INFECTION AND IMMUNITY, Mar. 1996, p. 1030–1034 Vol. 64, No. 3
0019-9567/96/$04.00+0

Antibody Responses to *Brucella abortus* 2308 in Cattle Vaccinated with *B. abortus* RB51

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Received 5 September 1995/Returned for modification 25 October 1995/Accepted 9 December 1995

Cattle vaccinated with *Brucella abortus* rough strain RB51 (SRB51) produced small amounts of serum immunoglobulin G (IgG) but no IgM antibody to smooth strain 2308 (S2308) bacteria and produced no IgG or IgM antibody to S2308 lipopolysaccharide (LPS). Western immunoblot analysis revealed that antiserum from SRB51-vaccinated cattle contained IgG antibody that reacted with S2308 proteins of 84 to <20 kDa. However, antiserum from the vaccinated cattle did not contain agglutinating *B. abortus* antibody in the tube agglutination test for brucellosis. These results suggest that SRB51-vaccinated cattle produced no antibody to S2308 LPS, although they did produce nonagglutinating IgG antibody that reacted with S2308 bacteria and bacterial proteins of 84 to <20 kDa.

Brucella abortus RB51 (SRB51) is a laboratory-derived lipopolysaccharide (LPS) O-antigen-deficient mutant of the virulent strain 2308 (S2308) of *B. abortus* (19). SRB51 is currently being evaluated as an alternative to *B. abortus* 19 (S19) as a vaccine for preventing brucellosis and abortions in cattle because unlike S19, it does not induce antibody to the *Brucella* LPS O-antigens that are detected by agglutination and other serodiagnostic tests for brucellosis (5–7, 20, 22). In addition, cattle vaccinated with SRB51 have both increased resistance to infection and decreased abortions that are induced by virulent S2308 (6, 7). Consequently, the replacement of S19 with SRB51 for use as a vaccine may facilitate the identification and removal of cattle with brucellosis from vaccinated herds.

Strain 45/20 (S45/20) is a rough strain of *B. abortus* which was developed by repeated passages in guinea pigs (13). In the 1940s, cattle vaccinated with live S45/20 were found to have good immunity to brucellosis with little to no agglutinating *B. abortus* antibody, which interfered with diagnosing brucellosis in vaccinated herds (12). However, the use of S45/20 as a vaccine was soon discontinued because S45/20 could revert to a smooth virulent form and cause abortions in vaccinated cattle (9, 28). In contrast with S45/20, SRB51 given to cattle or goats appears to be quite stable in that it does not revert to its parental S2308 smooth type (7, 16).

Studies with S45/20 in cattle have shown that this strain induces nonagglutinating immunoglobulin G (IgG) antibody which binds to smooth strains of *B. abortus* (2, 14). The significance of these nonagglutinating antibodies is unknown, although they have been implicated to act as blocking antibodies which may delay bacterial clearance and increase chronic *B. abortus* infections (14, 15). Cattle vaccinated with SRB51 have antibody to SRB51, but they do not have antibody which agglutinates smooth *B. abortus* (7, 19, 20, 22). However, the specificity of the antibody response in SRB51-vaccinated cattle has not been examined, and it is not known if SRB51 induces nonagglutinating *B. abortus* antibody similar to that found in cattle vaccinated with S45/20. The objectives of this study were to characterize the specificity of the antibody response in SRB51-vaccinated cattle and to determine if these cattle have nonagglutinating *B. abortus* antibody.

Vaccination of cattle. The cattle (polled Herefords) used in the experiments came from two separate SRB51 vaccine studies and consisted of 30 SRB51-vaccinated heifers, 6 S19-vaccinated heifers (positive controls), and 18 nonvaccinated heifers (negative controls). The SRB51 and S19 vaccines were prepared and dispensed in saline as described previously (22). Eight-month-old heifers were used in the first vaccine study. Six heifers were vaccinated with S19, 12 heifers were vaccinated with SRB51, and 6 nonvaccinated heifers were used as controls. All vaccinations (2 ml of 5×10^9 to 7×10^9 CFU) were given by subcutaneous injection in both the right and left axillary areas (1.0×10^{10} to 1.4×10^{10} total CFU). Ten-month-old heifers were used in the second vaccine study. Eighteen heifers were vaccinated by a subcutaneous injection of SRB51 (2 ml containing 6.8×10^9 CFU) in both the right and left axillary areas (1.4×10^{10} total CFU), and 12 non-vaccinated heifers were used as controls. Blood was obtained from all animals by jugular venipuncture before and at 4 and 10 weeks after vaccination. Blood was incubated for 12 h at 4°C before centrifugation. The serum was then aliquoted and stored at −70°C until assayed.

