Enzyme-Linked Immunosorbent Assay for Bovine Immunoglobulin Subclass-Specific Response to *Brucella abortus* Lipopolysaccharides

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An enzyme-linked immunosorbent assay was developed to follow the bovine response, by immunoglobulin class and subclass, to defined smooth and rough lipopolysaccharides (LPS) of *Brucella abortus*. Binding to smooth LPS of immunoglobulin G1 (IgG1) and IgG2 in sera from *Brucella*-infected animals was significantly greater than binding in sera from normal uninfected animals. Competition or steric blocking among IgM, IgG1, and IgG2 for binding sites on smooth LPS was shown to occur. Binding of IgM to *Brucella* smooth LPS with sera from uninfected animals was elevated above the assay control levels, and attempts to eliminate this nonspecific IgM binding were not successful. The same levels of nonspecific IgM binding were also seen with *Brucella* rough LPS, *Escherichia coli* LPS, and *Pseudomonas solanacearum* LPS. Sera from some, but not all, *Brucella*-infected animals showed elevated binding of IgG1 and IgM to both *E. coli* LPS and *Brucella* rough LPS as well as to *Brucella* smooth LPS. This was interpreted as specific antibody. Cross-reactions between *B. abortus* smooth or rough LPS and *E. coli* LPS could not be shown by immunodiffusion.

The bovine immune response to *Brucella abortus* is characterized by production of antibody first in the immunoglobulin M (IgM) class and then in the IgG class. This distribution in serum of classes of antibody produced has been shown to differ between cattle vaccinated with live *B. abortus* strain 19 and cattle infected with field strains of *B. abortus* (6). A quantitative study of immunoglobulin class- and subclass-specific response of vaccinated and infected cattle to whole *Brucella* cells has been conducted (5), but the system described in that paper is too complex for application to a diagnostic routine, and it does not provide for measurement of response to individual antigens.

The most commonly used serological tests for bovine brucellosis employ whole-cell antigens, and the immune responses to individual specific antigens of *B. abortus* have not been well characterized. The availability of well-characterized smooth and rough lipopolysaccharides (S-LPS and R-LPS, respectively) from *B. abortus* (20) now makes it possible to examine the immune response to purified LPS antigens.

The enzyme-linked immunosorbent assay

(ELISA) has been shown to be a very sensitive method for measuring immunoglobulin response (9, 15, 26). Application of the ELISA to diagnosis of bovine brucellosis has been described; an undefined soluble antigen from *Brucella* and a single enzyme conjugate with activity against both subclasses of IgG were used (26). The ELISA described in the present paper employed enzyme conjugates specific for bovine IgM, IgG1, and IgG2.

Diagnosis of bovine brucellosis has been complicated by the presence of a so-called "nonspecific" agglutinin for *Brucella* cells contained in the IgM fraction of bovine serum (17, 23) and by residual specific anti-*Brucella* antibody of the IgM class resulting from vaccination with *B. abortus* strain 19. For these reasons, various modifications of serological tests have been designed to minimize reactions of IgM and to measure IgG1 and IgG2 anti-*Brucella* antibodies (6, 10, 12, 21). The long-term objective of our work was to combine the use of defined antigens with a sensitive method for measuring specific immunoglobulins, which would clarify the bovine antibody response to *B. abortus* and lead to a useful diagnostic method. We report here some of the major parameters of the system, as well as diagnostic complications encountered.

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MATERIALS AND METHODS

Bovine test sera. Sera were obtained from animals in several herds infected with field strains of *B. abortus*. Two of these herds were in Florida, and one was in Wisconsin. Sera from animals experimentally infected with either *B. abortus* strain 2308 or *B. abortus* strain 19 were made available from an adult cattle vaccination experiment by the U. S. Department of Agriculture National Veterinary Services Laboratory, Ames, Iowa. Serological and bacteriological test results on the animals were known. Sera from the National Veterinary Services Laboratory also included some from mature, nonexposed animals.

Bacterial antigens. (i) S-LPS. Fermentor-grown cells of the smooth strain of *B. abortus* 1119-3 were obtained from the National Veterinary Services Laboratory. They were dried and extracted by the hot phenol-water method modified for *Brucella* by Redfern (M. S. Redfearn, Ph.D. thesis, University of Wisconsin, Madison, 1960) and Leong et al. (18), with additional changes as described by Moreno et al. (20). In brief, acetone-dried cells were extracted with equal volumes of phenol and water at 66°C for 30 min. LPS was precipitated from the phenol phase with methanol, dissolved in distilled water, and lyophilized.

