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Killing of *Brucella abortus* by Bovine Serum

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Studies of the serum bactericidal system in bovine brucellosis were undertaken to investigate the role of the humoral immune response in protection of cattle against the facultative intracellular parasite *Brucella abortus*. Fresh sera from normal control cattle, infected cattle, and cattle immunized with *B. abortus* cell envelopes were collected before treatment and during the course of immunization or infection. Normal fresh bovine serum or fresh agammaglobulinemic serum from colostrum-deprived calves was effective in killing smooth virulent *B. abortus* 2308, but rough strains RB51 (a rough mutant of strain 2308) and 45/20 were much more senitive to serum. The difference in susceptibility to serum was shown to be correlated with differences in lipopolysaccharide chemotype, with the more resistant strain 2308 having O polysaccharide and the more susceptible strains 45/20 and RB51 lacking O side chains. By treatment of fresh serum with $MgCl_2$ and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] killing was shown to occur via the classical pathway of complement activation. When antibody to *B. abortus* was present, killing of strain RB51 increased but killing of smooth strain 2308 decreased. The earliest antibody response in serum from infected animals did not interfere with killing. When affinity-purified bovine immunoglobulins specific for *B. abortus* smooth lipopolysaccharide were added to fresh normal bovine serum, immunoglobulin G1 (IgG1) and IgG2 isotypes blocked killing but IgM and IgA isotypes did not. Thus, it appears that serum from previously unexposed animals or animals early during infection can kill smooth *B. abortus*, an appropriate defense mechanism before the organism becomes intracellular. At later stages of infection, blocking antibodies predominate.

Host defense against infection with *Brucella abortus* is not yet fully understood. Control programs for brucellosis include immunization of cattle and slaughter of infected animals. Although the immune response is monitored by measurements of antibody titer, the role of antibody in protection is poorly defined. It is usually assumed that antibody is ineffective in defense because *B. abortus* is a facultative intracellular parasite (63). However, antibody may be involved in complement-mediated killing of the organism during the extracellular phase (26, 34) and may also be involved in opsonization and intracellular killing by phagocytes (10, 14). Several investigators have shown that passive immunization of mice with immune serum (2, 31, 33, 41, 50, 54, 62) or monoclonal antibody to O antigen (30, 40) is protective. Studies of the role of antibody in bovine brucellosis have been contradictory. Some studies indicate that fresh normal bovine serum kills *B. abortus* (22, 24–27). When serum was serially diluted in bactericidal assays, animals recovering from infection were shown to have high titers of bactericidal antibodies (24–27). In some of these studies, however, undiluted or minimally diluted serum from infected animals did not kill *B. abortus* (24, 26, 27). Another research group reported that plasma from a large number of infected animals always failed to inhibit growth of *B. abortus* even at high dilutions of plasma (with addition of bovine complement) (22).

To reexamine the role of bovine antibodies and complement in killing *B. abortus*, we studied the sensitivity of smooth (strain 2308) and rough (strains 45/20 and RB51) strains to killing by fresh serum from control, infected, and immunized cattle. Dilutions of bacteria were assayed in a standard volume of undiluted serum to keep complement components and other bactericidal factors in serum at constant concentrations similar to in vivo conditions (12, 35). Here we report differences in susceptibility of smooth and rough strains of *B. abortus*, the roles of classical and alternative complement pathways in killing, and the roles of antibody isotype and specificity in blocking killing of smooth strain 2308.

(This research was reported in part at the 38th Annual Brucellosis Research Conference, 1984, and as an abstract [L. B. Corbeil, Clin. Res. 35:425A, 1987].)

MATERIALS AND METHODS

Bacteria. Three strains of *B. abortus* were used in this study. Strain 2308 is a virulent smooth strain often used in challenge studies. Strain 45/20 is a rough strain used as a killed-cell commercial vaccine. Strain 45/20 is unstable and capable of reverting to the smooth form in vivo. It may contain minute quantities of lipopolysaccharide (LPS) O side chains which are insufficient to induce O antibodies after a single vaccination. Either this O antigen or core antigen appears to prime animals to produce detectable antibodies after subsequent injection of the same vaccine or contact with virulent field strains (3, 44, 61). Strain RB51 is a fully stable rough strain derived from 2308 (kindly provided by G. G. Schurig, Virginia Polytechnic Institute and State University, Blacksburg). Bacteria were grown on 5% bovine blood agar at 37°C. To ensure that smooth and rough

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characteristics were maintained, each strain was tested periodically for roughness by crystal violet uptake and acriflavin agglutination (1).

A rough UDP galactose epimerase-deficient mutant of *Escherichia coli*, J5 (71), was grown on tryptic soy agar (Difco Laboratories, Detroit, Mich.) and was included in some experiments as a positive control because of its ability to activate complement in the absence of antibody (5) and its extreme sensitivity to complement-mediated killing (12).

LPS. LPS from *Salmonella typhimurium* was obtained from Sigma Chemical Co., St. Louis, Mo., and LPS from each *Brucella* strain was extracred by modification of a rapid phenol-water micromethod previously described (23). Briefly, each strain was grown to a density of 10^9 CFU/ml (determined spectrophotometrically), and 10 ml of culture was treated with 1% Formalin and harvested. A 1-ml amount of hot (70°C) 45% aqueous phenol was added to the pellet from 10 ml of culture, stirred vigorously for 15 min, and cooled, and the phases were separated by centrifugation (12,000 × *g* for 10 min). The aqueous phase was removed, 500 μl of distilled water was added to the phenol phase, and the extraction was repeated. Aqueous sodium chloride (0.5 M final concentration) and 10 volumes of ethanol were then added to the pooled aqueous phases or phenol phases. After being cooled to −20°C, the precipitated LPS was sedimented by centrifugation at 5,000 × *g* for 10 min. The precipitate was resuspended in 100 μl of distilled water, and the precipitation was repeated. The final pellet was resuspended in 50 μl of distilled water and stored at −20°C. LPS preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel system described by Laemmli (29) was used, with a 15% separating gel containing 4 M urea. The LPS was stained by periodate oxidation and silver exactly as described by Tsai and Frasch (66). This procedure selectively stains LPS in the gel and not contaminating proteins.

