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Nylon Bead Enzyme-Linked Immunosorbent Assay for Detection of Sub-Picogram Quantities of *Brucella* Antigens

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An indirect sandwich enzyme-linked immunosorbent assay, using antibody covalently coupled to nylon beads, has been adapted for the detection of *Brucella* antigens. Optimum conditions were achieved by incubation of 1 ml of reaction mixture with a single bead, and by minimizing nonspecific interactions through the use of beads coated with purified bovine antibodies, preabsorption of third layer rabbit antibodies with normal bovine serum, and treatment of beads with normal goat serum before addition of the goat anti-rabbit enzyme conjugate. Beta-galactosidase was selected for use with clinical samples primarily because of low levels of endogenous enzyme in bovine leukocytes. Use of a fluorogenic substrate enhanced sensitivity 20-fold. Under these conditions, 100 fg of solubilized crude lipopolysaccharide or 8 to 10 *Brucella* cells was detectable in a fixed volume of 1 ml. A system was also devised for concentrating antigen which permitted ready detection of 2 pg of lipopolysaccharide in a volume of 50 ml (40 fg/ml). Attempts to detect lipopolysaccharide in the presence of concentrated serum or plasma were unsuccessful, but 10 brucellae added to a suspension of leukocytes from 100 ml of normal bovine blood were easily measured.

An important obstacle to eradication of bovine brucellosis is the inability to detect incubative infections, i.e., infections in animals manifesting neither serological evidence of exposure nor clinical signs of disease (23). Frequently, infection cannot be confirmed until after an animal has aborted, thereby allowing perpetuation of disease within the herd. A concerted effort has been made during the past several years to develop more sensitive antibody assays (12, 14, 18, 24, 27, 30, 31) to allow earlier detection of brucellosis. Another approach to diagnosing disease is direct detection of a microbial antigen (2, 3, 7, 28, 32, 33, 35, 36). We undertook to determine whether infection with *Brucella abortus* could be diagnosed earlier by detection of antigen than by the most sensitive antibody tests. The objective of this study was to develop a highly sensitive assay for *Brucella* antigens and to apply it for the detection of whole organisms or soluble antigens in large samples of bovine blood. Although *B. abortus* is commonly shed in milk, we concentrated on detection of antigen in blood because diagnosis of incubative infections is most imperative in nonlactating animals, e.g., pregnant heifers or cows in the last trimester of pregnancy. Detection of the organism or its antigens was attempted in the leukocyte fraction because it was hypothesized that *B. abortus*, a facultative intracellular parasite (20, 25), is conveyed through the bloodstream primarily within phagocytes.

MATERIALS AND METHODS

Antigens. Killed whole cells of *B. abortus* 2308 were prepared from organisms cultivated to log phase (34). Viable counts were made (34) on suspensions of washed cells (optical density at 610 nm, 0.14), which were then killed by the addition of concentrated Formalin solution to 0.5%. The relationship was then calculated between the optical density at 610 nm of the killed cell suspension and the actual concentration of organisms based on viable counts, considered an accurate representation of cell numbers since counts had been performed on cells in log phase. The killed cell suspension was adjusted to 1.0×10^9 cells per ml in sterile phosphate-buffered saline (PBS; pH 7.2), from which desired concentrations were prepared in dilution bottles. Suspensions of cells (6 to 7 ml) in an ice bath were sonicated in 15-ml rosette cooling cells (Branson Sonic Power Co., Danbury, Conn.) for 10 min at 60 W, using an ultrasound generator (model 1510, Bran-Sonic) (Braun Instruments, South San Francisco, Calif.) fitted with a 4-mm-diameter microprobe. Portions (1 ml) of whole or sonically disrupted cells in PBS mixed with Tween 20 (to 0.05% [vol/vol]) and normal bovine serum (to 1.5% [vol/vol]) were employed as antigens.