Agglutinating antibody. Serum samples were measured for agglutinating antibody by the *B. abortus* standard tube agglutination test (1). This analysis revealed that serum taken from the six S19-vaccinated cattle (positive controls) had titers ranging from 100 to 600 at 4 weeks after vaccination and titers from 0 to 100 at 10 weeks after vaccination. In contrast, serum from the 30 cattle at 4 and 10 weeks after vaccination with SRB51 had no measurable titer in the tube agglutination test. These results confirm previous findings that cattle vaccinated with SRB51 do not produce agglutinating *B. abortus* antibody when assayed with the standard tube agglutination test (6, 7, 20).

Antibody to S2308 LPS. The LPS of *B. abortus* is an immunodominant antigen (4), and agglutinating antibody is primarily made to this antigen (11). Therefore, even though SRB51-vaccinated cattle in the current study did not have agglutinating *B. abortus* antibody, these animals may have had nonagglutinating anti-LPS antibody. To assess this possibility, antibody (IgG and IgM) to the LPS of S2308 (i.e., the parental strain of SRB51) was measured by an enzyme-linked immunosorbent assay (ELISA) as previously described (27) and used either a goat anti-bovine IgG (H-chain specific, 1:500

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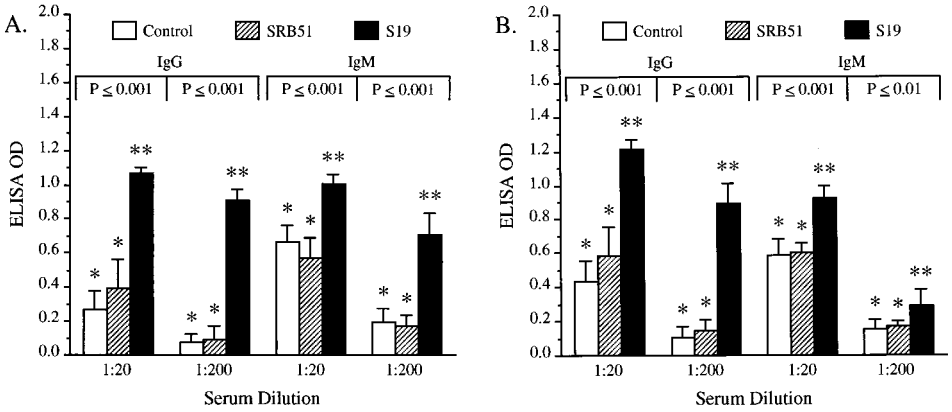

FIG. 1. Antibody to S2308 LPS in vaccinated cattle. Cattle were vaccinated with S19 or SRB51 at 8 months of age. Serum IgG and IgM to S2308 LPS was measured by an ELISA at 4 weeks (A) and 10 weeks (B) after vaccination. Results are expressed as mean OD ± SD for nonvaccinated control ($n = 6$), S19-vaccinated ($n = 6$), and SRB51-vaccinated ($n = 12$) cattle. $P \leq 0.001$ or $P \leq 0.01$ for ** versus * by analysis of variance and Fisher's protected least significant difference.

dilution) or goat anti-bovine IgM (H-chain specific, 1:500 dilution) horseradish peroxidase conjugate (Kirkegaard and Perry Laboratories, Inc, Gaithersburg, Md.). Results from the ELISA for cattle in the first vaccine study indicated that serum taken from the six S19-vaccinated cattle (positive controls) had significantly increased ($P \leq 0.01$) concentrations of both IgG and IgM to S2308 LPS at 4 and 10 weeks after vaccination compared with serum from six nonvaccinated control cattle (Fig. 1). In contrast, serum from 12 SRB51-vaccinated cattle at 4 and 10 weeks after vaccination had concentrations of IgG and IgM to S2308 LPS which were not different from those measured in serum from nonvaccinated control cattle (Fig. 1). The remaining 18 SRB51-vaccinated cattle which were examined in the second vaccine study also had no elevated serum antibody (IgG or IgM) to S2308 LPS compared with serum from 12 nonvaccinated control cattle (data not shown). Collectively, these results confirm a previous finding that SRB51 does not induce antibody to S2308 LPS when given to cattle (19). The same finding has also been reported for goats or mice given SRB51 (16, 25).

