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Conditions for Conducting the Indirect Hemolysis Test for Detection of Antibodies to *Brucella abortus*†

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Some conditions were examined for performing the indirect hemolysis test for bovine brucellosis. An antigen extracted by using dimethyl sulfoxide was used for all of the assays. Optimal results were obtained by using bovine erythrocytes coated with alkali-treated antigen at a concentration of 800 μg/ml. Exceeding this level did not give greater sensitivity. The sensitivity of the test could be decreased by increasing the number of coated erythrocytes used in the test. Evidence was also provided for the presence of heat-labile antibodies in the sera of vaccinated cattle. Heat treatment (58°C for 50 min) caused a reduction in titer of all sera tested. It was also shown that lysis of erythrocytes was complete in less than 60 min. Therefore, it would be possible to reduce the time needed for analysis. Non-alkali-treated ("native") antigen would bind to bovine erythrocytes, but it was less effective in the test than alkali-treated material. Erythrocytes coated with relatively large amounts of the native antigen were less suspectible to lysis than were cells which had been treated with lower concentrations.

There is considerable interest in distinguishing between antibodies arising from *Brucella abortus* strain 19 vaccination and the antibodies associated with infection with field strains. The complement fixation test is considered by some to be the most reliable means of making this distinction (2, 12). However, prozone reactions are frequently encountered with the complement fixation test, and there is the danger that sera possessing antibodies to *B. abortus* might appear to be negative (2, 13). In addition, some laboratories perform the cold complement fixation test, which requires an overnight incubation at 4 to 7°C (1). Since the complement fixation test must be performed with limiting amounts of complement, it is necessary to work with accurately titrated complement. Some workers consider this to be another disadvantage of the test (2, 13).

Plackett et al. have described an indirect hemolysis test (IHLT) for bovine brucellosis which they claim is superior to the complement fixation test because the prozone phenomenon was not observed. It could be carried out in 1 h, and excess amounts of complement were used (13). Their results also showed that antibody titers after vaccination of cattle with *B. abortus* strain 19 declined more rapidly than with other tests.

Plackett et al. did not fully explore the conditions for optimal performance of the IHLT (13). Carlson and Nicoletti have found that the sensitivity of the test is affected by the concentration of antigen used for coating the erythrocytes (personal communication). The antigen used for their studies was a dimethyl sulfoxide extract of *B. abortus* strain 1119-3. This finding prompted us to initiate the present study so that we could define the best test conditions for conducting the IHLT, using a dimethyl sulfoxide extract of *B. abortus* strain 1119-3.

MATERIALS AND METHODS

Bovine blood. Bovine blood was obtained by venipuncture and stored in modified Alsever solution at 4 to 7°C (4). The erythrocytes were tested with an antiserum specific for the J-antigen (15), and only J-antigen-negative erythrocytes were used for the IHLT.

Isotonic Veronal-buffered sodium chloride solutions. Isotonic Veronal-buffered sodium chloride containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (VBS), and 0.01 M ethylenediaminetetraacetic acid (EDTA) in VBS (EDTA-VBS) were used, and preparation methods have been previously described (8).

Antigen. The antigen used for coating bovine erythrocytes was extracted from 500 g (wet weight) of live *B. abortus* strain 1119-3 (kindly supplied by George Brown, U.S. Department of Agriculture, Ames, Iowa), using the dimethyl sulfoxide procedure described by Adams and by Plackett et al. (1, 13). This particular method for antigen extraction was selected because Carlson and Nicoletti were successful in performing the IHLT with a dimethyl sulfoxide antigen preparation, and results were equivocal when antigen produced by a hot phenol-water extraction procedure was used (personal communication).

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The crude dimethyl sulfoxide-extracted material was alkali treated before being coated onto bovine erythrocytes. The antigen preparation was diluted in 0.25 N NaOH, heated at 50°C for 60 min, and neutralized with 1.0 M acetic acid. The neutralized material was mixed with 3 volumes of ethanol and placed in an ice bath. The precipitate which formed was pelleted by centrifugation (12,000 × *g*), dissolved in a small volume of water, and dialyzed against water. The dialyzed antigen was lyophilized and stored at −20°C in a desiccator jar.

