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Characterization of *Brucella abortus* Soluble Antigen Employed in Immunoassay

DAVID T. BERMAN,[1]* BAMBI L. WILSON,[1] EDGARDO MORENO,[1] ROBERT D. ANGUS,[2] AND LOIS M. JONES[1]

Department of Veterinary Science, University of Wisconsin-Madison, Madison, Wisconsin 53706,[1] and Veterinary Services Laboratory, U.S. Department of Agriculture, Ames, Iowa 50010[2]

A soluble antigen extract of *Brucella abortus* (BASA) has been prepared by the National Veterinary Services Laboratories and furnished to a number of workers who are examining antibody-mediated and cell-mediated immune responses of cattle infected with *B. abortus*. Three lots of BASA were examined. There were quantitative but not qualitative differences among lots by content of protein, total carbohydrate, hexose, fatty acid, and 2-keto-3-deoxyoctonic acid. The presence of smooth lipopolysaccharide was demonstrated by the presence of 2-keto-3-deoxyoctonic acid and lipid, by *Limulus* lysate gelation activity, and by formation of characteristic lipopolysaccharide precipitates in immunoelectrophoresis. A polysaccharide antigen as well as two nonsurface antigens, A2 and C, were also identified. BASA is a satisfactory antigen for use in the enzyme-linked immunosorbent assay since the smooth lipopolysaccharide component bound to polystyrene and functioned in the test. Normal murine spleen cells showed a mitogenic response to BASA similar to that produced by purified smooth lipopolysaccharide. BASA has been used in other laboratories to stimulate peripheral blood leukocytes from cattle infected with *B. abortus*. Because BASA is a mixture of antigenic components shown to have mitogenic effects in the mouse system, questions on the nature of its stimulatory effect on bovine cells are raised.

Stabilization of the prevalence of bovine brucellosis nationally, and indeed an increase in its prevalence in the southern states during the past decade (15, 34), have stimulated increased research on the disease. Investigations on improvement of diagnostic methods have received particular attention, in part because cattle immunized with the attenuated strain 19 of *Brucella abortus* may have residual antibody specific for the smooth lipopolysaccharide (S-LPS) complex, which cannot be distinguished from that resulting from infection (34, 40). Approaches to the problem have included examination of both antibody-mediated (9, 31, 33) and cell-mediated immune responses (17-19, 21, 27) of cattle to a variety of soluble antigen extracts. Because most of the residual antibody from strain 19 vaccination is immunoglobulin M (IgM), various tests have been designed which detect antibody of the IgG class (2, 6, 26). These tests include the automatable enzyme-linked immunosorbent assay (ELISA) (7, 8, 22, 29, 36).

In a number of investigations with both cell-mediated immunoassays and ELISA, a soluble antigen extract of *B. abortus* (BASA) produced by the Reagents Section, National Veterinary Services Laboratories, U.S. Department of Agriculture has been used (7, 18, 29, 36). This extract is the supernatant fluid from autoclaved cells of *B. abortus* strain 1119-3, whose composition has been partially described (18). The objectives of the work reported here were (i) the chemical characterization of three different lots of BASA, (ii) the antigenic analysis of BASA relative to content of S-LPS and non-S-LPS antigens, (iii) the determination of the extent of variability of composition among the lots, and (iv) the examination of some of BASA's biological activities.

MATERIALS AND METHODS

Preparation of antigen extracts. BASA was prepared at the National Veterinary Services Laboratories, Ames, Iowa, from strain 1119-3 cells which had been propagated in a fermentor as described by Alton et al. (3). Harvested cells were suspended in double-distilled water at a ratio of 1 g (packed wet weight) to 4 ml of double-distilled water, shaken for 2 h, autoclaved for 20 min at 121°C, cooled, and centrifuged at 17,000 × *g* for 20 min at 4°C. The supernatant fluid was removed, stored at −25°C without preservative, and designated BASA.

An initial lot (lot 10) of BASA had been prepared and distributed to several research laboratories. A portion of lot 10 and portions of two subsequent lots (lots 12 and 13) were sent to Madison in the frozen state. A 40-ml amount of each lot was thawed and

lyophilized so that the chemical analyses could be referred to BASA on a dry weight basis. The yield of each preparation is given in Table 1. The dried preparations were stored in a desiccator at room temperature.