(ii) Purified *Brucella* LPSs. Purified S-LPS from *B. abortus* strain 1119-3 and purified R-LPS from *B. abortus* strain 45/20 adsorbed to bovine serum albumin were prepared as previously described (20).

(iii) Others. *Escherichia coli* LPS O 128:B12 (Difco Laboratories) and *Pseudomonas solanacearum* S-LPS (kindly provided by Luis Sequeira) were also used in these experiments.

Bovine immunoglobulins. The globulin fraction was precipitated with $(NH_4)_2SO_4$ from colostral whey as described by Hebert (16), and IgG1 was isolated from it by continuous gradient elution with 0.01 to 0.7 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.6) from diethylaminoethyl-Sephadex A50 (Pharmacia) as described by Duncan et al. (13). The gradient was applied after a protein peak was eluted with the starting buffer, and the ascending portion of the resulting broad peak, measured by absorbance at 280 nm, was collected and tested for the presence of IgG1.

Bovine IgG2 was isolated from the globulin fraction [precipitated with $(NH_4)_2SO_4$ from bovine serum] by ion-exchange chromatography as previously described (13). The initial peak eluted from diethylaminoethyl-Sephadex A50 with 0.01 M tris(hydroxymethyl)-aminomethane-hydrochloride (pH 8.6) was tested for the presence of IgG_2.

Bovine IgM was isolated by redissolving a distilled water precipitate of bovine serum in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) and collecting the ascending portions of the peak excluded from Sephadex G200 (Pharmacia).

Immunoglobulin fractions at 10 to 12 mg of protein per ml were tested for purity by immunoelectrophoresis in 0.8% high-electro-endosmotic agarose (Sigma Chemical Co.), using barbital buffer, pH 8.6. Each fraction showed a single precipitin line when tested with the appropriate commercial (Miles Laboratories,

Inc.) rabbit anti-bovine immunoglobulin serum and a single line when tested with an anti-whole bovine serum, prepared in guinea pigs, which detected all of the immunoglobulin classes when tested with whole serum. Protein content of immunoglobulin fractions was determined by the method of Lowry et al. (19).

Antisera. Isolated bovine immunoglobulins were subjected to immunoelectrophoresis, using commercial rabbit anti-bovine IgG1, IgG2, and IgM. The resulting IgM precipitin arcs and the most cathodal parts of the IgG1 and IgG2 arcs were excised from the agarose plates and washed extensively in 0.1 M phosphate-buffered saline, pH 7.2. The respective precipitates, each consisting of 40 immunoelectrophoresis arcs, were emulsified in Freund complete adjuvant (Difco) and injected into three guinea pigs each (7). The animals were given a second injection 1 week after the initial immunization, and antisera were collected by cardiac puncture after 3 to 4 additional weeks. The strongest precipitating antibody of each antiserum was directed against the immunoglobulin subclass injected, as determined by double immunodiffusion; varying amounts of cross-reacting antibodies against the other subclasses were also present.

Immunoadsorption. Immunoadsorbents were prepared by mixing bovine IgG1 and IgG2 preparations with bovine serum albumin (fraction V; Nutritional Biochemicals Corp.) at a ratio of 1:10 and polymerizing the mixtures with 97% ethyl chloroformate (Aldrich Chemical Co.) at a concentration of 1 ml/g of protein, as described by Avrameas and Ternynck (4). Fetal calf serum (Grand Island Biological Co.) was polymerized by ethyl chloroformate (0.6 ml/g of protein). Immunoadsorbents were stored at 4°C with 0.05% NaN_3.

The guinea pig anti-bovine immunoglobulin sera were adsorbed to subclass specificity with appropriate immunoadsorbents. Adsorption was carried out at room temperature with stirring for 2 h and then overnight at 4°C before the immunadsorbents were removed by centrifugation at 10,000 × *g* for 10 min. Subclass specificity was verified by double immunodiffusion against purified bovine immunoglobulin preparations.