Antibody to S2308 bacteria. The results from the tube agglutination assay and the S2308 LPS ELISA in the current study indicated that SRB51-vaccinated cattle have no agglutinating *B. abortus* antibody or S2308 LPS antibody. However, SRB51 and its parental S2308 contain the same outer membrane proteins (18, 19), and we previously noted that these two strains appear to contain most of the same antigenic proteins, as assessed by measuring lymphocyte proliferation in response to the proteins in either SRB51-vaccinated or S2308-infected cattle (21, 23). Therefore, it seems logical that SRB51-vaccinated cattle should have SRB51 antibody which binds to S2308 bacteria, presumably by recognizing outer membrane protein antigens which are shared with S2308. To assess this possibility, antibody to S2308 bacteria in SRB51 antiserum was measured by an enzyme immunoassay (EIA).

A total of 100 μl of 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 2×10^{10}, 2.5×10^{9}, or 5×10^{8} killed S2308 bacteria (gamma irradiation at 1.4×10^{6} rads) was added to 1.5-ml microcentrifuge tubes. Serum from each heifer vaccinated with S19 or SRB51 or prevaccination serum from each heifer was added to separate tubes containing the S2308 bacteria. Bacteria were incubated for 18 h at room temperature with the serum and then washed twice by centrifugation with PBS. Bacteria were resuspended in 100 μl of PBS, and 50 μl of a goat IgG-horseradish peroxidase conjugate specific for bovine IgG (H-chain specific) or bovine IgM (H-chain specific) was added at a final concentration of 2 μg/ml. Appropriate controls included incubating each concentration of S2308 bacteria with each of the conjugates alone. Tubes were incubated for 4 h at room temperature, and the bacteria were then washed three times by centrifugation with PBS. Bacteria were resuspended in 150 μl of PBS, and a 50-μl aliquot was removed from each tube and added to each of two flat-bottomed wells of a microtiter plate. The substrate solution (2-azino-3-ethylbenzthiazoline-6-sulfuric acid plus H_2O_2) was prepared as previously described (27), and 50 μl was added to each well. The optical density (OD) at 405 nm was read 20 min later with a 96-channel microtiter plate spectrophotometer (Vmax; Molecular Devices Corporation, Palo Alto, Calif.).

The results from the EIA indicated that serum taken from the six S19-vaccinated cattle (positive controls) in the first vaccine study had significantly increased ($P \leq 0.01$ or $P \leq 0.001$) concentrations of IgG and IgM to S2308 bacteria at 4 weeks after vaccination compared with prevaccination serum from the same six cattle (Fig. 2). In contrast, serum from 12 SRB51-vaccinated cattle at 4 weeks after vaccination had significantly increased ($P \leq 0.01$) concentrations of IgG but not IgM to S2308 bacteria compared with prevaccination serum from these animals (Fig. 2). The remaining 18 SRB51-vaccinated cattle in the second vaccine study also had elevated serum IgG but not IgM to S2308 bacteria at 4 weeks after vaccination compared with the concentrations of these antibodies in prevaccination serum (data not shown). Prevaccination serum and serum obtained at 10 weeks from SRB51-vaccinated cattle in the first and second vaccine studies did not have significantly different levels of IgG to S2308 bacteria (data not shown). These results and the negative results from the tube agglutination assay suggest that SRB51-vaccinated cattle have a low and transient (absent at 10 weeks) nonagglutinating IgG antibody response to S2308 bacteria. Similar results have been found for SRB51-vaccinated mice in that they also have a low and transient (absent at 12 weeks) antibody response to S2308 (25, 26).

A previous study noted that bovine IgG but not goat IgG can nonspecifically bind to intact S2308 bacteria by attachment to a cell surface protein (3). On the basis of this finding, several procedures were used in the current study to minimize the measurement of nonspecific binding of IgG to S2308 bacteria by the EIA. These included using a goat IgG conjugate which did not bind to S2308 bacteria in the EIA. In addition, the EIA