Preparation and composition of soluble extracts of brucellae employed for comparative purposes have been described in detail previously. LPS preparations included crude (f_5) and purified (f_{5p}) S-LPS, extracted in the phenol phase from *B. abortus* strain 1119-3, and crude (R3) and purified (R3a) rough LPS (R-LPS), extracted in the aqueous phase from *B. abortus* rough strain 45/20 (25). Crude S-LPS was obtained from *B. melitensis* strain 16M by hot saline extraction (31), and LPS-free polysaccharide B (poly B) was obtained by trichloroacetic acid extraction from rough *B. melitensis* strain B115 as described by Diaz et al. (9). LPS-free, protein-rich preparations extracted from rough *B. abortus* 45/20 have been described (31). They included a sonic extract and a hypertonic NaCl extract referred to as brucellin (24).

Two LPS preparations from *Escherichia coli* O128: B15 were purchased from Difco Laboratories; the phenol-water extract was used in the chemical analyses and in the *Limulus* lysate gelation activity (LLGA); the trichloracetic acid extract was used in the mitogen assays.

Chemical analyses. Protein content was determined by the method of Lowry (23) with bovine serum albumin as standard. Hexose determinations were performed by the anthrone method (14), and total carbohydrate was determined by the tryptophan sulfuric acid (11) method. The standard for the sugar assays was an equimolar mixture of D-glucose and D-mannose, which was selected on the basis of gas chromatographic analyses of *Brucella* LPS (unpublished data). In previously published assays of LPS, the standard was an equimolar mixture of D-glucose, D-galactose, L-rhamnose and D-mannose (25). Amide-linked fatty acids plus ester-linked fatty acids were measured by the procedure of Haskins (13) with tripalmitin as standard, and results were calculated as percent palmitic acid.

The method described by Ashwell (5) was used for estimation of 2-keto-3-deoxyoctonic acid (KDO) with a modification to account for the presence of interfering 2-deoxy-D-ribose or other 2-deoxy-aldoses (37). The standards were 2-deoxy-D-ribose (Sigma) and ammonium salt of KDO (Sigma) and were not subjected to preliminary hydrolysis. Precipitate formation was common with the complex extracts and was prevented by addition of dimethyl sulfoxide to assay mixtures and standards after addition of thiobarbituric acid (20). Absorbance was read at both 552 and 536 nm. The appropriate extinction coefficients were used to calculate percent KDO.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (39). The lower gel consisted of 15% acrylamide, and the stacking gel consisted of 4% acrylamide. A constant current of 10 mA was applied until the bromophenol blue tracking dye had traveled 8 cm. The gels were stained for protein with Coomassie brilliant blue R-250 (Sigma). The marker proteins (5 μg each) were phosphorylase b (molecular weight, 94,000), bovine serum albumin (molecular weight, 68,000), catalase (molecular weight, 58,000), fumarase (molecular weight, 49,000), aldolase (molecular weight, 40,000), carbonic anhydrase (molecular weight, 29,000), hemoglobin (molecular weight, 16,000), and cytochrome *c* (molecular weight, 12,000).

LLGA. Assays were performed as described by Sullivan and Watson (35) except that the rack of tubes was removed from the 37°C water bath after 1 h and held at 4°C for 15 min before determination of gelation. The test mixture consisted of 0.1 ml of *Limulus* lysate and 0.1 ml of antigen solution. Activity was

TABLE 1. *Chemical composition and LLGA of BASA, LPS from B. abortus, and commercial LPS from E. coli*