Conjugate. Anti-bovine IgM, IgG1, and IgG2, made specific by cross-adsorption, in 5-ml volumes were precipitated at 35% saturation of $(NH_4)_2SO_4$ (16). The individual immunoglobulin-rich fractions were dissolved in 1 ml of 0.01 M carbonate buffer (pH 9.5) and conjugated to 5 mg of horseradish peroxidase (type VI; Sigma) by the method of Nakane and Kawaoi (22), modified to eliminate the final gel filtration step for separation of bound and unbound enzyme, as suggested by Saunders et al. (26). The free enzyme did not interfere in the ELISA. The undiluted conjugate was stored at 4°C.

ELISA. The ELISA was performed as described by Engvall and Perlmann (15), with modifications for the peroxidase enzyme system. *B. abortus* S-LPS, *E. coli* LPS, and *P. solanacearum* LPS solutions at 10 ng/ml in 0.06 M carbonate buffer (pH 9.6) were dispensed into disposable polystyrene tubes (12 by 75 mm; Falcon Plastics) in 1-ml volumes. The tubes were then incubated at 37°C for 3 h. A *Brucella* R-LPS solution

at 10 ng/ml in barbital-acetate buffer, pH 4.6 (11), was incubated in the tubes at 75°C for 3 h (T. M. Buchanan, personal communication). Tubes containing the LPS solutions were stored at 4°C until use.

LPS was removed by suction, and the tubes were washed three times with phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 to reduce nonspecific binding. Bovine sera were diluted with phosphate-buffered saline (pH 7.2)–0.05% Tween 20 through a twofold series from 1:320 to 1:5,120, and 1 ml of each dilution was added to duplicate tubes for each subclass-specific conjugate. Assay controls included tubes with fetal calf serum at a 1:40 dilution, tubes without added serum, and tubes without fixed LPS. The tubes were incubated at room temperature for 5 h. The contents were removed, and three washings were performed as before, after which 1 ml of enzyme conjugate was added. The anti-IgM and anti-IgG1 reagents were used at a 1:750 dilution in phosphate-buffered saline (pH 7.2)–0.05% Tween 20, and the anti-IgG2 conjugate was diluted 1:250. These dilutions were selected on the basis of preliminary experiments, because they produced maximum differences in final optical density (OD) values between tests of sera from infected animals and assay controls, while remaining in the linear range of the spectrophotometer, and because they also conserved enzyme conjugates. After incubation at room temperature overnight, the tubes were again washed, and 1 ml of enzyme substrate was added. Working enzyme substrate was made up fresh and consisted of 1 ml of 1% (wt/vol) O-phenylenediamine (Eastman Kodak Co.) in methanol, 99 ml of distilled water, and 0.1 ml of 3% H_2O_2. The stock O-phenylenediamine solution could be kept at room temperature in the dark for up to 1 week. After incubation in the dark at room temperature for 30 min, 1 ml of 0.05 M H_2SO_4 was added to each tube to stop the reaction. The OD of the contents of each tube was measured at 490 nm.

The OD was thus proportional to the amount of conjugate bound, which was in turn proportional to the amount of anti-LPS of that specific immunoglobulin class present in the tested serum. The OD values, however, did not give a measure of the concentration of anti-*Brucella* LPS of a specific sublcass in the serum. A higher concentration of the anti-IgG2 conjugate was necessary to yield optimum OD differences between sera from infected animals and assay controls than was found with the anti-IgG1 reagent. This concentration difference could be a function of different levels of specific anti-*Brucella* LPS IgG1 and IgG2, or it could indicate inherent differences in the activity of the conjugates. Thus, direct comparison of OD values with the IgG1 and IgG2 conjugates cannot be assumed to indicate proportional concentration differences of anti-*Brucella* LPS of the IgG1 and IgG2 subclasses in serum used in the ELISA.

RESULTS

To ensure that the anti-*Brucella* immunoglobulin measured was directed against *Brucella* S-LPS rather than possible contaminants, the ELISA was performed with crude *Brucella* S-LPS (containing 30% protein) and purified *Bru-*

cella S-LPS (containing 6% protein), concurrently with identical serum dilutions from both *Brucella*-infected and nonexposed animals. OD values with each reagent were identical with both LPS preparations, indicating that the system measured only anti-LPS antibodies. Fixation of LPS to the tubes at pH 9.5 and washing away of unfixed LPS with a buffer containing Tween 20 probably resulted in removal of most of the proteins not covalently linked to the crude S-LPS (20). Crude *Brucella* S-LPS preparations were used exclusively after these tests.