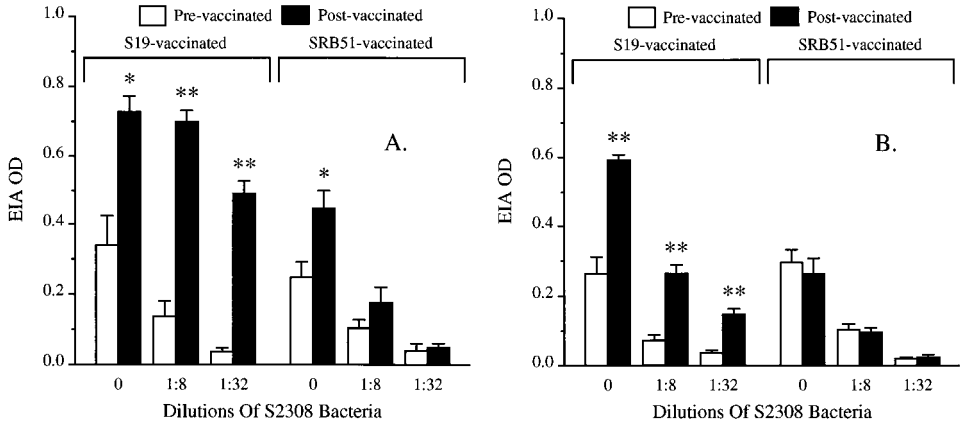

FIG. 2. Antibody to S2308 bacteria in vaccinated cattle. Cattle were vaccinated with S19 or SRB51 at 8 months of age. Serum IgG (A) and IgM (B) to S2308 bacteria was measured by an EIA at 4 weeks postvaccination. Results are expressed as mean OD ± SEM for S19-vaccinated (n = 6) and SRB51-vaccinated (n = 12) cattle. $P \leq 0.001$ for ** or $P \leq 0.01$ for * versus prevaccination serum by the paired Student t test.

was conducted with serum taken before and after cattle were vaccinated with either S19 or SRB51 so that the amount of nonspecific binding of IgG to S2308 would most likely be equal in the serum samples which were analyzed within each group of vaccinated cattle. Furthermore, total IgG concentrations were measured by a radial immunodiffusion assay (The Binding Site Inc., San Diego, Calif.) in serum taken before and after vaccination of the cattle with S19 (n = 6) or SRB51 (n = 12). The results from this assay indicated that mean IgG concentrations (± standard deviation [SD]) were not different in cattle before and after vaccination with S19 (18.0 ± 1.9 versus 20.1 ± 4.0 mg/ml) and in cattle before and after vaccination with SRB51 (20.6 ± 4.4 versus 23.4 ± 4.6 mg/ml). The presence of similar amounts of total IgG in the prevaccination and postvaccination serum samples suggests that nonspecific binding of IgG to S2308 was similar in both samples and that increased binding of IgG in postvaccination serum in the current study most likely resulted from specific binding of IgG to S2308.

Antibody to S2308 proteins. The specificity of the IgG antibody response to S2308 bacteria in cattle at 4 weeks after vaccination with SRB51 was determined by Western blot analysis. Proteins in whole-cell lysates from killed S2308 bacteria were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (22). The gel was placed in a transfer buffer (3) before electroelution (600 mA for 1 h) of the proteins onto a polyvinylidene fluoride membrane (Millipore, Bedford, Mass.). The membranes were blocked by incubation with a Tris-saline buffer (TSB; 20 mM Tris, 500 mM NaCl, pH 7.5) containing 3% nonfat milk and then incubated overnight at 4°C with each analyzed bovine serum sample which had been diluted 1:20 in TSB containing 0.3% nonfat milk. After washing, the membranes were incubated for 2 h with TSB containing 0.3% nonfat milk and a 1:1,000 dilution of a goat IgG-horseradish peroxidase conjugate specific for bovine IgG (H-chain specific). After additional washing, the bound conjugate was detected by adding TSB containing 0.06% 4-chloro-1-naphthol, 0.6% H_2O_2, and 10% methanol. The samples analyzed by Western blotting included (i) serum obtained from each of the 12 cattle (first vaccine study) and each of the 18 cattle (second vaccine study) at 4 weeks after vaccination with SRB51, (ii) pooled sera from 12 cattle in the first study or from 18 cattle in the second study, (iii) pooled sera from all 30 cattle in both studies, and (iv) pooled sera from 6 nonvaccinated cattle (negative control).