A trichloroacetic acid extract (5) of whole cells of *B. abortus* 2308 served as a source of soluble antigen. The extract was lyophilized after exhaustive dialysis against deionized water and was stored in a desiccator

at 4°C. Immunodiffusion (34) revealed the presence of two antigens, lipopolysaccharide and the native hapten of lipopolysaccharide, a related but not identical component (5, 22, 34). Contents of total hexose (35%), protein (15%), and nucleic acids (26%) were estimated by the methods of Dubois et al. (8) Peterson (26), and Kabat (16), respectively. Only the contribution of nucleic acids was omitted in calculating, on a dry weight basis, the concentration of antigen in the preparation, henceforth referred to as crude lipopolysaccharide (LPS_c). Desired concentrations of LPS_c were prepared in PBS containing 0.05% (vol/vol) Tween 20 and 1.5% (vol/vol) normal bovine serum (PBSTS).

In some experiments, LPS_c or sonically disrupted suspensions of cells in PBS were hydrolyzed in 1% (vol/vol) acetic acid for 45 min at 100°C under vacuum. The hydrolysates were cooled, neutralized with 3 M NaOH, and empolyed as antigens after the addition of Tween 20 and normal bovine serum.

Antisera. A 16-month-old heifer and four mature rabbits were immunized twice intramuscularly with a mixture of killed whole cells and crude cell envelopes (34) of *B. abortus* 2308 in incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.). One liter of blood was drawn from the heifer 10 days after each immunization. Rabbits were exsanguinated 10 days after the second immunization. Sera were stored at −20°C. Serum from the first bleed of the heifer developed a weak precipitin line with LPS_c. Serum from the second bleed and a pool of the rabbit antisera produced strong reactions with LPS_c and native hapten, as well as with other unidentified antigens in sonic extracts of strain 2308.

Globulin-rich fractions were prepared from the bovine serum (second bleed) and from a pooled sample of the rabbit antisera by $(NH_4)_2SO_4$ precipitation (19) and were stored at −20°C.

Bovine antibodies were purified on an immunoabsorbent prepared by coupling LPS_c to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals [9]). A globulin fraction from the first bleed was used since the majority of antibodies from the second bleed could not be eluted from the gel. Antibodies were eluted with 3 M sodium thiocyanate (4), concentrated by ultrafiltration, and stored at −70°C in 5-ml polypropylene vials (Walter Sarstedt, Inc., Princeton, N.J.).

In most experiments, the globulin fraction of the rabbit serum was preabsorbed with normal bovine serum. Whole bovine serum was coupled to CNBr-activated Sepharose 4B (9). A 1-ml sample (settled volume) of the gel was mixed with 1 ml of the globulin fraction (8 mg), and the contents were incubated for 16 h at 4°C on a tumble mixer before being centrifuged at 700 × *g* for 10 min at room temperature. The supernatant comprised the absorbed fraction.

Simulated clinical samples. Samples (100 ml) of heparinized blood were centrifuged at 2,000 × *g* for 20 min at 4°C. LPS_c dissolved in PBS was added to the desired concentration to the plasma fraction. Plasma was freed of lipid by treatment with 10% (vol/vol) ether, and Tween 20 was added to a concentration of 0.05% (vol/vol) before incubation with nylon beads. In some instances, tests were performed on diluted or heated plasma containing 0.1 M EDTA (6).

Leukocytes were obtained from the cellular fraction after repeated lysis of erythrocytes under hypotonic conditions. The final pellet of leukocytes (volume approximately 0.3 ml) derived from 100 ml of blood was resuspended in 1 ml of PBS containing a desired concentration of killed *Brucella* cells. The suspension was disrupted by sonication for 20 s and centrifuged at 500 × *g* for 5 min at room temperature to remove leukocyte debris, and the supernatant was retained. The pellet was washed with 0.3 ml of PBS and centrifuged as described above. The two supernatants were then combined and after the addition of Tween 20 to a final concentration of 0.05% (vol/vol), they were sonicated for 10 min. The final product (1.2 to 1.4 ml) was incubated with a single bead.

Enzyme-linked immunosorbent assay (ELISA). (i) Preparation of the solid phase. Antibodies were coupled to nylon beads, using a method similar to that of Hendry and Herrmann (13). Nylon 6/6 beads (6.4 mm in diameter, coarse finish, Precision Plastic Ball Co., Chicago, Ill.) were held in 3.5 M HCl (1 ml per bead) for 16 h at room temperature. The beads were rinsed in deionized water, and after neutralization of residual acidity with 0.1 M Na_2CO_3 and washing in PBS, glutaraldehyde (8% [vol/vol]; EM Grade; Electron Microscopy Sciences, Fort Washington, Pa.) was added at 0.5 ml per bead, and the beads were held for 2 h at room temperature. These procedures were performed with the beads either stationary or gently shaken (100 rpm; Junior Orbit Shaker, Labline, Ill.). After thorough washing with vigorous shaking in deionized water, the beads were allowed to stand in deionized water for 16 h at room temperature. Examination for traces of unbound glutaraldehyde was performed by washing the beads in a minimum volume of deionized water and analyzing the washings with modified Schiff reagent (Fisher Scientific Co., Pittsburgh, Pa.). Variations in the concentration of aldehyde groups among beads was assessed visually by staining with Schiff reagent.