Sera. Sera from *B. abortus* culture-positive cattle and from animals vaccinated with *B. abortus* strain 1119-3 were used in this study. Some of the culture-positive cattle were in a private dairy herd and were serologically positive for brucellosis when tested by the standard tube, rivanol, and complement fixation tests. Other culture-positive cattle were in a group which was challenged with a field strain of *B. abortus* (strain 2308) as part of a large vaccination trial being conducted at the U.S. Department of Agriculture laboratories at Ames, Iowa (supplied by B. Deyoe). These sera also gave positive results when assayed by the standard serological tests for brucellosis.

Serial samples were obtained from other cattle that had been vaccinated with *B. abortus* strain 1119-3. Sera from cattle that had received two different doses of antigen were obtained. One group was inoculated with a standard dose of 9×10^{10} bacteria, and another was inoculated with a reduced dose of 1×10^{9}. Both groups were challenged 8 months past vaccination with field strain bacteria.

Complement. Fresh frozen guinea pig serum (Pel Freeze, Rogers, Ark.) was used as a source of complement. This was absorbed three times at approximately 0°C with washed bovine erythrocytes (J-antigen negative) and stored at −70°C. The complement was titrated by the procedure of Mayer (9), and 10 CH_{50} units were used in the assay procedures (unless otherwise indicated).

Preparation of antigen-coated erythrocytes. Bovine erythrocytes were washed three times in VBS and suspended at a concentration of 3% (vol/vol) in the same diluent. A 1:15 dilution of the suspension was made with water, and the optical density of the hemolysate was determined at a wavelength of 541 nm. The concentration of cells in the suspension was adjusted so that a lysate prepared by diluting a sample of the suspension 1:15 with water had an optical density of 0.500 at a wavelength of 541 nm (9). The number of cells in the standardized suspension was determined by a hemacytometer and determined to be about 4.5×10^{8} cells/ml (standard deviation, 1.34×10^{7}).

Equal volumes of standardized bovine erythrocytes and alkali-treated antigen were mixed and incubated at 37°C for 60 min with constant, slow shaking. Antigen concentrations of 50, 100, 200, 400, 800, 1,600, 3,200 and 6,400 μg/ml were used. The cells were then washed twice with VBS and diluted to a concentration of 7.5×10^{7}/ml in VBS. Testing was carried out by mixing 0.2 ml of each preparation of coated cells with 0.2 ml of antiserum diluted serially with VBS. The mixture was incubated for 15 min at 37°C, with shaking, and 0.2 ml of adsorbed guinea pig complement was added to each tube. The completed tests were shaken for 60 min at 37°C, 2.4 ml of ice-cold EDTA-VBS was added to each tube, and they were centrifuged at 500 × *g* for 5 min at 0°C to pellet any unlysed cells. The optical densities of the supernatant fluid were determined at a wavelength of 414 nm. Controls consisted of bovine erythrocytes without antigen, spontaneous lysis, complete lysis, and a completed test without antiserum. The latter was to show that lysis required the presence of serum antibodies.

A microtiter test was used for some experiments. Sera were serially diluted in VBS, and 25-μl volumes were dispensed into wells of microtiter plates (Cooke Engineering Co., Alexandria, Va.). An equal volume of antigen-coated bovine erythrocytes (4.5×10^{7}/ml) was added to each dilution of serum. The mixtures were shaken at 37°C for 15 min, and then 25 μl of adsorbed complement (10 CH_{50} units) was added to each test. The plates were centrifuged at about 200 to 300 × *g* for 5 min and then examined. The endpoint for each serum was judged as the reciprocal of the highest dilution that produced 100% hemolysis of the antigen-coated bovine erythrocytes.

RESULTS

Optimal antigen concentration. The objective of this experiment was to determine the minimum concentration of coating antigen that would sensitize bovine erythrocytes for optimal use as targets in the IHLT.

The results (Fig. 1) indicated that the amount of hemolysis for any given dilution of antiserum increased as the concentration of coating antigen was increased, until a concentration of 800 μg/ml was reached. Cells treated with 800, 1,600, 3,200, or 6,400 μg of antigen per ml all lysed to about the same extent when exposed to various concentrations of antiserum and excessive amounts of complement. Therefore, 800 μg of alkali-treated antigen per ml was chosen as the optimal concentration for all subsequent experiments.