Western blot analysis revealed that SRB51-vaccinated cattle had IgG antibody mainly to the 29- to 20-kDa and <20-kDa proteins of S2308, because all 30 vaccinated cattle had antibody to either or both groups of these proteins (Fig. 3). Responses to the other S2308 proteins were less common, in that 63% of the 30 vaccinated cattle had IgG antibody to proteins of 84 to >53 kDa, 53% had antibody to proteins of 53 to >35 kDa, and 67% had antibody to proteins of 35 to >29 kDa (Fig. 3). Pooled sera from SRB51 vaccinated cattle in the first (n = 12) or second (n = 18) vaccine study and pooled sera from all 30 cattle in both studies had IgG antibody which recognized S2308 proteins of the same molecular masses (84 to <20 kDa) (Fig. 4).

The results from the Western blot analysis demonstrated that the nonagglutinating *B. abortus* IgG antibody reacted mainly with S2308 proteins of 29 to 20 kDa and <20 kDa. Similar results have been found in cattle vaccinated with S45/20 in that they have nonagglutinating *B. abortus* IgG antibody which reacts primarily with outer membrane proteins (OMP) of 27, 14, 12, and 10 kDa of S45/20 (17). Nonagglutinating IgG antibody also occurs in cattle that have been chronically infected with smooth *B. abortus* S1119 (15). The significance of these antibodies in cattle vaccinated with S45/20 or chronically infected with S1119 is unknown. However, it has been speculated that they may delay clearance of *B. abortus* in cattle, because mice given the bovine antibodies by passive transfer have a slower clearance of *B. abortus* following infection (14).

S2308 and SRB51 contain the same OMP (18), and bovine SRB51 antiserum reacts with S2308 OMP (19). In the current study, antibody to the intact S2308 bacteria in SRB51-vaccinated cattle probably resulted from antibody to one or more SRB51 OMP which reacted with the corresponding OMP in S2308. Rough and smooth strains of *B. abortus* contain at least seven surface-exposed OMP (10, 16, 19, 25 to 27, 31 to 34, 36 to 38, and 89 kDa). However, two of these OMP (31 to 34 and 89 kDa) in smooth *B. abortus* are not accessible for binding by IgG antibody, presumably because of steric hindrance by the presence of the LPS O-side chain (8). On the basis of these findings, the OMP of 31 to 34 and 89 kDa can probably be excluded as possible surface antigens on the intact S2308 bacteria which reacted with bovine SRB51 antiserum in the current study. As previously noted, steric hindrance by the LPS O-side chain is thought to prevent binding of IgG to certain surface antigens on smooth *B. abortus* (8). Steric hindrance by the LPS O-side chain in the current study may also explain why