Sera from 26 animals infected with *B. abortus* and from 16 nonexposed animals were examined in the ELISA against *Brucella* S-LPS by using the anti-IgG1 and anti-IgG2 conjugates. The OD values with the dilution series of sera from infected animals were high compared with the assay controls, although OD values with the anti-IgG2 conjugate were not as high as those with the anti-IgG1 conjugate. OD values from the dilution series of sera from nonexposed animals were always at or below the maximum values for the assay controls with both conjugates (Fig. 1).

Tests with bovine sera and the anti-IgM reagent with *Brucella* S-LPS gave substantially different results. Sera from infected animals showed high OD levels similar to those observed with the anti-IgG1 reagent, but OD values with sera from all nonexposed animals were also significantly higher than the assay controls (Fig. 2).

Examination of a dilution series of sera from eight infected animals with particularly high IgG1 levels by the ELISA against S-LPS with all three reagents showed that there is an interaction between the subclasses of antibody in the system. As the mean OD values with the anti-IgM reagent dropped with dilution of the test sera, the mean OD values with the anti-IgG1 and anti-IgG2 conjugates rose (Fig. 3). Serum from each animal showed significant increases in IgG1 and IgG2 binding from one dilution to the next. The range of differences in values among individual sera at each dilution was great enough to produce the overlapping of standard deviations of the mean OD values shown in Fig. 3.

To explore the nature of the IgM binding with sera from nonexposed animals, the pH of the immunoglobulin-binding step of the ELISA was lowered to pH 4.2 by diluting serum in 0.1 M acetate buffer (pH 4.2) plus 0.85% NaCl to dissociate nonspecific antibody-LPS complexes. OD values with the anti-IgM reagent were increased with all sera tested against *Brucella* S-LPS, including several that had been consistently low in the standard procedure.
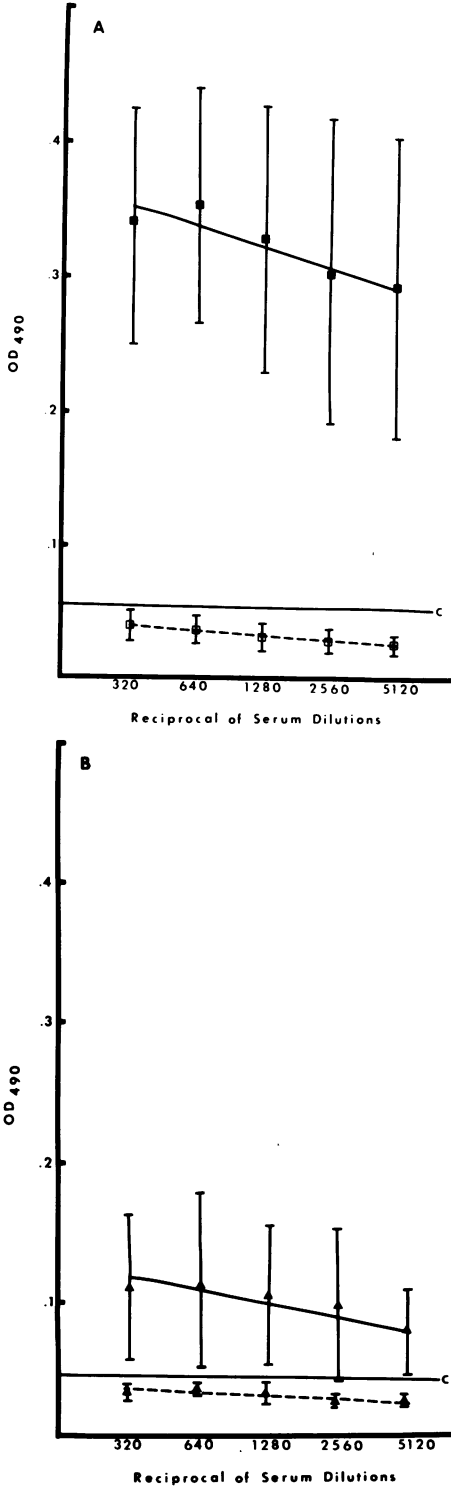
Sera from seven uninfected cattle were tested