Optimal erythrocyte concentration. A concentration of 7.5×10^{7} bovine erythrocytes per ml was arbitrarily chosen for the IHLT. This concentration was somewhat less than the one used by Plackett et al. (13), and we considered the possibility that the sensitivity of the test (e.g., ability to detect antibodies) might be influenced by the number of coated erythrocytes used in the test. Therefore, bovine erythrocytes were sensitized with antigen (800 μg/ml) at a concentration of 4.5×10^{8}/ml and then adjusted to concentrations of 4.5×10^{8}/ml, 2.25×10^{8}/ml, 7.5×10^{7}/ml, and 3.75×10^{7}/ml in VBS. Each of the different cell preparations was then used to perform the indirect hemolysis test, using the procedure described in the foregoing section. Various dilutions of an antiserum from a culture-positive cow were used with each cell suspension.

The sensitivity of the test increased as the

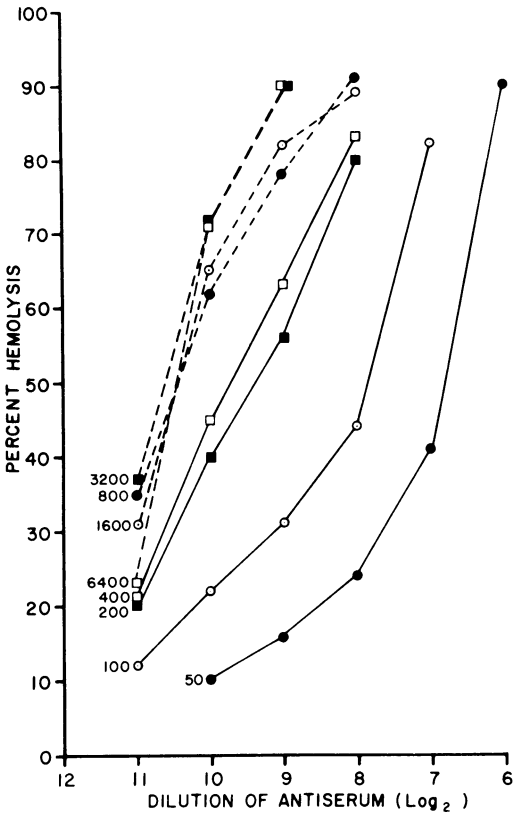

FIG. 1. *Complement-mediated hemolysis of bovine erythrocytes coated with different concentrations of alkali-treated Brucella antigen. The number to the left of each curve designates the concentration (in micrograms per milliliter) of alkali-treated antigen used.*

number of cells in the test decreased (Fig. 2). The percentage of cells lysed was greatest with the two lowest cell concentrations. The cell concentration originally chosen for the IHLT (7.5×10^7/ml) gave maximum sensitivity. The use of fewer cells (3.75×10^7/ml) did not appreciably alter the results.

The increased IHLT sensitivity was not a result of differences in the density of antigen per cell because all of the preparations had been coated with antigen at a concentration of 4.5×10^8/ml before the cells were readjusted to different concentrations. The possibility was considered that the amount of complement used for testing was limiting (despite our intention to use excess amounts), because limitation of complement could result in decreased lysis at all concentrations of antibodies as the number of coated erythrocytes was increased. Therefore, the same experiment was repeated with twice as much complement in each of the reaction mixtures (20 CH_{50} units). The results were very similar to those obtained in the previous experiment. There was a small increase in hemolysis at each antiserum concentration, but the trend was the same as in Fig. 2. Increasing the cell density decreased the sensitivity of the test, and this was probably not a function of limitation of complement.