Immunoglobulins diluted to either 100 μg/ml (for globulin fractions) or 50 μg/ml (for purified antibodies) in PBS were added to the beads and incubated for 4 h at room temperature with gentle shaking at 100 rpm. Unbound protein was removed by washing the beads with PBS, and unreacted aldehyde groups were blocked by incubation with either 2% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) or 0.5 M lysine (free base; Sigma) in PBS for 2 h at room temperature. In some experiments, beads treated with lysine were washed and incubated in PBS containing 5% normal bovine serum for a further 2 h. The beads were washed thoroughly in PBS and stored at 4°C in sterile PBS. In some instances, the washed beads were lyophilized in PBS containing 5% normal bovine serum, divided into groups of 10, and stored at −20°C with desiccant. All beads were rinsed in PBS before use.

(ii) Assay procedure. Antibody-coated nylon beads were added to fixed sample volumes in 5-ml polypropylene vials (Sarstedt) or, if volumes exceeded 1.4 ml, in 25-ml glass Erlenmeyer flasks. All glassware was acid cleaned. Samples were placed on a rotary shaker at 250 rpm for 16 h at 4°C.

For concentration of antigen, 50-ml samples were dispensed into vertically mounted barrels of 60-ml polypropylene syringes, each containing a single bead. The tips of four barrels were connected by rubber tubing and polypropylene 'Y' connectors (Fisher) to produce a single outlet which was joined to a peristal-

tic pump (Pharmacia). The liquid in the barrels was drawn out over the stationary beads at a flow rate of either 2.1 ml/h for 24 h or 1.05 ml/h for 48 h at 4°C.

After incubation, beads were transferred to glass test tubes and washed three times with PBS containing 0.15% (vol/vol) Tween 20 (PBST*). A 1-ml sample of a 1:500 dilution of rabbit anti-*Brucella* globulins in PBSTS was added to each tube containing one or more beads. After incubation for 45 min at 37°C, the beads were washed three times with PBST*, followed by the addition of 1 ml of conjugate diluted in PBSTS. In some experiments, beads were incubated with 1 ml of 2% normal goat serum in PBS containing 0.05% (vol/vol) Tween 20 for 45 min at 37°C and were washed three times with PBST* before the addition of conjugate. Conjugates consisted of goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (1:2,000 dilution; Cappel Laboratories, Cochranville, Pa.) or to β-galactosidase (1:1,000 dilution; Zymed Laboratories, Burlingame, Calif.). Beads were incubated for 45 min at 37°C, washed three times with PBST*, and transferred to separate tubes for addition of substrate.

Peroxidase activity was assayed by the addition of 0.6 ml of 2,2'-azino-di-(3-ethyl benzothiazoline) sulfonic acid (ABTS) (diammonium salt; Sigma) [15]). Beads were incubated at room temperature, and the reaction was stopped after 25 min with an equal volume of 0.1 M hydrofluoric acid. Readings were performed in a spectrophotometer (model DU, Beckman Instruments, Inc., Fullerton, Calif.).

Beta-galactosidase activity was assayed by the addition of 0.5 ml of either the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma [10]) or the fluorogenic substrate 4-methyl-umbelliferyl-β-D-galactoside (4MUG; Sigma [1]). Beads were incubated at 37°C, and the reaction was terminated after 45 min by the addition of 80 μl of 3 M Na_2CO_3. The absorbance of o-nitrophenol was measured at 405 nm (17) in an ELISA Reader (Microtiter, Dynatech Laboratories, Inc., Alexandria, Va.). For the fluorometric assay, the reaction mixture was diluted 100-fold in 0.5 M Na_2CO_3, and fluorescence was measured in a fluorometer (Turner, model 112, Sequoia-Turner Corp., Mountain View, Calif.) equipped with a temperature-stabilized door (25°C), using an excitation radiation of 360 nm (Corning 7-60 filter) and an emission wavelength of 448 nm (Wratten 2A and 48 filters [17]).