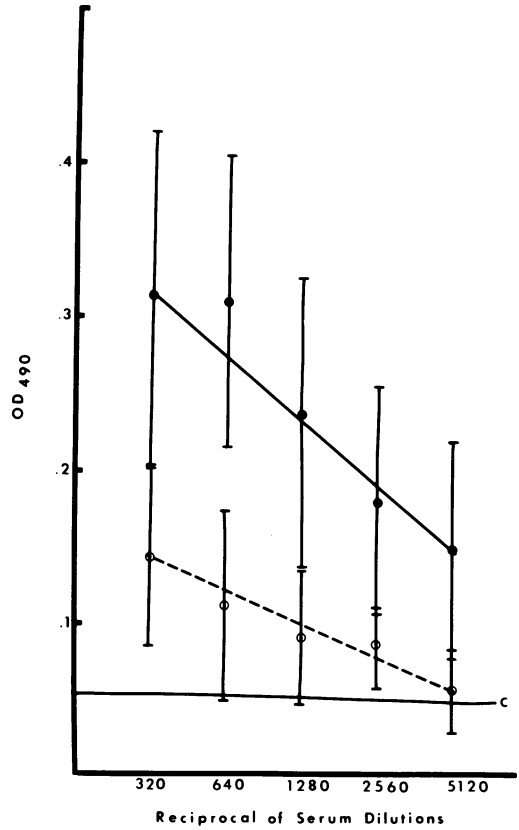FIG. 2. *Titration of bovine IgM antibody to Brucella S-LPS as measured by ELISA. Symbols: ●, means of values obtained with sera from 26 infected animals; ○, means of values obtained with sera from 16 nonexposed animals. Vertical bars represent two standard deviations. The horizontal line C shows the maximum level of assay controls. OD_{490}, OD at 490 nm.*

in the ELISA with *Brucella* R-LPS, *E. coli* LPS, and *Pseudomonas* LPS to test the hypothesis that nonspecific IgM binding was unique to *Brucella* S-LPS. As Fig. 4 shows, levels of IgM binding to *E. coli* LPS were significantly higher than assay controls with sera from all animals. IgM binding to *E. coli* LPS with sera from two animals was extremely high; these sera also showed IgM binding to *Brucella* R-LPS. Tests of sera from the other five animals showed IgM binding to *Brucella* R-LPS to be greater than

FIG. 1. *Titration of bovine IgG antibody to Brucella S-LPS as measured by ELISA. Solid symbols indicate means of values obtained with sera from 26*

infected animals, and open symbols indicate means of values obtained with sera from 16 nonexposed animals. Vertical bars represent two standard deviations. IgG1 and IgG2 responses are shown in (A) and (B), respectively. The horizontal line C shows maximum level of assay controls. OD_{490}, OD at 490 nm.*
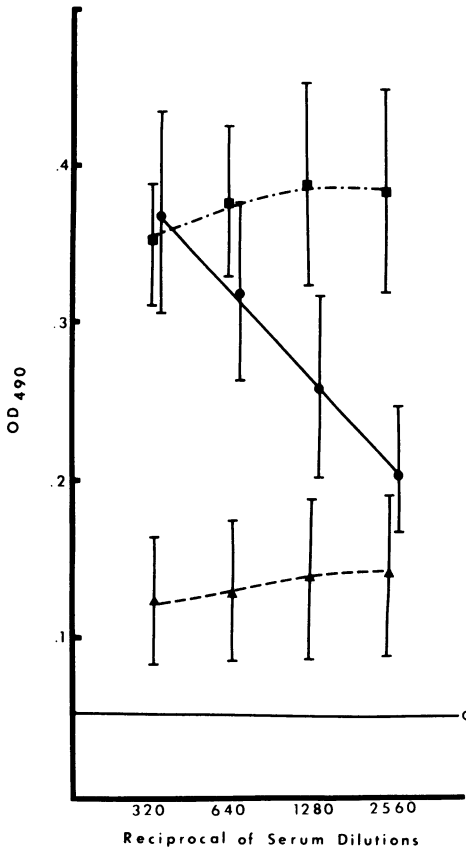
FIG. 3. *Titration of three classes of bovine antibody to Brucella S-LPS from sera with high IgG1 levels as measured by ELISA. Symbols indicate mean values obtained with IgG1 (■), IgM (●), and IgG2 (▲) reagents and sera from eight infected animals. Vertical bars represent two standard deviations. The horizontal line C shows the maximum level of assay controls. OD$_{490}$, OD at 490 nm.*