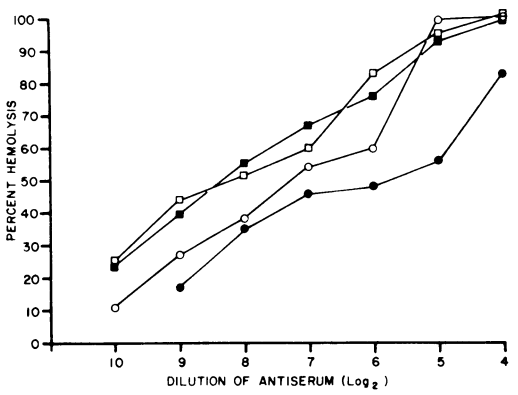

FIG. 2. *Effect of cell concentration on the extent of hemolysis in the indirect hemolysis test. Cells were treated with alkali-treated antigen at a concentration of 800 μg/ml, and 10 CH_{50} units of complement were used. Symbols: ●, 4.5×10^8 cells/ml; ○, 2.25×10^8 cells/ml; ■, 7.5×10^7 cells/ml; □, 3.75×10^7 cells/ml.*

Effect of heat on antibody titers detectable by the IHLT. Sera for serological testing are commonly "heat inactivated" to destroy complement before assays for antibodies are performed. However, some bovine immunoglobulins are supposedly heat labile (3), and this procedure could destroy some antibodies specific for the antigens that are capable of adsorbing to bovine erythrocytes.

Since a large number of assays were carried out with several different sera, the microtiter assay was used instead of the tube test which has been described for the preceding experiments. Sera from representative cattle of the low-dose vaccination group and the high-dose vaccination group were selected for the heat inactivation experiment. All of the sera had antibody titers, demonstrated by the IHLT, using unheated sera. Samples, taken from each selected cow at weekly intervals after vaccination, were divided in two. One part of each was heated for 50 min at 58°C before titration by the micro-IHLT. The other part of each serum was titrated without prior heat treatment. The results of the experiment are summarized in Table 1. Antibody titers of all sera were reduced by heat treatment. These results suggested that the sera contained heat-labile antibodies. However, 8 of

TABLE 1. *Effect of heating[a] on IHLT titers[b] of sera from cattle receiving a single dose of B. abortus strain 1119-3 on 26 April 1978*

Animal no.	Bleeding date (1978)	Antibody titer[c]	
		Unheated	Heated
4	5/10	32	0
	5/31	64	4
	7/05	256	32
	8/16	512	32
	8/25	128	16
	12/13	32	16
9	6/07	8	0
	6/21	8	0
	7/19	4	0
	9/13	0	0
	12/13	0	0
10	5/31	16	0
	6/21	8	0
	7/19	16	0
	8/16	0	0
	9/13	2	0
50	5/18	16	4
	5/24	16	4
	5/31	16	0
	6/07	16	2
	6/14	32	2
	6/21	4	2
	7/05	8	0
	7/19	8	2
51	5/10	32	16
	6/07	16	4
	6/21	8	0
	7/05	26	2
	8/16	8	0
	10/25	4	0
20	5/10	32	0
	5/31	32	8
	6/14	32	4
	7/05	16	0
	8/16	2	0
	9/13	2	0
	10/25	4	0
62	5/03	32	32
	5/31	32	4
	6/14	32	4
	7/05	32	2
	8/16	16	0
19	5/18	4	0
	5/31	4	0
	6/21	8	0
	7/05	32	2
	8/02	8	2
	6/14	2	0
61	5/10	4	0
	5/31	16	4
	6/14	16	4
	7/05	16	4
	8/16	8	2
	9/13	2	2
	10/25	4	2
70	5/10	8	4
	5/24	32	4
	6/24	8	4
	7/05	16	4
	8/16	4	2

[a] Heated for 50 min at 58°C.
[b] Micro-IHLT. Each titer is the average of two determinations.
[c] Reciprocal of the highest dilution of antiserum causing complete lysis in the micro-IHLT.

10 animals had residual antibody titers even after heat treatment, which suggests that some of the antibodies were heat stable.

Kinetics of hemolysis by the IHLT. The IHLT as described by Plackett et al. was incubated for 60 min at 37°C after the addition of complement (13). Studies were conducted to determine whether this incubation time and temperature were optimal. A kinetic analysis was made to determine the rate of hemolysis generation, using bovine erythrocytes sensitized with 800 μg of alkali-treated *B. abortus* 1119-3 antigen per ml, 10 CH_{50} units of adsorbed guinea pig complement, and three different limiting dilutions of an antiserum specific for the *B. abortus* antigen. Equal parts of coated erythrocytes and antiserum were incubated together at 37°C for 15 min. Then a third volume of complement was added, and 0.6-ml samples were taken at timed intervals. The samples were mixed with 2.4 ml of ice-cold 0.01 M EDTA-VBS and centrifuged, and the optical densities were determined at a wavelength of 414 nm. Percent hemolysis was determined for each sample, and the values were plotted on the ordinate of linear paper, with time of sampling shown on the abscissa (Fig. 3). The results show that hemolysis reached a maximum between 15 and 30 min after initiation of the reaction when antibodies were limiting. Therefore, the 60-min incubation period that has been previously used by others (13) is sufficient for the completion of this test.