Bactericidal assay. The assay system used serial dilutions of a bacterial suspension in undiluted serum rather than a standard suspension of bacteria in dilutions of serum to ensure that all bactericidal factors in serum were present in adequate concentrations (12, 35). Organisms were scraped from the surface of blood agar plates after 24 h of growth, since preliminary data indicated that *B. abortus* grown for 24 h was slightly more susceptible to serum killing than 48-h cultures. Harvested bacterial cells were suspended at an OD_{610} of 0.125 in tryptose-phosphate buffer (7) at pH 8.0 (to reduce autogglutination of rough strains which occurred at neutral pH). Tenfold dilutions of this suspension were incubated with equal volumes (50 μl) of fresh undiluted bovine serum in a 96-well microtiter plate at 37°C in air. Parallel samples were incubated in heated serum (56°C for 30 min) and in tryptose-phosphate buffer. Heated serum and buffer controls were expected to give similar results, ensuring that viable-cell-count decreases were not due to agglutination. If these controls did not give similar results, the assay was repeated. Microtiter plates were incubated for 3 h (unless otherwise indicated), since preliminary data indicated that much less killing was detected at 1 h. After this incubation period, two 25-μl drops from each dilution were placed separately on relatively dry blood agar plates. When the drops were absorbed into the agar, plates were inverted and incubated for 48 h in 10% CO_2 at 37°C. Colony counts from dilutions yielding between 10 and 100 colonies per drop were used for calculations. Duplicates were averaged and the log of the CFU per milliliter in fresh serum was subtracted from the log CFU per milliliter in heated serum. The results are expressed as the $\log_{10}$ of the number of bacteria killed by 1 ml of serum or the "logs killed" (12, 35).

Serum. Serial samples of complement-preserved serum were obtained from cattle in order to study the progression of immunological events in chronological fashion with an autologous complement source. In a first experiment (70), 24 2-year-old Holstein heifers which were serologically negative for brucellosis were bled five times at monthly intervals to obtain baseline values. Eleven of these heifers were then vaccinated on days 107 and 246 with 5 mg of rough strain 45/20 cell envelopes in adjuvant (70). Cell envelopes from this rough vaccine strain were used to elicit antibodies against outer membrane proteins rather than primarily against smooth LPS, as is characteristic of infected animals (57, 58). Each dose of vaccine contained cell envelopes (5 mg), trehalose dimycolate (5 mg), a butyryl derivative of muramyl dipeptide (5 mg), spermidine (100 μl), mineral oil (1.8 ml), and 0.4% Tween 80 solution (8.2 ml). Six animals were vaccinated with adjuvant alone. Six of the 11 principals and three of the adjuvant controls were pregnant. Another group of controls consisted of nonvaccinated pregnant heifers. In a second experiment, three animals were infected by conjunctival inoculation of 10^7 organisms of virulent smooth *B. abortus* 2308 after 5 preinfection serum collections. In both experiments blood was collected monthly after infection or vaccination for up to 10 months. All blood samples were held for 2 h at room temperature and 2 h at 4°C before serum was removed from the clot and frozen at −70°C to preserve complement. All samples were then lyophilized and kept at −20°C until tested for bactericidal activity. In a few experiments, freshly preserved serum from neonatal colostrum-deprived calves was used as a standard homologous complement source. In these experiments, agammaglobulinemia was confirmed by $ZnSO_4$ turbidity (36) and radial immunodiffusion (38). Normal bovine serum obtained from two steers was pooled and frozen within 4 h of collection to preserve complement as above. Sera with positive *Brucella* agglutination titers from cattle infected with field strains of *B. abortus* served as a pooled source of positive serum.

Antibody determinations. Agglutination titers were determined by the standard diagnostic serological tests for brucellosis (1). All sera were screened by the card agglutination test. Tube and rivanol agglutination tests (1) were only done if the card test was positive. These tests detect primarily antibodies specific for smooth LPS (S-LPS) (58). The rivanol agglutination test measures immunoglobulin G (IgG) antibody activity because IgM is precipitated by rivanol.

Antibodies to group 2 (porin) and group 3 outer membrane proteins (67) were measured by kinetics-based enzyme-linked immunosorbent assay (ELISA) with purified outer membrane proteins of strain 45/20 as antigens and normalized slopes of the reaction rates as an indication of antibody activity (69, 70). For measurement of antibodies of each immunoglobulin isotype in sera from infected animals, the kinetics-based ELISA was modified to utilize a deoxycholate extract as antigen and horseradish peroxidase conjugates of goat anti-bovine IgG1, IgG2, IgM, and IgA. Specificity of conjugates had been confirmed by ELISA against purified bovine immunoglobulin isotypes as described previously (46). By using background levels of antibody as a baseline, antibody titers could be estimated because a direct relationship exists between increases in slope values and $\log_2$ antibody titer (69). Thus, in a prior study of porin antibodies by the kinetics-based ELISA method, increases in slope values of 100, 200, 300, and 400% (i.e., two- to fivefold) over baseline values represented approximate titers

of 16, 246, 3,900, and 60,000, respectively (69). Thus it could be estimated, for example, that following the second vaccination with cell envelopes, the peak titer of antibodies to group 3 proteins (approximately a fivefold increase in slope) was about 60,000 (see Fig. 2a), whereas the peak antibody titer to the same antigen in *B. abortus*-infected heifers (approximately a twofold increase) was about 16 (see Fig. 5a).

Complement pathway determination. To determine the pathway of complement killing of *B. abortus*, EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Sigma Chemical Co.] and $MgCl_2$ were used to inhibit the classical but preserve the alternative complement pathway. EGTA and $MgCl_2$ were each dissolved in tryptose-phosphate buffer at a concentration of 86 mM so that when used as the diluent in the bactericidal assay, the final concentration was 43 mM, as shown to be effective by MacDonald et al. (32) for bovine complement. Although this is approximately four times the concentration of EGTA-$MgCl_2$ used in most other studies, it appears not to be inhibitory to the alternative pathway, since we showed alternative-pathway-mediated killing of some *Haemophilus somnus* isolates by the same procedures (12).