assay controls but never as great as the IgM binding to *E. coli* LPS. OD values with IgM reagent and *Pseudomonas* LPS were very close to assay control levels with sera from all animals tested. IgG1 binding to all LPS preparations was at or below the level of assay controls. When R-LPS and S-LPS from *Brucella* were tested concurrently with identical dilutions of serum from nonexposed animals, IgM binding was found to be higher with the R-LPS than with S-LPS.

To confirm that IgM binding to the LPSs was nonspecific, sera from eight animals infected with *B. abortus* were tested in the ELISA against *E. coli* LPS, *Brucella* R-LPS, and *Pseudomonas* LPS. IgM and IgG1 binding to *Pseudomonas* LPS was at or below the level of assay controls with all sera. Sera from all eight animals

showed IgM binding to *E. coli* LPS and *Brucella* R-LPS at levels above the assay controls (Fig. 5). Sera from some *Brucella*-infected cows (cows 69, 75, and 82) showed extremely high IgM binding to *E. coli* LPS. This high IgM binding was associated with IgG1 binding greater than assay controls, indicating a specific reaction against *E. coli* LPS. Serum from cow 75 before its exposure to *B. abortus* had a high level of IgM binding to *E. coli* LPS (Fig. 4). Serum from cow 69, with specific reactions to *E. coli* LPS, showed elevated binding of both IgM and IgG1 against *Brucella* R-LPS, as did serum from cow 20, which did not respond specifically to *E. coli* LPS. Serum from cow 69 precipitated with *E. coli* LPS in immunodiffusion but not with *Brucella* R-LPS.

An attempt to demonstrate an antigenic relationship between *Brucella* S-LPS and *E. coli* LPS by immunodiffusion in 0.5% agarose was not successful. The limited diffusion of the LPS molecules caused the lines of precipitation to form too close to the wells for the arcs to approach and make possible any visible cross or spur formation.

Absorptions of serum from infected cow 69 were carried out with S-LPS and *E. coli* LPS. Portions of serum were mixed separately with either *Brucella* S-LPS or *E. coli* LPS at 5 mg of LPS per ml of serum, allowed to react at room
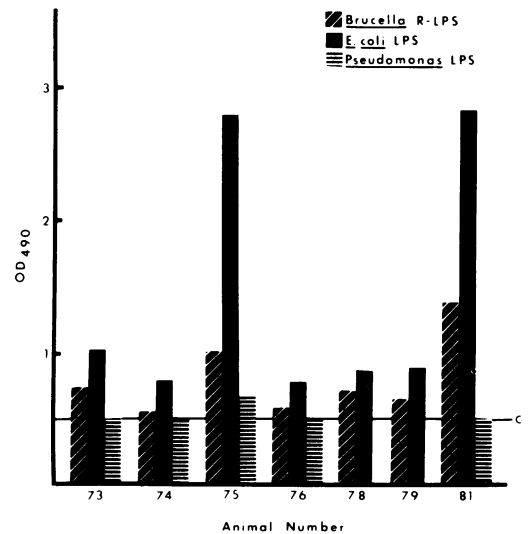