Statistical treatment of results. All samples were assayed in triplicate, and comparisons between test and control samples were made with the *t* test (29).

RESULTS

Preparation and storage of beads. The concentration of aldehyde groups varied appreciably on beads that were treated while stationary with acid and glutaraldehyde, but it was homogeneous on beads gently shaken in these reagents. All beads used for these studies were therefore prepared with shaking.

Of the reagents tested for binding to unreacted aldehyde groups, only lysine followed by bovine serum produced beads with satisfactorily low background absorbance.

Beads coated with the globulin fraction of serum could be stored in PBS at 4°C for several months without loss of activity. Under the same conditions, the activity of beads coated with purified antibodies was lost after 3 days. However, such beads lyophilized in the presence of 5% normal bovine serum and stored in a desiccator at −20°C retained activity for 3 to 4 months.

Optimal conditions for the assay. A comparison was made of the efficiency of detecting a fixed quantity of antigen in different volumes of fluid and with different numbers of beads (Table 1). The data presented are representative of two experiments. When two or three beads were employed, they were maintained as a group throughout the assay. Volumes of third-layer, fourth-layer, and substrate reagents were kept constant regardless of the number of beads. Incubation of a single bead in 1 ml of solution produced a greater increase in absorbance than with one bead in 5 ml of solution containing the same quantity of LPS_c (Table 1). The potential advantage of using more than one bead to detect antigen in limiting concentrations was offset when substrate volumes were kept constant, because the nonspecific background absorbances associated with each bead were additive and masked the small increases due to antigen

TABLE 1. Determination of optimum conditions for the detection of LPS_c by ELISA[a]

No. of beads	Reaction vol (ml)	E_{405}[d]		Increase in absorbance (%)[e]
		Test	Control	
1	1[b]	0.235 ± 0.015	0.132 ± 0.007	78.0
1	5[c]	0.175 ± 0.025	0.127 ± 0.008	37.8
2	5[c]	0.248 ± 0.008	0.260 ± 0.026	0
3	5[c]	0.405 ± 0.023	0.393 ± 0.016	3.1

[a] The enzyme was horseradish peroxidase, and the substrate was ABTS plus H_2O_2.
[b] Test samples contained 10 pg of LPS_c.
[c] Test samples contained a total of 10 pg of LPS_c at 2 pg/ml.
[d] Mean of three samples ± standard deviation from a single experiment.
[e] Calculated from the formula [(mean of test − mean of control)/(mean of control)] × 100.

TABLE 2. Treatments producing reduction of nonspecific interactions in ELISA[a]

Treatment[b]	Background absorbance at 405 nm		Reduction in absorbance (%)[e]
	Mean ± SD[c]	Grand mean[d]	
A	(0.146 ± 0.008)(0.158 ± 0.045)(0.152 ± 0.013)	0.152	
B	(0.076 ± 0.008)(0.068 ± 0.016)(0.082 ± 0.008)	0.075	50.6
B + C	(0.065 ± 0.010)(0.063 ± 0.008)	0.064	57.9
B + C + D	(0.053 ± 0.007)(0.058 ± 0.008)(0.065 ± 0.005)	0.059	61.2

[a] The enzyme was horseradish peroxidase and the substrate was ABTS plus H_2O_2.

[b] Treatments: A, $(NH_4)_2SO_4$ fraction of bovine anti-*Brucella* antiserum as first layer; B, Purified anti-*Brucella* antibodies as first layer; C, Rabbit anti-*Brucella* antibodies preabsorbed with normal bovine serum; D, Pretreatment of beads with 2% normal goat serum.

[c] Data in parentheses are from single experiments.

[d] Calculated from the mean of separate experiments, excluding standard deviations.

[e] Based on treatment A, i.e., [(grand mean of treatment A − grand mean of other treatments)/(grand mean of treatment A)] × 100.

(Table 1). Thus, for maximum sensitivity, a single bead was employed with volumes as close as possible to 1 ml.