Activity of purified bovine immunoglobulin isotypes in killing. Bovine IgG1, IgG2, and IgM from serum as well as IgA from colostral whey were purified by isotype-specific affinity chromatography (46). These immunoglobulins were purified further by affinity chromatography to contain only antibody to *B. abortus* S-LPS (60). All were freeze-dried and reconstituted to 0.3 mg/ml. To determine the effect of anti-S-LPS antibodies of each isotype on killing of *B. abortus*, 40 μl of purified antibodies was added to 60 μl of fresh normal bovine serum. Serial twofold dilutions were made in fresh normal bovine serum or heated normal bovine serum (agglutination control) in a microtiter plate in duplicate. Equal volumes of diluted bacteria were added and mixed, and the covered plate was incubated at 37°C for 3 h. Bactericidial activity was determined by duplicate plate counts as above. The assay was repeated four times on different days to ensure reproducibility.

Statistical analyses. Data were analyzed by the ANOVA and ONEWAY procedures described in the SSPS manual (45). To determine significance of differences in serum killing during the course of immunization or infection, a set of orthogonal contrasts was used so that the bactericidal activity of serum collected on day 0 was compared with that of the second serum sample (day 10), then the first two samples (days 0 and 10) were compared with the third (day 35), and then the first three samples (days 0, 10, and 35) were compared with the fourth (day 70), and so on in this manner to yield a sequential analysis of day effects. For studies of the role of isotypes of anti-LPS antibodies in killing *B. abortus*, a two-way analysis of variance was used.

RESULTS

Bactericidal assays with fresh normal bovine serum or fresh serum from an agammaglobulinemic calf demonstrated killing of *B. abortus*, although rough strains 45/20 and RB51 were much more sensitive than smooth strain 2308 (Fig. 1). *E. coli* J5 was also killed very efficiently by the normal bovine serum and agammaglobulinemic calf serum. In the two cattle experiments, baseline data from five pretreatment samples also showed that *B. abortus* smooth strain 2308 and its rough mutant (RB51) were killed by fresh serum which contained little or no antibody against outer membrane proteins or the LPS O side chain. Again, the rough strain was killed much more efficiently than the smooth strain (Fig. 2–5).

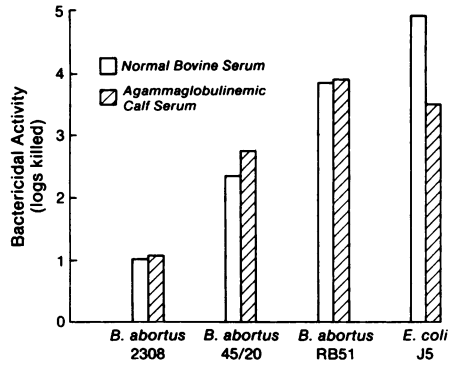

FIG. 1. Killing of smooth *B. abortus* 2308, rough *B. abortus* 45/20, rough *B. abortus* RB51, and rough *E. coli* J5 by fresh complement-preserved sera, including agammaglobulinemic colostrum-deprived neonatal calf serum and normal adult bovine serum. These data are from a representative experiment which was repeated with similar results.

The complement pathway involved in killing was determined by incubation with EGTA-$MgCl_2$, which inactivates the classical pathway. Killing of all three strains was essentially eliminated in the presence of EGTA-$MgCl_2$ (Fig. 6).

SDS-PAGE analysis of the LPSs of the three *B. abortus* strains was consistent with the results in other reports (43) and confirmed the smooth and rough designation of the strains. The LPS of smooth *B. abortus* 2308 partitioned exclusively in the phenol phase, as noted by Moreno et al. (43), and produced high- and low-molecular-weight bands (Fig. 7, lane 3). The diffuse character of the high-molecular-weight band representing O side chains (Fig. 7, lane 3) was observed in S-LPS of *B. abortus* by Moreno et al. (43) and stands in contrast to the ladder pattern produced by S-LPS of *S. typhimurium* (Fig. 7, lane 1). The LPSs of strains 45/20 and RB51 partitioned into both aqueous and phenol phases and produced only low-molecular-weight bands (Fig. 7, lanes 4–7) characteristic of rough LPS of *B. abortus* (43). When LPS purified on a larger scale was treated with proteinase K to remove contaminating protein, the gel patterns did not differ from those in Fig. 7. However, Dubray and Limet (13) recently demonstrated that with appropriate sample loads and under optimal conditions of electrophoresis, fine patterns could be resolved in protein-free LPS of smooth *B. abortus* and *B. melitensis*.

Sera from cattle were tested in two experiments to determine the effect of antibody responses on bactericidal activity during the course of immunization or infection. Experiment 1 was designed to ensure that approximately half of the animals in each group were pregnant in order to test the effect of pregnancy on immunity (70). Inspection of the data revealed no differences due to pregnancy, so data for nonpregnant and pregnant animals in each group were pooled. After infection or immunization of cattle, antibody titers increased as expected. Heifers immunized with cell envelopes of rough *B. abortus* strain 45/20 produced strong antibody responses to outer membrane proteins (Fig. 2a). The majority of heifers produced no detectable reactions against S-LPS, as determined by the agglutination test. Low agglutination titers were detected in 12 of the 162 samples tested, with the greatest response at 2 weeks after the second vaccination. In contrast, animals infected with virulent