FIG. 4. *IgM antibody response to B. abortus R-LPS, E. coli LPS, and P. solanacearum LPS with sera from seven animals not previously exposed to Brucella, as measured by ELISA. Values shown are averages from duplicate tests of sera at a dilution of 1:320. The horizontal line C shows the maximum level of assay controls. OD$_{490}$, OD at 490 nm.*
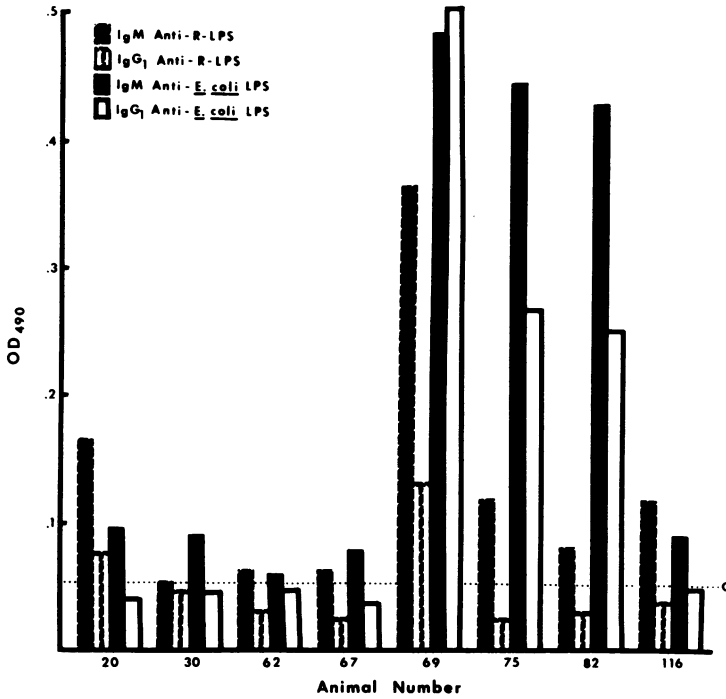
FIG. 5. *IgM and IgG1 antibody responses to B. abortus R-LPS and E. coli LPS with sera from eight animals infected with B. abortus. Values shown are averages from duplicate tests of sera at a dilution of 1: 320. The horizontal broken line C shows the maximum level of assay controls. OD₄₉₀, OD at 490 nm.*

temperature for 3 h and overnight at 4°C, and centrifuged to remove precipitate before use in the ELISA. The absorbed sera were then tested in the ELISA with *Brucella* S-LPS. Absorption with *Brucella* S-LPS removed all original activity of the serum against S-LPS, whereas absorption with *E. coli* LPS removed a small amount of IgM activity but had no effect on IgG1 activity against *Brucella* S-LPS (Table 1).

DISCUSSION

The presence of nonspecific agglutinins for *Brucella* cells in bovine serum has long been accepted. The agglutinins were studied extensively and found to be heat sensitive (3), to have sedimentation coefficients of 13.8S to 16.6S (25), and, more recently, to sediment with IgM in sucrose density gradients (23). The nonspecific agglutinin could be absorbed from some sera by organisms other than *Brucella*. With other sera such absorptions did not lower the titer of anti-*Brucella* agglutination (17). Absorption from serum of nonspecific agglutinin for smooth *Brucella* cells by rough *Brucella* cells has been reported (1). Using the radial immunodiffusion method, Beh observed a reduction in the IgM content of serum from nonexposed cattle after

TABLE 1. *Effect of absorption of bovine anti-B. abortus serum with Brucella S-LPS or E. coli LPS on binding of IgM and IgG1 to Brucella S-LPS*

Specific reagent	OD with:[a]			
	Unabsorbed serum	Serum absorbed with *Brucella* S-LPS	Serum absorbed with *E. coli* LPS	Assay controls
IgM	0.309	0.036	0.188	0.030
IgG1	0.280	0.045	0.262	0.035

[a] Values shown are average values for OD at 490 nm from duplicate tests with the 1:640 serum dilution.

absorption with *B. abortus* cells (5). His data can be interpreted as supporting our demonstration of nonspecific binding with the more sensitive ELISA method. The nonspecific agglutinin must be differentiated from specific early IgM anti-LPS from infected animals, as well as from that which may persist in noninfected animals after vaccination (2, 23). It seems probable from the data presented here that the nonspecific IgM agglutinin is reacting with the LPS molecules on the cell surface.

The nature of the reaction of *Brucella* S-LPS

and IgM from nonexposed cattle is not known. Lowering the pH to 4.2, at which specific IgM binding as seen in agglutination is diminished and nonspecific antibody-antigen reactions have been shown to dissociate (24), caused an increase in binding with the anti-IgM reagent. This suggests that the binding may be the result of a charge-charge attraction that becomes stronger under acid conditions.

Nonspecific IgM binding was also observed with the anti-IgM reagent and *B. abortus* R-LPS, *E. coli* LPS, and *P. solanacearum* LPS. These data suggest that the nonspecific reaction is not mediated through the *O* side chains, which are absent from the R-LPS and are different in sugar content among *Brucella* S-LPS, *E. coli* LPS, and *Pseudomonas* LPS. The view that nonspecific binding is a function of the lipid or polysaccharide core is supported by the observation of greater nonspecific IgM binding to RLPS than to S-LPS with the same serum dilutions.