Three treatments were employed in an attempt to reduce nonspecific absorbance (Table 2). The net reduction of nonspecific absorbance was approximately 61%, and subsequent studies were performed using all three modifications.

Titration of *Brucella* antigens. Titration curves of LPS_c with the β-galactosidase system are shown in Fig. 1. The reaction patterns produced by both substrates were similar and were characterized by a plateau between 2×10^3 and 2×10^6 fg of LPS_c per ml. This region of the curves was reproduced in two additional experiments, using concentrations between 2×10^3 and 2×10^7 fg/ml. Concentrations below 2×10^3 fg/ml were repeated in three additional experiments, using 4MUG as substrate (Table 3). The minimum concentration of LPS_c detectable was 100

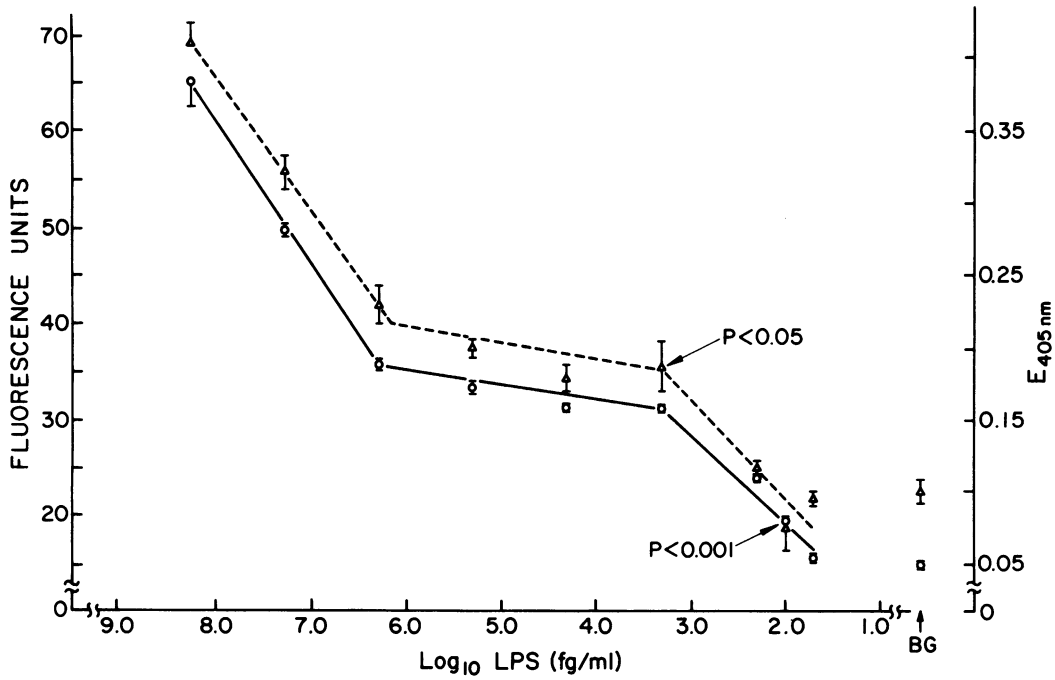

FIG. 1. Titration of *Brucella* LPS_c by ELISA, using β-galactosidase as the enzyme. Symbols: △---△, E_{405} with ONPG as substrate; ○—○, fluorescence units with 4MUG as substrate. Each point is the mean of three readings; bars represent standard deviations. BG, Background values (fluorescence or absorbance) of control samples.

TABLE 3. Limits of sensitivity in detection of LPS_c by ELISA, using β-galactosidase as the enzyme and 4MUG as the substrate

Expt no.	Fluorescence units[a] for the following concn of LPS (fg/ml)				
	0 (Control)	50	100	200	2,000
1	4.33 ± 0.577	5.16 ± 1.610[b]	5.00 ± 0[b]	5.66 ± 0.288[c]	9.00 ± 0.866[d]
2	6.16 ± 0.153	6.06 ± 0.153[b]	6.83 ± 0.150[d]	7.73 ± 0.208[e]	11.01 ± 1.100[d]
3	9.16 ± 1.040	8.66 ± 0.288[b]	10.30 ± 0.289[b]	13.50 ± 0.500[d]	18.40 ± 1.650[d]
4	14.06 ± 0.156	15.63 ± 0.930[b]	18.56 ± 0.550[e]	23.9 ± 1.510[e]	31.16 ± 0.814[e]

