative response to S2308 proteins of 27 to 18 kDa by LN cells from S2308-infected cattle in the current study. Studies have not proven that lymphocytes from S19-vaccinated or S2308-infected cattle recognize and proliferate in response to the same protein antigens of *B. abortus*. In the absence of such evidence, identifying immunodominant antigens during clinical infections but not after vaccination with S19 might be more useful in isolating *B. abortus* antigens that are critical for immunity to brucellosis.

REFERENCES

1. **Brooks-Alder, B., and G. A. Splitter.** 1988. Determination of bovine lymphocyte responses to extracted proteins of *Brucella abortus* by using protein immunoblotting. Infect. Immun. **56**:2581–2586.

2. **Brooks-Worrell, B. M., and G. A. Splitter.** 1992. Sodium dodecyl sulfate- and salt-extracted antigens from various *Brucella* species induce proliferation of bovine lymphocytes. Infect. Immun. **60:** 2136–2138.
3. **Brooks-Worrell, B. M., and G. A. Splitter.** 1992. Antigens of *Brucella abortus* S19 immunodominant for bovine lymphocytes as identified by one- and two-dimensional cellular immunoblotting. Infect. Immun. **60:**2459–2464.
4. **Cordes, D. O., and M. E. Carter.** 1979. Persistence of *Brucella abortus* infection in six herds of cattle under brucellosis eradication. N. Z. Vet. J. **27:**255–259.
5. **Enright, F. M.** 1990. The pathogenesis and pathobiology of *Brucella* infection in domestic animals, p. 301–320. *In* K. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.
6. **Nicoletti, P.** 1990. Vaccination, p. 283–300. *In* K. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.
7. **Nicoletti, P., and A. J. Winter.** 1990. The immune response to *Brucella abortus*: the cell-mediated response to infections, p. 83–95. *In* K. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.
8. **Phillips, M., B. L. Deyoe, and P. C. Canning.** 1989. Protection of mice against *Brucella abortus* infection by inoculation with monoclonal antibodies recognizing *Brucella* O-antigen. Am. J. Vet. Res. **50:**2158–2161.
9. **Schurig, G. G., C. Hammerberg, and B. R. Finkler.** 1984. Monoclonal antibodies to *Brucella* surface antigens associated with the smooth lipopolysaccharide complex. Am. J. Vet. Res. **45:**967–971.
10. **Schurig, G. G., R. M. Roop, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan.** 1991. Biological properties of RB51: a stable rough strain of *Brucella abortus*. Vet. Microbiol. **28:**171–188.
11. **Smith, R., III, L. G. Adams, B. A. Sowa, and T. A. Ficht.** 1990. Induction of lymphocyte responsiveness by the outer membrane-peptidoglycan complex of rough strains of *Brucella abortus*. Vet. Immunol. Immunopathol. **26:**31–48.
12. **Stevens, M. G., S. C. Olsen, and N. F. Cheville.** Comparative analysis of immune responses in cattle vaccinated with *Brucella abortus* strain 19 or strain RB51. Vet. Immunol. Immunopathol., in press.
13. **Sutherland, S. S., and J. Searson.** 1990. The immune response to *Brucella abortus*: the humoral immune response, p. 65–81. *In* K. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Inc., Boca Raton, Fla.
14. **Verstreate, D. R., M. T. Creasy, N. T. Caveney, C. L. Baldwin, M. W. Blab, and A. J. Winter.** 1982. Outer membrane proteins of *Brucella abortus*: isolation and characterization. Infect. Immun. **35:**979–989.
15. **Wyckoff, J. H., III, J. L. Howland, and A. W. Confer.** 1993. Comparison of *Brucella abortus* antigen preparations for in vitro stimulation of immune bovine T-lymphocyte cell lines. Vet. Immunol. Immunopathol. **36:**45–64.
16. **Young, E. J.** 1983. Human brucellosis. Rev. Infect. Dis. **5:**821–842.
17. **Zhu, Y., S. C. Oliveira, and G. A. Splitter.** 1993. Isolation of *Brucella abortus ssb* and *uvrA* genes from a genomic library by use of lymphocytes as probes. Infect. Immun. **61:**5339–5344.