Preparation	Protein (% of dry wt)	Total carbohydrate (% of dry wt)	Hexose (% of dry wt)	FAA + FAE[a]	KDO (% of dry wt)	LLGA[b] (ng/ml)	Dry wt (mg/ml)	Protein[c] (mg/ml)
BASA lot 10	20.5	16.6[d]	14.3[d]	13.9	0.58	2	11.43	8.9
BASA lot 12	16.7	8.9[d]	6.4[d]	13.0	0.86	2	10.49	7.2
BASA lot 13	16.0	10.6[d]	7.5[d]	14.3	0.28	2	8.03	6.0
f_5-crude S-LPS[e]	24.0	10.7[f]	ND[g]	19.0	0.67[h]	1		
f_{5p}-pure S-LPS[e]	6.3	11.6[f]	ND	26.4	0.86[h]	0.1		
R3-crude R-LPS[e]	1.3	13.6[f]	ND	10.0	1.5[h]	10		
R3a-pure R-LPS[e]	1.5	6.5[f]	ND	27.0	3.0[h]	1		
LPS from *E. coli*	4.4	18.4[f]	ND	43	1.7	0.2		

[a] FAA, Amide-linked fatty acids; FAE, ester-linked fatty acids. The numbers represent percent, calculated as palmitic acid, of dry weight.

[b] Lowest concentration needed to form a solid gel.

[c] Micro-Kjeldahl analyses performed at National Veterinary Services Laboratory, Ames, Iowa.

[d] Standard is equimolar mixture of D-glucose and D-mannose.

[e] Data for this preparation previously published (25).

[f] Standard is equimolar mixture of D-glucose, D-galactose, L-rhamnose, and D-mannose.

[g] ND, Not done.

[h] Corrected from those previously published (25).

expressed as the lowest antigen concentration in nanograms per milliliter needed to form a solid gel.

Serological analysis. Barbital buffer (pH 8.6) and 0.8% agarose were employed in immunoelectrophoresis. For double immunodiffusion, gels were prepared with 1% agarose in borate buffer (pH 8.3) containing 10% sodium chloride to enhance precipitation with bovine antisera (9).

Sera from *B. abortus*-infected cattle, hyperimmune rabbit sera, and monospecific sera were those previously described by Schurig et al. (31).

ELISA. BASA was used in comparison with a preparation of S-LPS from *B. abortus* strain 1119-3 in the ELISA described by Lamb et al. (22) with sera from infected cows. Separate enzyme conjugates specific for IgM, IgG_1, and IgG_2 were used. The S-LPS preparation contained 30% protein by the Lowry method (23) and had been employed by Lamb et al. (22) at a concentration of 10 ng/ml of carbonate buffer. In the present study, tests were also done with S-LPS concentrations of 10 ng, 100 ng, 1 μg, and 10 μg/ml. Dried BASA lot 10 was reconstituted in carbonate buffer at 10 mg/ml and employed in the ELISA at 1:1,000 dilution, as reported by Saunders et al. (29), and also at 1:100,000 dilution.

Mitogen assay. Assays of mitogenic activity of extracts for mouse spleen cells were done as described by Moreno and Berman (24) with spleen cells from the endotoxin-resistant C_3H/HeJ (38) and the congenic, endotoxin-sensitive $C_3H/HeAU$ mice provided by R. Auerbach (Zoology Department, University of Wisconsin).

Culture medium was RPMI-1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml (GIBCO), 5×10^{-5} M 2-mercaptoethanol, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Microbiological Associates, Walkersville, Md.).

Antigen extracts were dissolved in culture medium with the aid of sonication (Sonicator 180, British Instruments Inc.) at a concentration of 2.5 mg/ml and filtered through 0.45-μm-pore size filters (Millipore Corp., Bedford, Mass.). A volume of 0.05 ml of antigen extract diluted in medium was placed in triplicate wells of flat-bottomed microtiter plates (Linbro 1S-MRC-96-TC). Control wells had medium alone. Concanavalin A (Con A) (Sigma) was used as a control mitogen in all experiments. Spleen cells obtained from two or three mice, 7 weeks of age, were pooled, washed two times with 0.1 M phosphate-buffered saline (pH 7.4) at 4°C, and suspended in culture medium at 2.5×10^6 cells per ml. A volume of 0.2 ml (5×10^5 cells) was placed in each well. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air for 42 h. Two microcuries of [^{3}H]thymidine (New England Nuclear, Boston, Mass.; specific activity, 2.0 Ci/mmol) was added to the wells for 6 h before harvesting. The cells were harvested on glass filter paper with a multimanifold sample harvester, washed with 0.85% saline solution, and finally dried for 12 h at 37°C. [^{3}H]thymidine uptake was measured by liquid scintillation counting in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) and recorded in counts per minute.