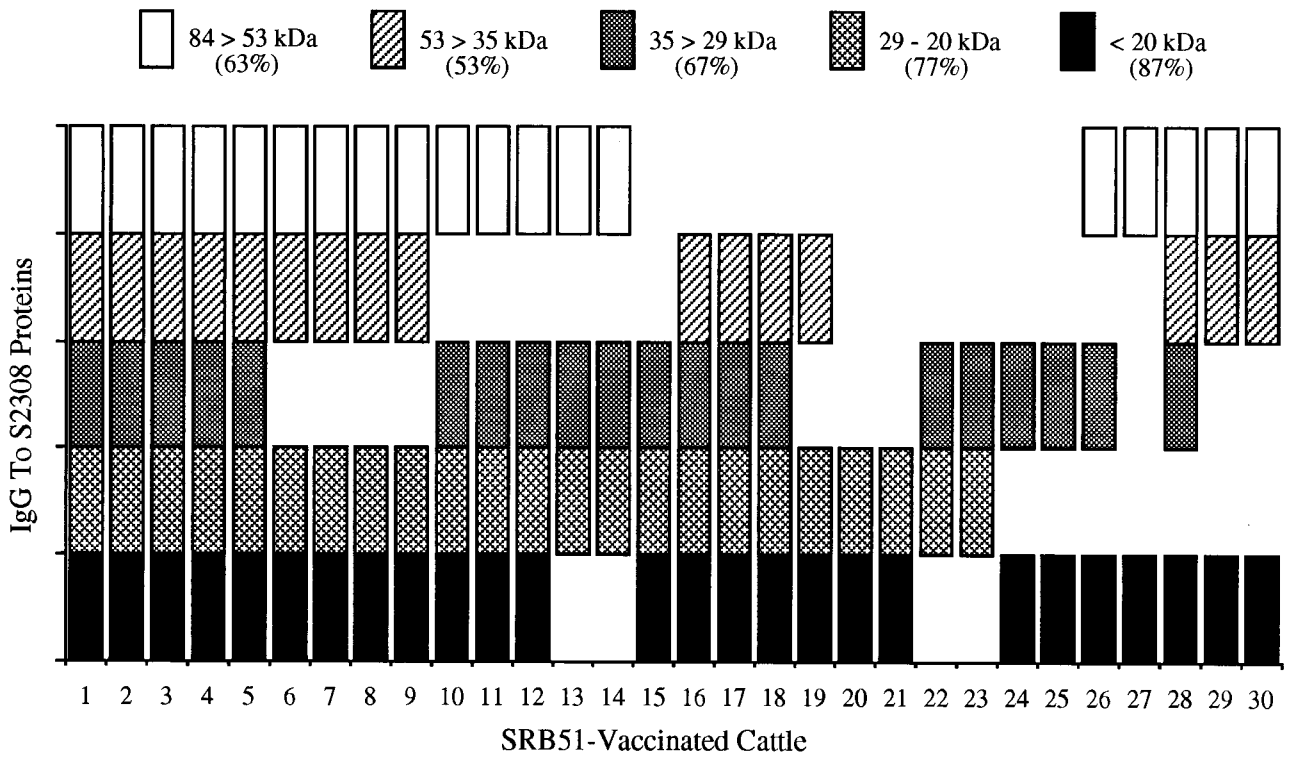

FIG. 3. Schematic representation of Western blot analysis, indicating S2308 antigens recognized by IgG antibody in 30 cattle vaccinated with SRB51. Antibodies to five groups of S2308 antigens (84 to >53 kDa, 53 to >35 kDa, 35 to >29 kDa, 29 to 20 kDa, and <20 kDa) are indicated. Numbers in parentheses indicate the percentage of the 30 cattle which had antibody to the specified group of S2308 antigens.

IgG in bovine SRB51 antiserum was able to bind to smooth *B. abortus* yet failed to agglutinate smooth *B. abortus* in the tube agglutination assay. Furthermore, hindrance of antibody binding by the O-side chain would be the most likely explanation for our previous finding that mice vaccinated with SRB51 have lower antibody titers to smooth S2308 than to rough SRB51 (26) when assessed by a dot ELISA. Similar results were obtained by retrospective analysis of sera from the 30 SRB51-vaccinated cattle in the current study. These vaccinated cattle had dot ELISA titers ($\log_{10} \pm$ SD) to S2308 at 4 (1.1 ± 0.6) and 10 (1.2 ± 0.7) weeks which were significantly lower than the titers to SRB51 at 4 (2.6 ± 0.5) and 10 (2.4 ± 0.4) weeks ($P \leq 0.01$ by the paired Student t test).

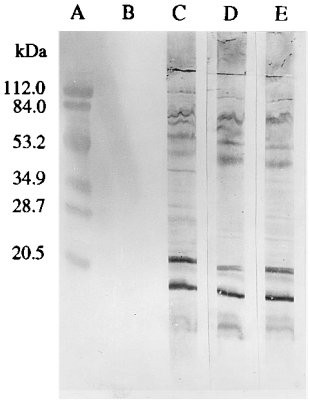

FIG. 4. Western blot analysis indicating S2308 antigens recognized by IgG antibody in cattle vaccinated with SRB51. Lane A, prestained molecular size standards of 112, 84, 53.2, 34.9, 28.7, and 20.5 kDa. Proteins reacted with sera include: lane B, pooled sera from 6 nonvaccinated cattle; lane C, pooled sera from 12 SRB51-vaccinated cattle in the first vaccine study; lane D, pooled sera from 18 SRB51-vaccinated cattle in the second vaccine study; lane E, pooled sera from all 30 SRB51-vaccinated cattle.

The significance of an IgG antibody response to S2308 in SRB51-vaccinated cattle in the current study is not clear, because the response was low and had a short persistence and the IgG was nonfunctional (nonagglutinating). It is not known if the IgG to S2308 in SRB51 antiserum can act as an opsonin. However, it is unlikely that the IgG can fix complement, because bovine SRB51 antiserum does not react in the complement fixation test for brucellosis (20). Current evidence indicates that vaccination of mice with SRB51 induces immunity to infection with S2308 by cell-mediated but not by antibody immune responses (10, 24, 25). It is likely that protective immunity in cattle vaccinated with SRB51 also results mostly from cellular immune responses, because vaccinated cattle in the current study had little to no antibody which reacted with S2308 bacteria, and yet, as previously noted, these cattle do have lymph node lymphocytes which proliferate in response to S2308 bacteria and certain bacterial proteins (22, 23).

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