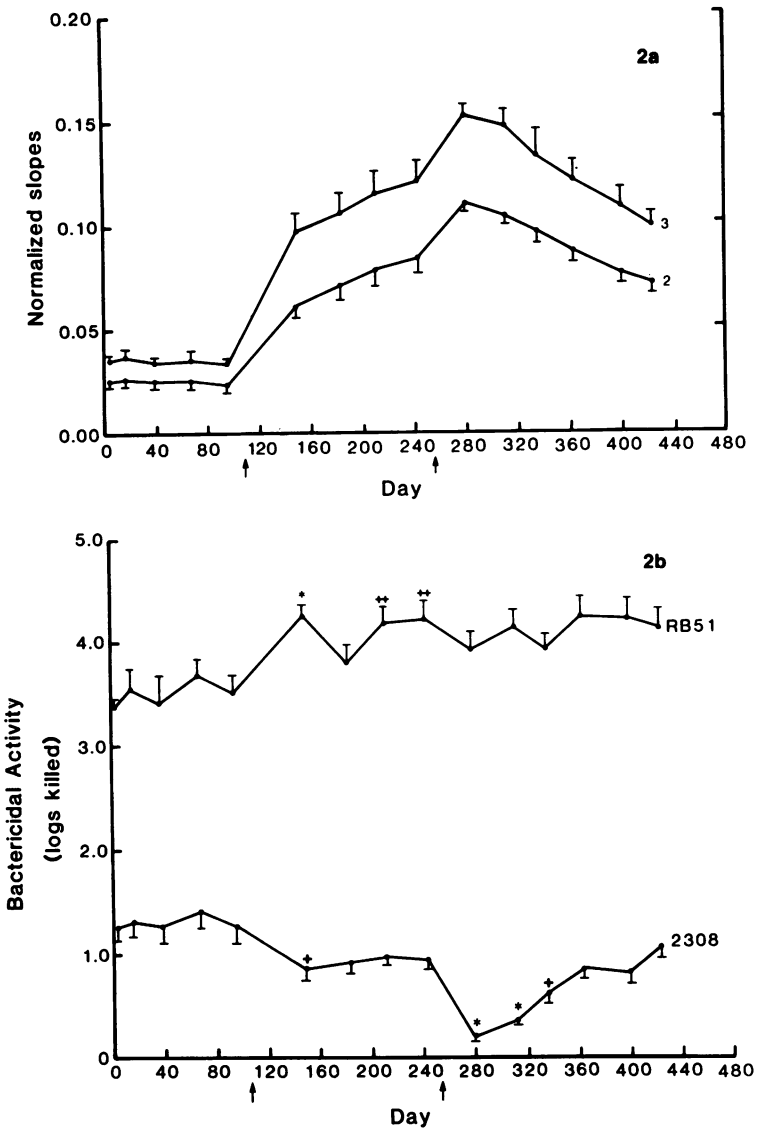

FIG. 2. Experiment 1: antibody responses and complement-mediated killing of *B. abortus* by serum from 11 cattle immunized with strain 45/20 cell envelopes in adjuvant on days 107 and 246 (arrows). Five serum samples were taken before the first immunization on day 107. Values represent means ± standard error. (a) Antibody activity by ELISA against porin (line 2) or group 3 proteins (line 3) presented as normalized slopes. (b) Killing of rough (RB51) and smooth (2308) *B. abortus*. Values significantly different from preceding values are indicated: ++, $P < 0.05$; +, $P < 0.01$; *, $P < 0.001$.

strain 2308 showed only low levels of antibodies to outer membrane proteins, whereas prompt, strong, and sustained responses occurred to S-LPS (Fig. 5a).

In bactericidal assays with rough strain RB51, a significant increase in killing coincided with increased antibody concentrations in sera from 11 heifers immunized with cell envelopes (Fig. 2b). A significant relationship between antibody and killing of strain RB51 was not detected with serum from three infected animals, due to higher standard deviations (Fig. 5b). Changes in killing patterns of smooth strain 2308 were much more striking. In five preinoculation samples from each animal in each group (Fig. 2b and 5b), approximately 1 log of killing was observed. However, after the first immunization with cell envelopes, killing of *B. abortus* 2308 decreased significantly ($P = 0.005$) (Fig. 2b). This was the time at which increasing antibody concentrations to outer membrane proteins (Fig. 2a) and S-LPS were first detected. A second and more precipitous decline in killing was detected in the first ($P < 0.001$), second ($P < 0.001$), and third ($P = 0.007$) serum samples taken after the second immunization (Fig. 2b). These decreases coincided with anamnestic antibody response to outer membrane proteins (Fig. 2a) and, in three of the heifers, to S-LPS. Serum from infected animals demonstrated an even greater decrease in killing of strain 2308 (Fig. 5b), which was correlated with increases in antibody concentrations (Fig. 5a). The first samples taken after infection contained increased levels of IgM agglutinins, which had no effect on killing of strain 2308. However, in the second samples after infection, when both tube and rivanol agglutination titers were high, killing was significantly lower than on the preceding sampling days ($P = 0.001$). From the third sampling after infection onward, killing decreased