Coating bovine erythrocytes with non-alkali-treated antigen. The IHLT as de-

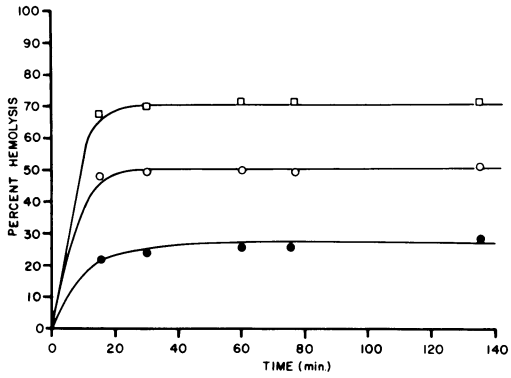

FIG. 3. *Rate of hemolysis by the IHLT. Cells were treated with 800 g of alkali-treated antigen per ml, and 10 CH_{50} units of complement were used with the dilutions of antiserum:* (□) *1:128;* (○) *1:256;* (●) *1:512.*

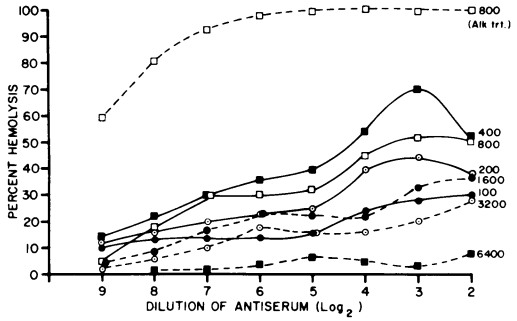

FIG. 4. *Complement-mediated hemolysis of bovine erythrocytes coated with different concentrations of "native" Brucella antigen. The number to the right of each curve designates the concentration (in micrograms per milliliter) of native antigen used. The upper curve shows the amount of hemolysis obtained when alkali-treated antigen (800 μg/ml) was used.*

scribed by Plackett et al. employed lipopolysaccharide containing antigen which had been alkali-treated before coating onto erythrocytes (13). Studies were conducted to determine whether this treatment was necessary. The alkali treatment is harsh, and some antigens in the crude preparation might be destroyed. In addition, a considerable amount of time could be saved if the alkali step could be omitted.

Bovine erythrocytes were coated with various concentrations of native antigen (not treated with alkali), using the protocol previously described. The cells were washed and used in the IHLT with 10 CH_{50} units of complement and an antiserum from a culture-positive cow. As a control, cells coated with 800 μg of alkali-treated antigen per ml were assayed simultaneously. Cells coated with non-alkali-treated antigen were lysed by antibodies and complement. However, the results indicated that the native antigen might be anti-complementary (Fig. 4). The percent hemolysis increased as the amount of coating antigen was increased, until a concentration of 400 μg/ml was used. Cells treated with concentrations of 800 μg of antigen or more per ml were less susceptible to lysis. There was a maximum of 10% lysis of cells which had been treated with antigen at a concentration of 6,400 μg/ml. In contrast, bovine erythrocytes which had been coated with alkali-treated antigen at a concentration of 800 μg/ml were completely lysed at several antiserum dilutions.

Bovine erythrocytes which had been treated with native antigen were tested by passive hemagglutination for the presence of surface-bound antigen. The various cell preparations were thoroughly washed with VBS and standardized to 4.5×10^7 cells/ml in the same diluent. Equal volumes of coated cells and serial dilutions of the antiserum used in the preceding experiment were mixed. The mixtures were placed at room temperature, and the cells were allowed to settle. The results (Fig. 5) showed that all of the cells had detectable antigen(s) on their surfaces. Furthermore, the titer of the antiserum increased as the amount of coating native antigen was increased.