[a] Mean of three samples ± standard deviation.
[b] Difference between test samples and controls not significant; $P > 0.05$.
[c] Difference between test samples and controls significant at $P < 0.05$.
[d] Difference between test samples and controls significant at $P < 0.01$.
[e] Difference between test samples and controls significant at $P < 0.001$.

fg/ml (two of four experiments, at $P < 0.001$ and $P < 0.01$; Fig. 1; Table 3). The minimum concentration of LPS_c detectable with ONPG or ABTS was 2 pg/ml, based on significant differences between tests samples and controls ($P < 0.05$ or lower; three experiments). Hydrolysis of LPS_c with acetic acid caused no increase in sensitivity.

An assay of whole and sonicated organisms at concentrations between 4 and 10^5 cells per ml, using 4MUG, is shown in Fig. 2. The lowest concentration of whole organisms detectable ($P < 0.05$) was 10^3 cells per ml (Fig. 2 and two additional experiments). Four assays were made of sonicated cells at 10 (or fewer) cells per ml (Table 4). Sonication lowered the limit of detection by approximately 100-fold so that 10 bacteria were detectable per ml ($P < 0.05$ to < 0.001 in every instance), whereas 8 bacteria were detectable per ml ($P < 0.05$) two of three times

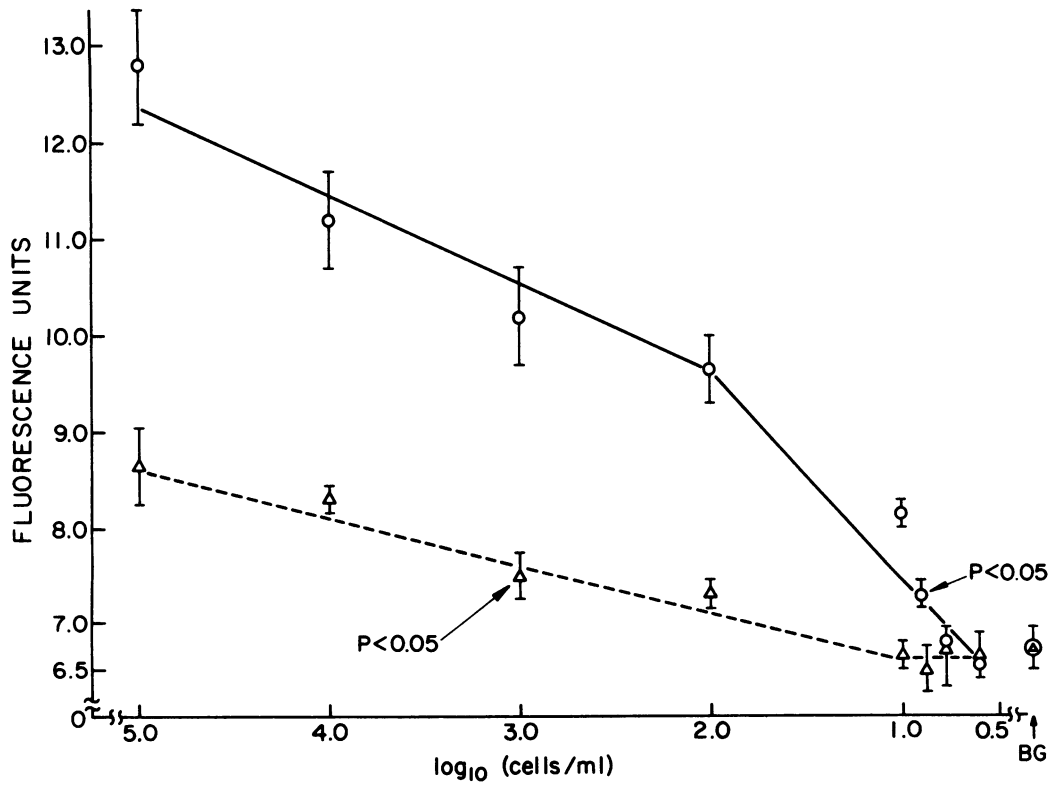

FIG. 2. Titration of brucellae by ELISA, using β-galactosidase as the enzyme and 4MUG as the substrate. Symbols: △---△, whole organisms; ○—○, sonically disrupted organisms. Each point is the mean of three readings; bars represent standard deviations. BG, Background fluorescence.