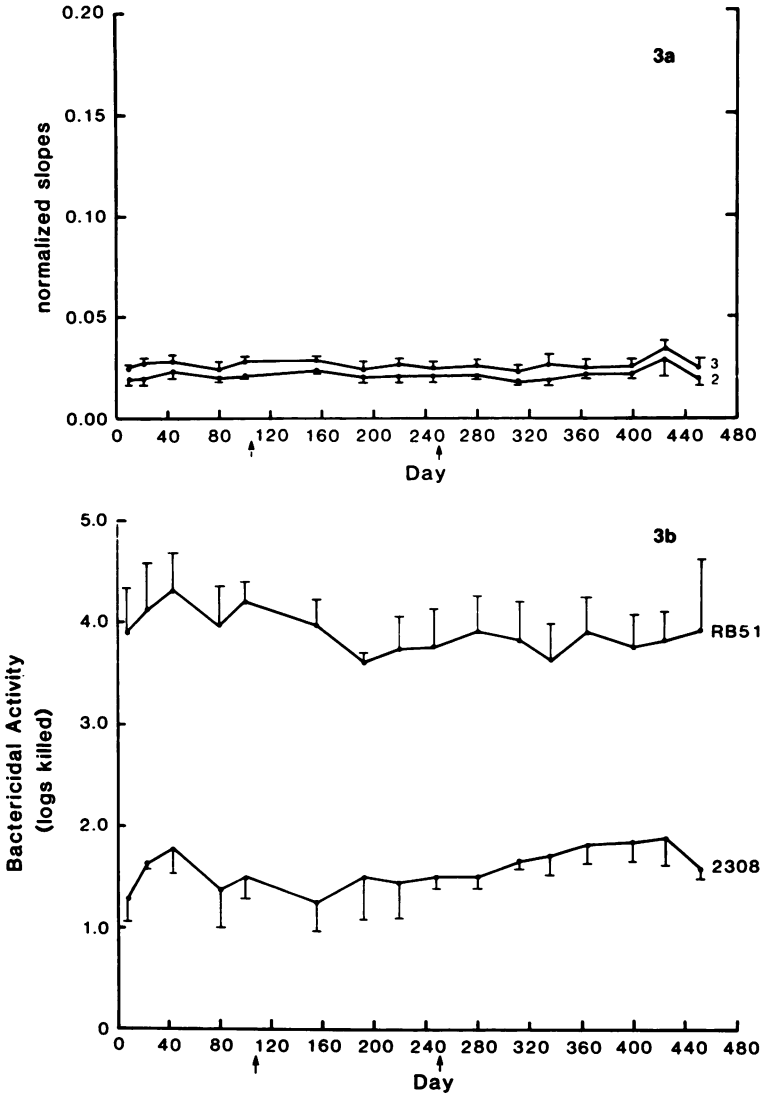

FIG. 3. Experiment 1: antibody responses and complement-mediated killing of *B. abortus* by serum from six cattle immunized with adjuvant alone on days 107 and 246 (arrows). Five serum samples were taken before the first immunization on day 107. Values represent means ± standard error. (a) Antibody activity to porin (line 2) and group 3 proteins (line 3) by ELISA presented as normalized slopes. (b) Killing of rough (RB51) and smooth (2308) *B. abortus*.

further and was significantly different from that in preceding samples ($P < 0.001$). On the last sampling days, only one animal remained in the experiment, so statistical comparisons were not made. By the third sampling period, most of the anti-S-LPS antibody was IgG (Fig. 5a). Very low increases in antibodies to outer membrane proteins (titers estimated at 16 or less) were detectable during this period (Fig. 5a). Thus, the time of lowest bactericidal activity coincided with times of highest anti-S-LPS IgG antibodies. Both adjuvant-vaccinated and nontreated control groups, which had no antibody responses (Fig. 3a and 4a), showed little variation in bactericidal activity for either strain throughout the experimental period (Fig. 3b and 4b).

The above data suggested that the isotypes of antibodies in the sera of infected animals were related to differences in blocking killing of strain 2308 (Fig. 5b). Therefore, isotype-specific antibody responses to *B. abortus* in these sera were determined by kinetics-based ELISA (70) with a deoxycholate extract which contained both outer membrane proteins and S-LPS. With the first samples taken after infection, strain 2308 was killed as efficiently as with preinfection sera (Fig. 5b), and IgM activity, as measured by ELISA, was higher than activity of other isotypes (Fig. 8). At the second sampling period, when killing had decreased relative to killing by preceding samples ($P < 0.001$) (Fig. 5b), IgM had increased but so had IgG1 and IgG2 antibodies (Fig. 8). From the third sampling period on, IgG1 and IgG2 antibodies predominated (Fig. 8), and negligible killing activity for smooth *B. abortus* was demonstrated (Fig. 5b). Mean IgA levels peaked at the third sampling time but remained low throughout (Fig. 8).

To determine directly the role of anti-S-LPS antibodies of each isotype in killing (or blocking of killing), affinity-purified bovine anti-*B. abortus* S-LPS antibodies of each isotype were serially diluted in the presence of a standard amount of bovine complement and tested for killing of *B. abortus* 2308. The addition of IgA or IgM antibodies to fresh normal bovine serum produced no significant change in

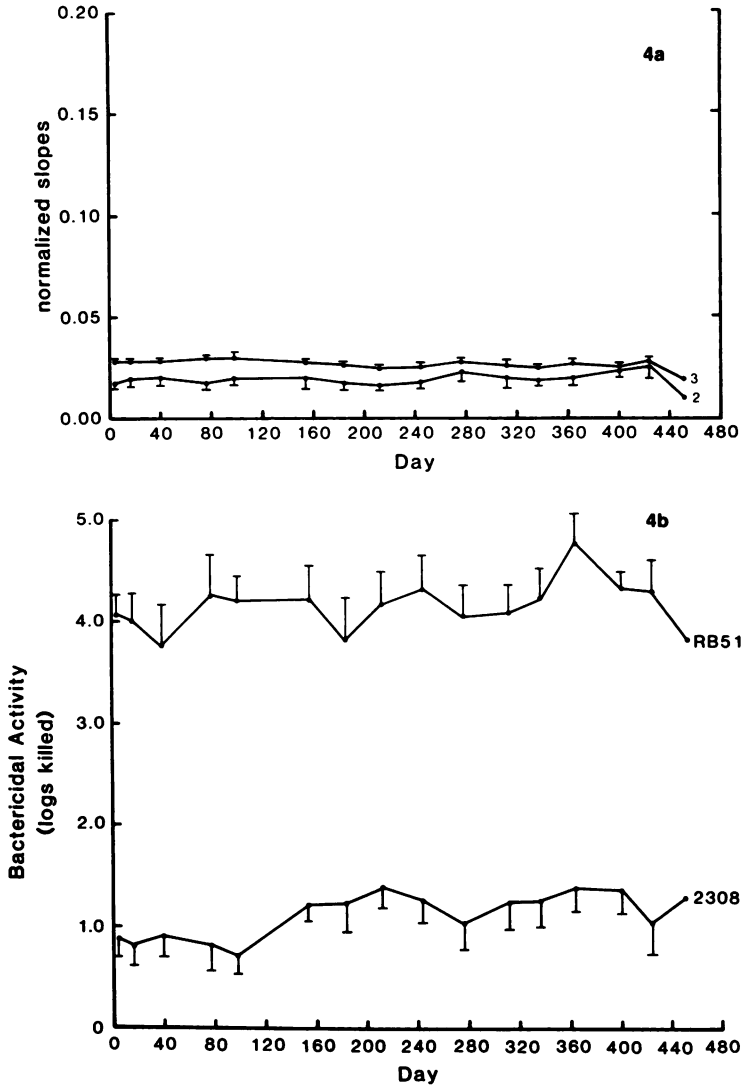

FIG. 4. Experiment 1: antibody response and complement-mediated killing of *B. abortus* by serum from four noninfected nonimmunized cattle. Values represent mean ± standard error. (a) Antibody to porin (line 2) and group 3 proteins (line 3) by ELISA presented as normalized slopes. (b) Killing of rough (RB51) and smooth (2308) *B. abortus*.

killing (Fig. 9). However, addition of IgG1 and IgG2 antibodies lowered killing values significantly ($P < 0.01$) (Fig. 9) to the range of killing seen with postinfection sera (Fig. 5b) or postimmunization sera (Fig. 2b). Moreover, the decrease in killing caused by IgG2 antibodies was significantly greater than that achieved by IgG1 antibodies ($P = 0.01$), as shown by two-way analysis of variance (Fig. 9).