RESULTS

Chemical analytical data and LLGA, given in Table 1, show the extent of variation among the three lots of BASA in protein, total carbohydrate, and hexose content. All three lots contained lipid and KDO and had the same LLGA. The data obtained with crude *B. abortus* S-LPS (f_5) were similar to that seen with the three lots of BASA, with the exception of LLGA and content of protein and lipid.

The presence of S-LPS in BASA was also demonstrated by immunodiffusion (Fig. 1). On the basis of dilution to extinction of the S-LPS line, as well as LLGA, BASA lot 10 contained approximately half the quantity of S-LPS as f_5.

Attempts were made to show identity of the S-LPS component with different preparations of smooth *Brucella*, including crude S-LPS from *B. melitensis*; the latter diffuses better than S-LPS from *B. abortus*. However, the S-LPS components did not diffuse far enough for the lines to join (Fig. 2). Poly B, the second component in Fig. 1, showed complete identity with poly B in other S-LPS preparations (Fig. 2), as well as with poly B prepared from a rough strain of *B. melitensis* which lacks S-LPS (Fig. 3).

BASA lot 10 was also examined for the presence of antigens distinct from the S-LPS com-

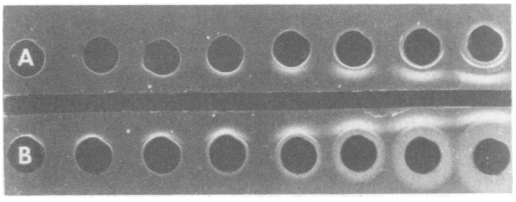

FIG. 1. *Immunodiffusion of doubling dilutions of BASA lot 10 (A) and B. abortus crude S-LPS f_5 (B) reacted with serum from a cow with brucellosis (center trough). The highest concentration of each antigen was 20 mg/ml in the well to the right. The diffuse lines closest to the serum trough are characteristic of poly B. The sharp lines closer to the antigen wells are S-LPS. At lower antigen concentrations, the S-LPS line is in the edge of the well.*

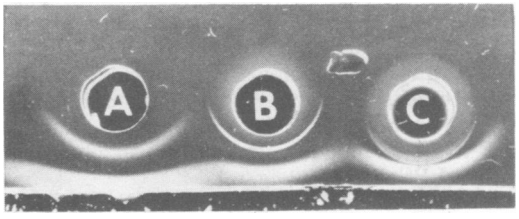

FIG. 2. *Immunodiffusion of B. melitensis crude S-LPS (A), BASA lot 10 (B), and B. abortus crude S-LPS (C) reacted with serum from a cow with brucellosis.*

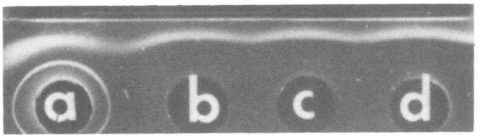

FIG. 3. *Immunodiffusion of BASA lot 10 (a) and three different preparations of LPS-free poly B (b, c, d) reacted with serum from a cow infected with B. abortus. The ring of precipitate around well A is attributed to the S-LPS.*

plex, which had been identified by means of immunoelectrophoresis and immunodiffusion (31). The use of reference sera from the previous study showed that antigens A2 and C are present in BASA; there were also additional antigenic components which could not be identified. As shown in Fig. 4, BASA and the LPS-free extracts have a common component which develops reactions of identity with infected cow sera and with monospecific anti-A2 serum. The infected cow serum pool also reveals a second line with BASA, but not with the protein antigens prepared from rough *Brucella* strains.

Antigen C had been identified as positively charged on the basis of its migration in immunoelectrophoresis of sonic extracts or protein extracts of rough brucellae (31). The presence of antigen C in BASA was inferred from formation of a precipitate in immunoelectrophoresis in a position corresponding to that of antigen C in 45/20 sonic extract (Fig. 5). The S-LPS lines are also evident near the antigen wells charged with S-LPS (f_5) and BASA, but not with 45/20 sonic extract. The lines produced by the anodally migrating antigens in BASA were diffuse, as contrasted with the well-defined lines produced by the antigens in the 45/20 sonic extract. Jones et al. (16) observed a similar loss of definition of antigens in protein extracts which had been heated to 100°C.