DISCUSSION

We have examined several conditions that can be varied when using the IHLT for the detection of antibodies to *B. abortus*. In this study we utilized the dimethyl sulfoxide method for extraction of lipopolysaccharide from *B. abortus*, strain 1119-3 (1, 13). This preparation served very well as our coating antigen, but we do not know whether antigens other than lipopolysaccharide were present and capable of binding to bovine erythrocytes. It is possible that non-lipopolysaccharide antigens were present on erythrocytes, since heat-stable antibodies were detected in some antisera. Heat-stable bovine immunoglobulins are believed to be of the immunoglobulin G class (3). Several studies have revealed that lipopolysaccharides are T cell-independent antigens which stimulate the exclusive production of immunoglobulin M antibodies (4, 11, 14). This has been reported for *B. abortus* lipopolysaccharide as well as for the lipopolysaccharides of the *Enterobacteriaceae* (14). In the latter studies, some strains of mice synthesized 2-mercaptoethanol-resistant antibodies during a secondary immune response to *Brucella* lipopolysaccharide but not to *Escherichia coli* lipopolysaccharide. It is possible that cattle re-

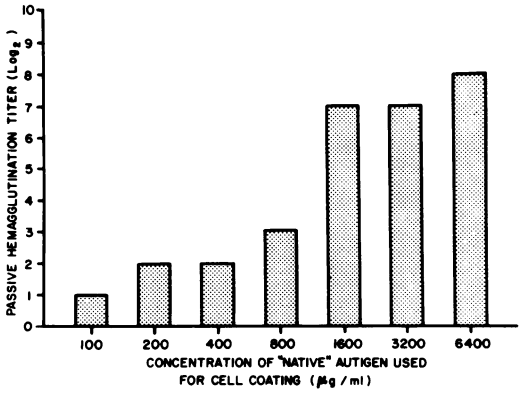

FIG. 5. *Titration of an antiserum specific for B. abortus with bovine erythrocytes which were treated with different concentrations of native antigen.*

spond differently to antigenic stimulation by lipopolysaccharides.

The appearance of immunoglobulin G immunoglobulins reactive with cell-bound antigens could reflect the presence of substances other than lipopolysaccharides on the cells. Studies are under way in this laboratory to identify the classes of immunoglobulins which are functional in the IHLT, as described here, and to analyze the dimethyl sulfoxide preparation for multiple cell-binding antigens.

Our experiments have revealed that for maximum sensitivity the optimal antigen concentration for coating bovine erythrocytes, using the described conditions, is 800 µg of alkali-treated material per ml. The sensitivity of the test did not increase as the concentration of coating antigen was increased beyond this level. We also learned that a cell concentration of between 2.25×10^7 and 4.5×10^7 per ml was optimal for performing the test. Increasing the cell number resulted in a decrease in percent lysis, even though excess amounts of complement were used.

Plackett et al. reported that the IHLT was not subject to the prozone phenomenon (13). However, in these studies, some sera were found which were anticomplementary at high concentrations, but this effect could be diluted out. It is possible that the anticomplementary phenomenon is caused by non-complement fixing antibodies in the sera which compete with complement fixing antibodies for antigenic sites.

The question of specificity of the IHLT has not been considered in this study. There is a possibility that substances which cross-react with non-*Brucella* antigens can bind to bovine erythrocytes and are detected by antibodies in the sera of the vaccinated or infected animals. This will be the subject of future research in this laboratory.

The IHLT as originally described was incubated for 60 min at 37°C (13). However, our experiments have revealed that the test goes to completion between 15 and 30 min at 37°C, using excess amounts of complement. Therefore, it would be possible to reduce the time for analysis.

An interesting observation was the apparent anticomplementary activity of cell-bound native antigen. Interactions of bacterial lipopolysaccharides with the complement system have been well documented (7, 10). However, these studies have revealed that the mechanism is complement activation through either the classic or alternative pathways. Since the antigen coated bovine erythrocytes did not lyse when exposed to antibodies and excessive amounts of complement, there is some question as to whether or not native *B. abortus* antigen causes inhibition of complement activity through either of the mechanisms described above.

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