TABLE 4. Limits of sensitivity in detection of *Brucella* cells by ELISA, using β-galactosidase as the enzyme and 4MUG as substrate

Expt no.	Fluorescence units[a] for the following concn of sonicated brucellae (cells per ml)				
	0 (Control)	4	6	8	10
1	4.00 ± 0.500	4.16 ± 0.280[b]	ND	5.16 ± 0.288[c]	5.53 ± 0.577[d]
2	6.60 ± 0.400	6.55 ± 0.760[b]	6.80 ± 0.577[b]	7.33 ± 0.288[c]	8.16 ± 0.488[e]
3	7.00 ± 0.500	ND	ND	ND	8.63 ± 0.730[c]
4	4.66 ± 0.285	4.30 ± 0.288[b]	4.33 ± 0.288[b]	5.0 ± 0.500[b]	5.36 ± 0.288[d]
5	13.83 ± 0.289	ND	ND	ND	17.50 ± 0.500[f]

[a] Mean of three samples ± standard deviation. ND, Not determined.
[b] Difference between test samples and controls not significant; $P > 0.05$.
[c] Difference between test samples and controls significant at $P < 0.05$.
[d] Difference between test samples and controls significant at $P < 0.02$.
[e] Difference between test samples and controls significant at $P < 0.01$.
[f] Difference between test samples and controls significant at $P < 0.001$.

(Fig. 2; Table 4). As with LPS_c, mild acid hydrolysis of sonicated organisms failed to increase sensitivity.

Detection of *Brucella* antigens in simulated clinical samples. Analysis of extracts of normal bovine leukocytes derived from 100 ml of blood with the β-galactosidase system produced low background values (6 to 8 fluorescence units with 4MUG; 0.10 to 0.15 absorbance unit with ONPG), whereas the background absorbance with equivalent extracts in the horseradish peroxidase system ranged from 0.7 to 1.0 absorbance unit. Subsequent studies were therefore performed with β-galactosidase and its fluorogenic substrate.

Ten *Brucella* cells were detected with essentially the same efficiency when added to a suspension of leukocytes from 100 ml of blood as in PBSTS alone (Table 5). In contrast, it was not possible to detect picogram quantities of LPS_c added to the plasma fraction from 100 ml of blood. Attempts to concentrate LPS_c from the fluid phase of plasma by ethanol precipitation after deproteinization (6) proved unsuccessful. An affinity binding method for concentration of antigen was therefore employed. LPS_c (2 pg) in 50 ml of PBSTS was drawn by a pump over a single antibody-coated bead over a period of 24 or 48 h. Recovery of antigen after this was done at the faster rate was poor, but the slower rate

TABLE 5. Detection of *Brucella* antigens added to bovine leukocytes, plasma, and serum by ELISA, using β-galactosidase as the enzyme and 4MUG as the substrate

Medium[a]	Reaction vol (ml)	Antigen	Length of incubation (h)	Increase in fluorescence over control (%)	
				Individual expts[c]	Mean[d]
PBSTS[b]	1	10 brucellae	16	(25.5)(26.5)(22.8)(28.1)(38.2)	28.2 ± 5.9
LE	1.2–1.4	10 brucellae	16	(18.4)(24.0)(37.5)	26.6 ± 9.8
PBSTS[b]	1	LPS_c (2 pg)	16	(100.1)(93.2)(105.8)(124.2)(64.0)(110.0)	99.6 ± 20.3
PBSTS	50	LPS_c (2 pg)	24	(16.5)(21.4)	18.9
PBSTS	50	LPS_c (2 pg)	48	(53.5)(44.6)	49.0
Pl + T	50	LPS_c (2 pg)	48	(2.8)(1.7)	2.2
Pl + T	1	LPS_c (2 pg)	16	(0)(15.5)(3.6)	6.3 ± 8.1
Pl (50%) + T	1	LPS_c (2 pg)	16	(5.6)(0)	2.8
Pl (25%) + T	1	LPS_c (2 pg)	16	(0)(10.7)	5.4
Pl (12.5%) + T	1	LPS_c (2 pg)	16	(18.1)(54.0)	36.1
Pl (56°C) + T	1	LPS_c (2 pg)	16	(0)(34.0)	17.0
S (56°C) + T	1	LPS_c (2 pg)	16	(0)(17.9)(1.3)	6.4 ± 9.9
S + T	1	LPS_c (2 pg)	16	(12.6)(6.3)(13.6)	10.8 ± 3.9