Sera taken from cattle before, and 4 to 20 weeks after, conjunctival inoculation with a virulent strain of *B. abortus* were tested in the ELISA with two concentrations of BASA and four concentrations of S-LPS. In general, with all three conjugates (anti-IgM, anti-IgG_1 and anti-IgG_2), the levels of binding of antibody to BASA at a 1:100,000 dilution and to S-LPS at 10 ng/ml were equivalent. The optical densities in these tests were low. Higher levels of binding were observed with BASA at 1:1,000 dilution, and these were equivalent to those obtained with S-LPS at 1 or 10 μg/ml.

As both the S-LPS preparation and BASA had high protein content, it is possible that antibody in the serum might be binding to components in addition to S-LPS. To examine this question, serum from an infected cow before and after absorption with living smooth *B. abortus* cells or with sonic extract from rough *B. abortus* 45/20 cells was tested. The results in Table 2 show that absorption with living smooth cells removed agglutinins, anti-S-LPS precipitins, and antigen binding capacity in ELISA with either the IgM or the IgG_1 reagents. In contrast, serum absorbed with sonic extract from rough cells retained its agglutinins, precipitins to S-LPS, and its binding capacity in the ELISA with both IgM and IgG_1 reagents.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the three lots of BASA is illustrated in Fig. 6. The absence of sharp protein

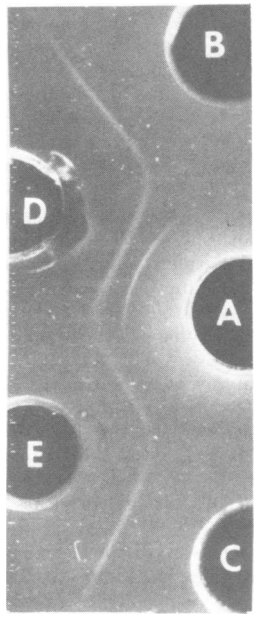

FIG. 4. *Immunodiffusion of BASA lot 10 (A), B. melitensis B115 protein antigen (B), and B. abortus 45/20 sonic extract (C) reacted with a pool of sera from cows infected with B. abortus (D) and monospecific anti-A2 serum (E).*

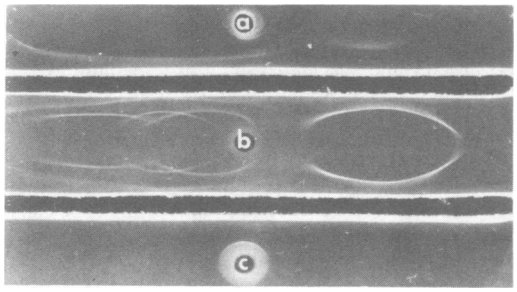

FIG. 5. *Immunoelectrophoresis of BASA lot 10 (a), 45/20 sonic extract (b), and B. abortus crude S-LPS F_5 (c) reacted with serum from a cow infected with B. abortus.*

TABLE 2. *Serological tests with serum from a B. abortus-infected cow*

Serum absorbed with:	Card test	Tube agglutination titer	Precipitation lines in immunodiffusion with:			OD[a] in ELISA with 1/320 dilution of test serum	
			S-LPS	BASA	Sonicate	IgM	IgG_1
Not absorbed	+	++1:6,400	+	+	+	0.73	0.93
Live cells *B. abortus* smooth strain 19	−	−1:100	−	−	+	0.06	0.06
B. abortus rough strain 45/20 sonic extract	+	++1:6,400	+	+	−	0.69	0.92

[a] Optical density at 490 nm.