Sera from some infected animals that had elevated IgM and IgG1 against *Brucella* S-LPS also showed IgM elevated above nonspecific levels and specific IgG1 antibodies against *E. coli* LPS and *Brucella* R-LPS. The response to common core determinants present in both *Brucella* S-LPS and R-LPS might be expected in animals infected with *B. abortus,* thus explaining the elevated levels of anti-R-LPS. However, elevated anti-*E. coli* LPS antibodies were not expected to accompany the elevated anti-S-LPS antibody levels in these animals. Cross-reactions between *Brucella* S-LPS and *E. coli* LPS could not be demonstrated by immunodiffusion, but this is not a sufficiently sensitive method to exclude the existence of such cross-reactions.

The absorption experiment indicates that there is no cross-reaction between LPS from *E. coli* and LPS from *B. abortus.* Absorption with *E. coli* LPS resulted in only a slight decrease of IgM binding to *Brucella* S-LPS, which can be accounted for by the nonspecific binding of IgM to both LPS preparations. These data suggest that the cows had been exposed previously to *E. coli* and that after exposure to *B. abortus* they developed elevated specific anti-*E. coli* levels of both IgG1 and IgM along with the elevated anti-*Brucella* S-LPS. This could occur either by an adjuvant effect from *Brucella* infection or by activation of all existing clones to similar LPS antigens (14).

There are several possible mechanisms for the observed elevation of IgG1 and IgG2 binding which accompanied the drop in IgM binding over a dilution series of sera from infected cows. Competition may occur between antibody classes and subclasses for binding sites on the LPS. At low dilutions, IgM present in fairly large amounts would compete with the IgG subclasses. Upon further dilution, the IgM of lower affinity would not compete as effectively and IgG would bind to the LPS sites. Steric hindrance could also play a role in this phenomenon. Pentameric IgM is large enough to prevent IgG binding to surrounding LPS sites or to block sterically detection of bound IgG by the anti-IgG reagents. Either or both mechanisms could produce the observed effects.

An ELISA that measures the response to more than one class or subclass of immunoglobulin gives a more complete understanding of this assay system than do earlier experiments in which single-class reagents were used. The competition for antigenic sites or steric blocking between the various subclasses of immunoglobulins shown here may be an explanation for reports of the occurrence of prozones in the ELISA. Bruins et al. (8) used only an anti-IgG reagent with a rough *Salmonella* system and noticed a prozone in which IgG binding was seen to rise over a series of dilutions of sera obtained early in immunization. The prozone was not observed with sera obtained later in the course of immunization. Early in immunization there would be higher IgM-IgG ratios of specific anti-LPS; a dilution of the IgM molecules of lower affinity would allow IgG to bind or be detected at higher dilutions. Later in immunization, when IgM levels drop, IgM would not block or compete as effectively with IgG and prozones would not be seen. A similar prozone effect has been described in an ELISA with rat serum, *E. coli* LPS, and both anti-IgM and anti-IgG reagents. The effect was abolished by heating serum at 56°C for 30 min (27). The same heat treatment did not prevent prozones or the competition effect in our bovine system and increased variability among replicate serum samples.

The ELISA using the anti-IgG1 and anti-IgG2 conjugates may prove to be useful for the diagnosis of bovine brucellosis. Sera from animals with no prior exposure to *B. abortus* consistently show low levels of IgG1 and IgG2 binding in the range of assay controls. That control level can be readily differentiated from the elevated IgG1 and IgG2 binding seen with sera from animals infected with *Brucella.*

Nonspecific binding of bovine IgM to *Brucella* LPS as well as to LPS from other species was demonstrated in the ELISA. Since the test conditions could not be modified to eliminate this binding and since residual IgM anti-*Brucella* titers from vaccination are also seen with this sytem, the ELISA using the anti-IgM conjugate

has not solved this diagnostic dilemma.

The competition or blocking between antibody classes and subclasses for binding sites makes the ELISA a much more complicated system than was previously thought. This competition should not interfere with use of the anti-IgG1 and -IgG2 systems as potential diagnostic methods, although misleadingly low readings may result if an unusually high specific ratio of IgM to IgG is present in the serum, as occurs in recently infected animals.

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