DISCUSSION

B. abortus was equally susceptible to killing by fresh normal bovine serum and agammaglobulinemic calf serum, indicating that complement-mediated killing of *B. abortus* can occur in the absence of either natural or acquired antibody. Rough strains were much more susceptible to serum killing than smooth strains, as would be expected. Although both LPS and outer membrane proteins have been implicated in serum resistance of gram-negative bacteria (64), LPS appears to be the major factor determining the differences in serum susceptibility between smooth and rough *B. abortus* strains. The three *B. abortus* strains did not differ in outer membrane protein profiles (56), but the more serum-sensitive rough strains lacked the O side chains that were present in *B. abortus* 2308 and *S. typhimurium* (Fig. 7). That *B. abortus* with S-LPS is more serum resistant than *B. abortus* with rough LPS is consistent with data on other smooth and rough gram-negative bacteria, including enteric species (64) and *Campylobacter fetus* (52).

The complement pathway involved in killing was determined by treating serum with EGTA-$MgCl_2$. Since virtually no killing activity remained after EGTA-$MgCl_2$ treatment, it is likely that classical pathway activation was responsible (47, 48). This is consistent with data showing that *B. abortus* LPS does not activate the alternative pathway of complement (20). It may be that lipid A activation of the classical pathway was involved, since *B. abortus* lipid A has been reported to activate the complement cascade (42) and lipid A of other gram-negative bacteria binds and activates C1 to initiate the classical pathway (5). However, one cannot

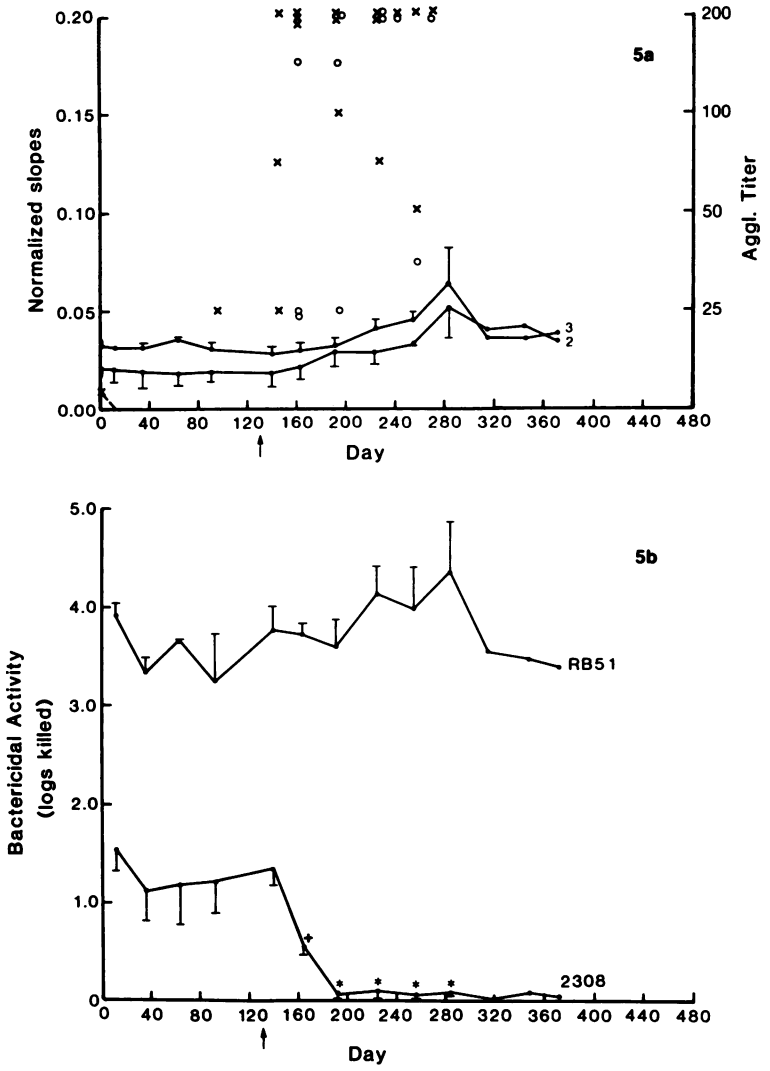

FIG. 5. Experiment 2: antibody response and complement-mediated killing of *B. abortus* by serum from three cattle infected with strain 2308. Five serum samples were taken before infection on day 129 (arrow). Values represent means ± standard error, except for agglutination data, for which individual animal titers are presented. (a) Antibody to porin (line 2) and group 3 proteins (line 3) by ELISA presented as normalized slopes. Tube agglutination titers (×) and rivanol agglutination titers (○) represent S-LPS antibody activity. Note that no agglutination titers are given for before day 93 or after day 320. Sera from these dates were negative by the card screening test, so no tube agglutination or rivanol titers were determined. (b) Killing of rough (RB51) and smooth (2308) *B. abortus*. Values significantly different from preceding values are marked: +, $P < 0.01$; *, $P < 0.001$.

generalize on this subject. In recent studies with *Haemophilus influenzae* type b, the alternative pathway was responsible for killing (59), but in this system anticapsular antibody was required. In the absence of anticapsular antibody, complement-mediated killing occurred but not by the alternative pathway (59). In contrast, an encapsulated strain of *E. coli* was shown to be killed primarily by antibody-dependent activation of the classical pathway (65), whereas an unencapsulated rough strain of *E. coli* activated the classical pathway independently of antibody (5). Clearly the mechanism of complement activation by bacteria varies with the surface characteristics of the organisms.