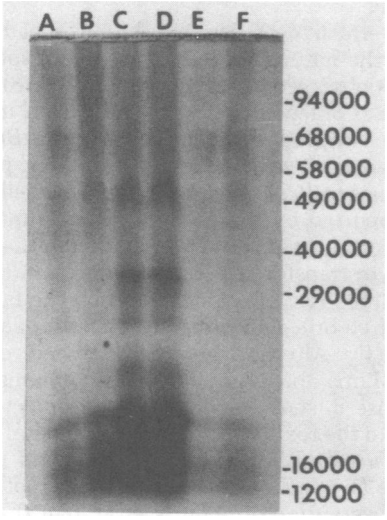

FIG. 6. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of marker proteins and BASA in 10% polyacrylamide gel. Preparations and concentrations were BASA lot 13, 0.3 mg, (A); lot 13, 0.6 mg (B); lot 10, 0.3 mg, (C); lot 10, 0.6 mg, (D); lot 12, 0.3 mg; (E); and lot 12, 0.6 mg (F).*

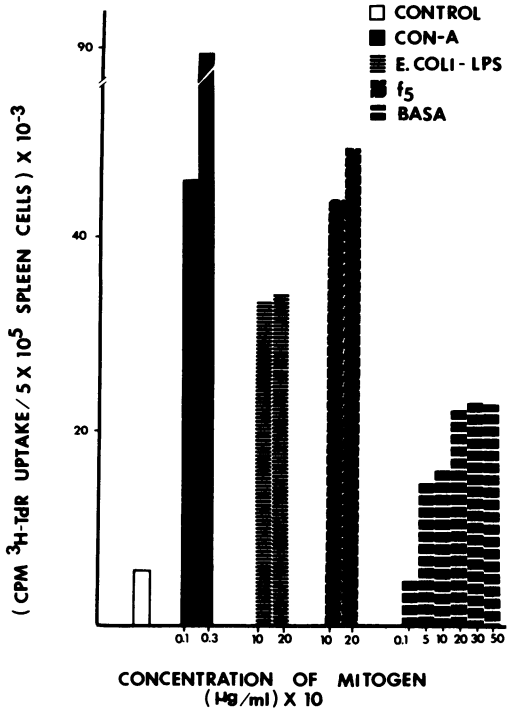

FIG. 7. *[^{3}H]thymidine incorporation by C3H/HeJ mouse spleen cell cultures containing concanavalin A, trichloroacetic acid-extracted E. coli LPS, B. abortus LPS (f_5), or BASA.*

bands in the gels is attributable, at least in part, to the relatively high content of S-LPS and to the presence of salt in these preparations. There were quantitative differences related to the differences in protein content among the three preparations, but qualitative differences were not apparent.

The mitogenic response of spleen cells of the endotoxin-resistant C_3H/HeJ mice to BASA lot 13 and to other mitogens is shown in Fig. 7. Similar results were obtained with spleen cells of the congenic, endotoxin-sensitive C_3H/HeAU mice (data not shown). The maximum stimulation level was reached at a concentration of 200 μg of BASA per ml; higher concentrations of antigen did not inhibit or significantly increase the uptake of [^{3}H]thymidine. The dose-response profile induced by BASA was similar to those produced with f_5 and f_{5p}, however, the counts per minute obtained were lower than with these endotoxin preparations from *B. abortus* and *E. coli*. The standard error was below 10% of the total counts for each preparation.

DISCUSSION

The presence of S-LPS in BASA was demonstrated by its content of KDO and lipid, LLGA, and by formation of characteristic LPS precipitates in immunoelectrophoresis and immunodiffusion. Poly B (9) was also seen in immunodiffusion. In addition to S-LPS and poly B, BASA was shown to contain the nonsurface antigens A2 and C which had been identified previously in protein-rich *Brucella* extracts but not in crude S-LPS preparations (31). These four antigens in BASA are among the principal ones to which

infected cows produce antibody (9, 31, 33).

It appears that the S-LPS component in BASA is the antigenic component which functions in the ELISA test. Lamb et al. (22) have shown that crude S-LPS containing 30% protein and purified S-LPS containing 6% protein gave identical results in ELISA with a variety of serum samples. The absorption experiment described in the present study shows that the antibody measured in ELISA is directed against surface antigens which are most probably S-LPS rather than against protein components. Lamb et al. (22) proposed that protein present in f_5 did not bind to the polystyrene, and Stemshorn (Ph.D. thesis, University of Guelph, Guelph, Ontario, Canada, 1979) was unable to demonstrate binding of protein A2 to polystyrene for use in an ELISA. Thus, ELISAs either with S-LPS preparations or with BASA apparently measure antibody of the same specificity as that detected in the agglutination and complement-fixation tests. BASA has the advantage of being easier to prepare than S-LPS.