[a] Abbreviations: PBSTS, PBS containing 0.05% (vol/vol) Tween 20 and 1.5% (vol/vol) normal bovine serum; LE, Extracts of leukocytes from 100 ml of normal bovine blood; Pl, Delipidated normal bovine plasma; S, Normal bovine serum; T, Tween 20 to a final concentration of 0.05% (vol/vol).
[b] Optimum conditions giving maximum recovery of antigens.
[c] Calculated from [(mean of test − mean of control)/(mean of control)] × 100. A value within parentheses corresponds to data from a single experiment.
[d] Mean percent increase from individual experiments. Standard deviations are stated when more than two experiments were carried out.

produced a mean increase in fluorescence over the control of nearly 50% (Table 5; $P < 0.02$ and < 0.01 for the two experiments). However, LPS_c added to 50 ml of delipidated plasma could not be detected in this manner (Table 5). Preliminary experiments had shown that neither the presence of heparin nor prior treatment with ether interfered with the binding of LPS_c to antibody-coated beads. Detection of 2 pg of LPS_c was not possible even in 1-ml volumes of plasma or serum, although some antigen could be measured when plasma was diluted to 12.5%. Heating of plasma or serum at 56°C for 30 min in the presence of Tween resulted in little or no improvement in detection of LPS_c (Table 5).

DISCUSSION

The ELISA employing antibodies covalently coupled to nylon beads (13) has proven sufficiently sensitive to enable ready detection of 100 fg of soluble *Brucella* antigens or as few as eight *Brucella* cells. Like Hendry and Herrmann (13), we found that covalent coupling of antibody to nylon beads provided much greater sensitivity than noncovalent attachment to polystyrene beads (M. T. Creasy, unpublished data). In our hands, shaking of the beads during acid and glutaraldehyde treatments and scrupulous removal of unbound glutaraldehyde were essential prerequisites for uniform binding of antibody without denaturation. Beads treated with lysine followed by normal bovine serum to bind unreacted aldehyde groups exhibited background staining much lower than that achieved with lysine alone, or with 2% bovine serum albumin as used by Hendry and Herrmann (13).

The sensitivity of the assay with one bead was substantially diminished by increasing the sample volume, despite continual rotation of the bead during the 16-h incubation period, and adding more beads to larger volumes of sample was not helpful (Table 1). The sensitivity of the assay for large volumes was greatly amplified by drawing fluid over a stationary bead at a rate not exceeding 1 ml/h (Table 5). By this procedure, concentrations of LPS_c below 5 fg/ml may be detectable in a 50-ml sample. Such a concentration system, based on affinity binding, may be applicable for the detection of low concentrations of antigens or antibodies in a variety of biological fluids or extracts. A further improvement in sensitivity may be possible by utilization of radiolabeled substrates (11), although the disadvantages of incorporating radioisotopes into an ELISA system must be considered.

The concentration of *Brucella* antigens as whole cells, cell fragments, or in soluble form in the fluid phase of the blood of infected animals is not known. In this study, the detection of LPS_c in low picogram concentrations was not possible in undiluted plasma or serum (Table 5). Inhibitory factors may have been involved (D. L. Persons and E. H. Gerlach, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, E24, p 80). Detection of microbial polysaccharide antigens in blood serum has been reported after various methods of deproteinization (6) which were unsuccessful in our hands in producing the required protein-free supernatant. Furthermore, the value of deproteinization in separating *Brucella* LPS_c is questionable, since *Brucella* LPS_c is tightly complexed with bacterial proteins (21) and probably would be removed along with the blood proteins.

The use of beads permitted assay in a single sample (1.2 to 1.4 ml) of the total leukocytes derived from 100 ml of blood. Preliminary experiments (V. Y. Perera and A. J. Winter, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C160, p. 338) indicate that *Brucella* antigens can be identified by this technique in circulating leukocytes of experimentally infected cattle. An evaluation of antigen detection as an ancillary system to antibody detection systems for identifying cattle with incubative infections is currently under way.

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