In cattle immunized with *B. abortus* cell envelopes, serum killing of rough strain RB51 increased when antibody activity increased, as has been reported for other gram-negative bacteria (64). However, in animals immunized with cell envelopes or infected with a virulent strain of *B. abortus*, the presence of antibodies was associated with a decrease in killing of smooth strain 2308, suggesting that antibody interfered with killing. A similar blocking effect of immune serum on killing of *B. abortus* has been noted by Hall et al. (17, 18), who demonstrated decreased bactericidal activity in serum from human patients with chronic brucellosis and showed that blocking antibodies in immune rabbit serum were of the IgA isotype (18). Wilkinson (68) found blocking antibodies in IgA and IgG but not in IgM fractions of serum from human patients with chronic brucellosis. More recently, IgA-blocking antibodies were demonstrated in serum from patients convalescing from meningococcal infections (16), and IgG antibodies were found to block serum bactericidal activity against gonococci (28, 35). The significance of these findings is emphasized by data showing that deficiencies in serum bactericidal activity are correlated with increased susceptibility to both meningococcemia and gonococcemia

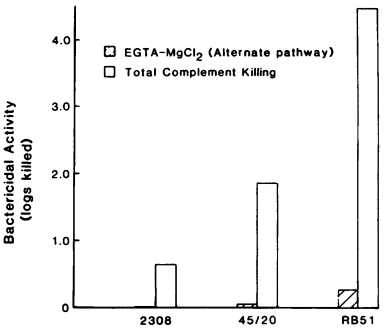

FIG. 6. Killing of *B. abortus* by normal bovine serum (open bars) or EGTA-$MgCl_2$-treated normal bovine serum (striped bars) after 3 h of incubation.

(51). In experimental brucellosis, passive transfer of antibrucella sera has been reported to promote the survival of *B. abortus* in mice (15) and chickens (14). However, both immune serum (2, 33, 40, 41, 50, 54, 62) and monoclonal O antibodies (30, 41) have been shown to protect mice against brucellosis. Thus, antibodies can either enhance or diminish *B. abortus* infection in vivo.

Antibody titers were detected in infected cattle before the decrease in bactericidal activity (Fig. 5), indicating that there was no blocking activity in the earliest antibody response. The identity of the nonblocking antibody as IgM was indicated by its precipitation by rivanol (Fig. 5a) and by the lack of inhibition of killing by purified S-LPS antibody of the IgM isotype (Fig. 9). The lack of increase in complement-mediated killing by nonblocking bovine IgM may be related to the capacity of *B. abortus* to bind the Fc region of a subpopulation of bovine IgM (49). In contrast, both IgG1 and IgG2 anti-S-LPS antibodies did block killing by fresh normal bovine serum (Fig. 9), similar to findings with *Neisseria gonorrhoeae* (28, 35, 55). The reason for blocking activity by bovine IgG1 and IgG2 antibodies is not clear, because both isotypes can fix bovine complement (37). It is noteworthy, however, that IgG2 antibodies were the more efficient blockers (Fig. 9) and that bovine IgG2 antibodies have been associated with prozone phenomena in *B. abortus*-specific agglutination tests (11) and complement fixation tests (39).

The specificity of blocking antibodies was studied by comparing killing by normal serum and serum from infected

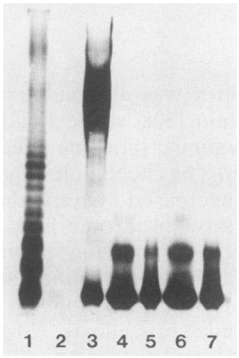

FIG. 7. PAGE profile of phenol-water-extracted LPS by SDS-PAGE and silver staining. Lane 1, Aqueous phase *S. typhimurium* LPS; lane 2, aqueous-phase *B. abortus* 2308; lane 3, phenol phase *B. abortus* 2308; lane 4, aqueous phase *B. abortus* 45/20; lane 5, phenol phase *B. abortus* 45/20; lane 6, aqueous phase *B. abortus* RB51; lane 7, phenol phase *B. abortus* RB51. Silver staining was done by the method of Tsai and Frasch (66).

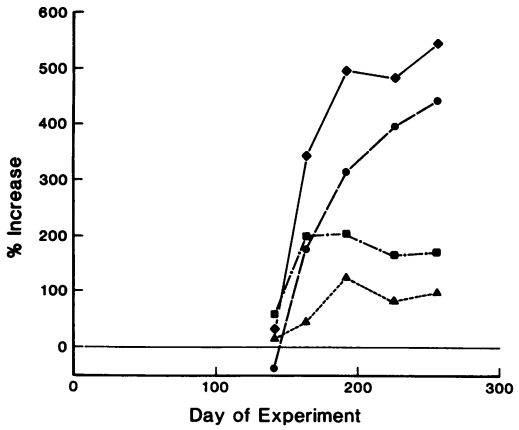

FIG. 8. Isotypes of antibodies to *B. abortus* in sera from cattle in experiment 2. Symbols: ◆, IgG1; ●, IgG2; ■, IgM; ▲, IgA. Reactions were determined by kinetics-based ELISA with a deoxycholate extract of *B. abortus* as the antigen. Data points represent percent increase in antibody concentration over the mean of four preinfection samples.

or immunized animals. Normal bovine serum is known to contain IgM antibodies cross-reacting with *B. abortus* LPS (51). Sera from infected animals contain predominantly IgG1 antibodies (3, 4, 9, 51), reacting with smooth LPS (57, 58). Animals immunized with cell envelopes of rough strain 45/20 produced high levels of antibodies to porins and group 3 antigens (Fig. 2a) and low concentrations of antibodies to S-LPS. The main inhibitory effect of these sera in killing of smooth strain 2308 (Fig. 2b) occurred when both outer membrane protein and S-LPS antibodies were at their highest levels (Fig. 2a). However, blocking effects were more complete and much more sustained in the infected heifers (Fig. 5b), which had high titers of S-LPS antibodies but little increase in antibodies to outer membrane proteins (Fig. 5a). The predominance of the antibody response to S-LPS in cattle infected with *B. abortus* has been noted previously (57, 58; C. L. Baldwin, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1983). These results indicated that anti-S-LPS antibody may have been primarily responsible for blocking killing, and experiments with affinity-purified anti-S-LPS antibody (Fig. 9) strongly supported this idea. This stands in contrast to the gonococcal system, in which antibodies to outer membrane proteins blocked killing by fresh normal human serum of strains from disseminated gonococcal infection (55).