Spellman and Reed (32) reported that crude *B. abortus* S-LPS was mitogenic for murine spleen cells, and Moreno and Berman (24) confirmed this with purified phenol-water-extracted LPS, either from smooth or rough *B. abortus*. The data in that report support the position that mitogenic stimulation by purified *Brucella* LPS of spleen cells of C_3H/HeJ mice is attributable to the unique composition of *Brucella* lipid A (25) and not to the presence of an endotoxin protein. In addition, they (24) observed that two LPS-free protein preparations extracted from rough *B. abortus* by different methods (31) showed different biological activity. The sonic extract was mitogenic for murine spleen cells; the brucellin was not. These observations are extended by the present demonstration of a mitogenic effect by BASA on murine spleen cells, with a dose-response curve similar to that produced with *Brucella* S-LPS.

In their studies on in vitro stimulation of bovine peripheral blood leukocytes (PBL) by *Brucella* antigens, Kaneene et al. (17) found that the PBL from cows infected with *B. abortus* demonstrated greater thymidine uptake when cultured with BASA than when cultured with LPS-free brucellin provided by us. Greater stimulation was observed with BASA even when the two antigen preparations were used at equal protein concentrations. Although they recognized the presence of endotoxin in BASA on the basis of LLGA, they discounted its significance and suggested that "... the protein fraction was primarily responsible for stimulating the sensitized lymphocytes to undergo blastogenesis" (18). The basis for this conclusion is unclear, and their data can be interpreted equally well if it is postulated that LPS contributes to the stimulation. Significantly, brucellin produces typical delayed-type hypersensitivity reactions in *Brucella*-infected cows, goats, and guinea pigs (3) and is not mitogenic for normal mouse spleen cells. As shown in the present studies, BASA is mitogenic for normal murine cells at concentrations equal to those used by Kaneene et al. in their bovine PBL stimulation assay. At those concentrations, there is at least 100 μg of LPS per ml.

Several hypotheses may be advanced to explain the nature of the bovine PBL response to BASA observed by Kaneene et al. (17, 18). Some of the proliferation seen in PBL from infected cattle cultured with LPS-containing *Brucella* antigens such as BASA may represent expansion of clones of *Brucella* LPS-committed cells. This is supported by the observation of Kaneene et al. (18) that maximum stimulation in their lymphocyte transformation system occurred in cultures incubated for 6 days and by the fact that LPS-free brucellin produced less stimulation of PBL (17). Alternatively, the increased response of PBL may be one manifestation of nonspecific increase in sensitivity of *Brucella*-infected animals to the toxic effect of enterobacterial as well as *Brucella* LPSs reported by several investigators (1, 10). Consistent with this hypothesis is the observation of Schurig et al. (30) that PBL from *Campylobacter*-infected cows, but not those from normal animals responded to *E. coli* LPS. Also, it is known that in vitro transformation responses of lymphocytes from humans and other animals during severe infection or after vaccination (4, 12, 28) are largely governed by nonspecific factors. Because of this, studies of lymphocyte responses to antigens and bacterial products should include several controls in addition to cells from normal and infected subjects. Determination of the responses of cells from *Brucella*-infected cattle to purified LPS from *E. coli* or *Salmonella*, in addition to purified *Brucella* LPS, and the responsiveness to BASA of PBL from cattle infected with organisms other than *Brucella* would be appropriate tests of this hypothesis.

The possibility of using the lymphocyte stimulation response as a means of detecting early *Brucella* infection is worth pursuing (19), but it has not yet been proved rigorously that the response is specific for *Brucella*.

Our demonstration that BASA contains a mixture of components which are mitogenic for normal mouse spleen cells emphasizes the necessity of clarifying the nature of the responses of bo-

vine PBL to BASA. Without examination of the response to defined antigens, conclusions on the mechanisms of the in vitro stimulation tests, with such a complex mixture of antigens, should be considered as tentative.

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