Our findings that bovine serum bactericidal activity to smooth *B. abortus* is independent of antibody and that anti-S-LPS antibodies of both IgG isotypes block killing are consistent with those of Huddleson et al. (22). They found that fresh plasma from infected animals not only failed to prevent multiplication but also inhibited killing when added to bactericidal fresh normal bovine plasma. This inhibition was postulated to be due to antibodies which did not bind complement (22). Later, Huddleson (21) reported that serum collected 7 days after infection of guinea pigs with *B. abortus* was bactericidal in the presence of complement, but serum collected 30 days after infection was not bactericidal at low dilutions. This prozone effect was shown to be due to antibodies which did not mediate complement killing of *Brucella* (22). Irwin and Beach also reported substantial levels of bactericidal activity in sera of normal cattle and noted prozones in bactericidal tests with some sera from infected cattle (26). The absence of killing observed at lower

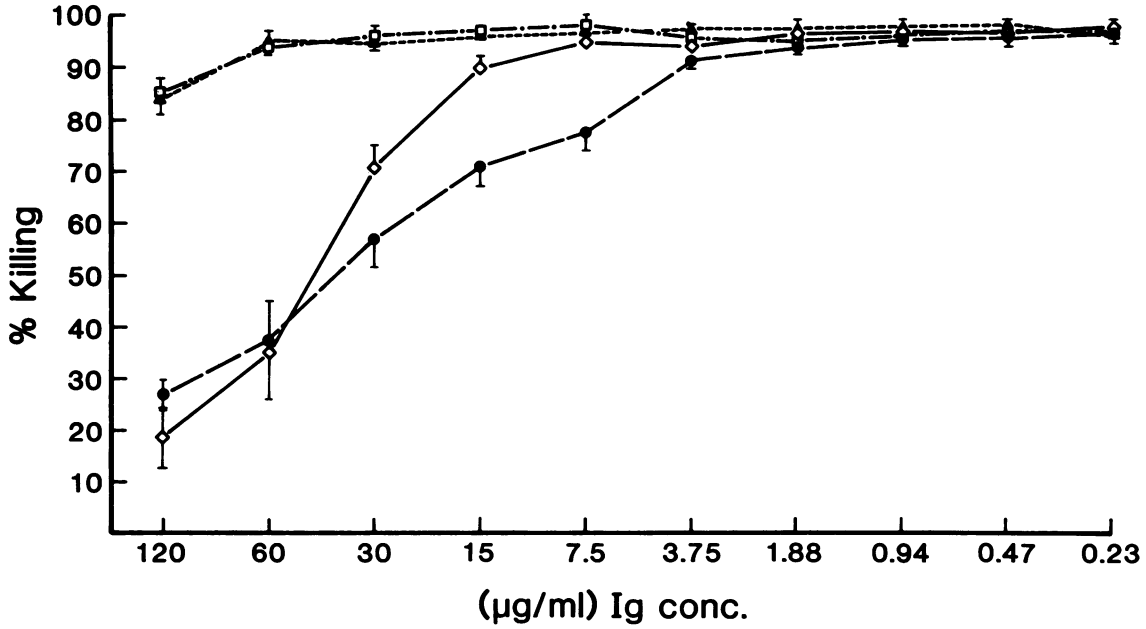

FIG. 9. Effect of purified antibody of each bovine isotype on killing of *B. abortus* 2308 by fresh normal bovine serum after 3 h of incubation. Isotypes used: ◇, IgG1; ●, IgG2; □, IgA; ▲, IgM. % Killing (i.e., the percentage of bacteria killed) = [(CFU per milliliter in heated serum − CFU per milliliter in fresh serum)/CFU per milliliter in heated serum] × 100. The experiment was repeated four times, with results presented as mean ± standard errors. Effects of IgG1 and IgG2 were significantly different from those of IgM and IgA ($P < 0.01$). Effect of IgG2 was significantly different from that of IgG1 ($P = 0.01$), as determined by a two-way analysis of variance.

serum dilutions (26) may have been due to IgG-blocking antibodies (Fig. 9). However, in this (26) and other early reports (24, 25, 27), increases in bactericidal activity were noted in the sera of many cattle following vaccination with strain 19 or infection with virulent *B. abortus* strains. The method employed by these workers for measuring bactericidal activity differed from ours in several respects. Most notably, they incubated overnight (as opposed to 3 h) and although undiluted serum was included, they used serum dilutions to achieve endpoints. We cannot exclude the possibility that dilution of sera in our study would have revealed the presence of antibody-mediated bactericidal activity, although the inhibition assay with decreasing quantities of purified S-LPS antibodies did not demonstrate an antibody concentration at which killing exceeded that achieved by normal serum (Fig. 9). While this issue has not been resolved, it is our view that the effect of undiluted serum on bacterial killing, as measured in this study, is the most accurate reflection of events which occur in vivo (12, 35).

These results may be related to the protective functions of the humoral immune response in natural *B. abortus* infections. Although the blocking activity of bovine anti-S-LPS antibodies appears to be contradictory to the protective role of O antibodies in mice (30, 41), these observations may be readily reconciled. Complement-mediated killing of *B. abortus* could occur very early in infection in cattle, before the formation of IgG-blocking antibodies. The serum bactericidal effect is likely to be most appropriate for host defense at this early period, when the number of bacteria is small and before *B. abortus* has become intracellular. Later in infection, IgG antibodies which block serum killing may thereby protect the host against the release of excessive quantities of endotoxin. Even though high titers of blocking antibodies may be associated with prolonged infection and even abortion (26), the same antibodies may also serve crucial functions in opsonization and enhancing intracellular killing by phagocytes.

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