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Utilization of a Specific In Vitro Lymphocyte Immunostimulation Assay as an Aid in Detection of Brucella-Infected Cattle Not Detected by Serological Tests

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Studies using the in vitro lymphocyte stimulation test (LST) were conducted with cattle in a dairy herd with a high percentage of reactors to several serological tests for brucellosis. Lymphocytes were prepared from peripheral bovine blood by the Ficoll-diatrizoate technique. Lymphocytes were cultured using microtitration culture plates. *Brucella abortus* soluble antigen, at a concentration of 4.4 μg/culture, was added to the appropriate wells of microtitration culture plates and incubated for 6 days. The lymphocyte stimulation responses were measured by assaying for [^{3}H]thymidine incorporation into DNA. Seroagglutination tests were conducted simultaneously with the LST, and tissues were collected after slaughter of the cattle for bacteriological culture to isolate *B. abortus*. All 21 animals studied were serologically negative for anti-brucella antibodies. Two of the 21 animals were classified as infected with *Brucella* by the LST, and *B. abortus* biotype 1 was isolated from tissues of these same two animals. The LST exhibited significant sensitivity and specificity in this study, and more observations of this nature might strengthen the application of this assay as an aid in the diagosis of brucellosis.

In our previous reports (10-12) it was stated that the in vitro lymphocyte stimulation (LST) correlated with infection in the animals tested. Reference was made to the effect that this test might be used as an aid in the diagnosis of bovine brucellosis, especially where serological test results were doubtful. This experiment was designed to evaluate the LST as an aid in the diagnosis of bovine brucellosis among cattle not detected as reactors by serological tests in a herd infected with a field strain of *Brucella abortus* as determined by isolation of brucellae.

MATERIALS AND METHODS

History of the animals. The animals used in this study came from a single dairy herd of 47 head of cattle. The owner of this herd had discontinued vaccination against brucellosis 6 years previously. In the fall of 1977, on routine testing, this herd had a suspicious milk ring test reaction, which led to tracing the source of the milk to the affected herd. The herd was further tested using the standard and supplemental blood serum agglutination tests. On the basis of these tests, 26 lactating cows were designated reactors and were sent to market for slaughter. The remaining 21 lactating cows were bled a few days later, and blood was collected for both serological tests and the LST. The animals were slaughtered the next day as part of the herd depopulation procedure, and the following tissues were collected for isolation of *B. abortus*: retropharyngeal, supramammary, lumbar, internal and external iliac lymph nodes, spleen, milk from the rear quarters of the udder, udder tissue, part of the wall of the body of the uterus and ovary, and, when the cow was pregnant, fetal membranes, fetal spleen, and stomach.

The bacterial culturing and typing for these samples were done at the National Animal Disease Laboratory, Ames, Iowa. The serological tests were conducted at the Federal Brucellosis Laboratory at St. Paul, Minn., and the LSTs were conducted at the College of Veterinary Medicine, University of Minnesota, St. Paul. This was a single-blind study since none of the laboratories knew the status of the animals from which samples came.

Collection of blood. Approximately 30 ml of blood was collected by jugular venipuncture from each animal. Twenty milliliters of each blood sample was placed into sterile tubes containing heparin (50 U/ml; Upjohn Co., Kalamazoo, Mich.). The remaining 10 ml was placed into tubes and allowed to clot, and serum was collected for later use in the standard and supplemental agglutination tests for humoral antibodies.

Preparation of lymphocyte suspension. After collection of blood, heparinized blood was subjected to the same procedures as previously reported (10, 11).

Culture medium, antigen, mitogen, and cell cultures. RPMI 1640 (Biolabs) was used as the cul-

ture medium. *B. abortus* soluble antigen was used at a concentration of 4.4 μg/culture. The mitogen concanavalin A (Miles-Yeda, Rehovot, Israel) was used at a concentration of 2 μg/culture. The culturing of cells, conditions of culturing, [*methyl*-^{3}H]thymidine (Schwarz/Mann, Orangeburg, N.Y.) labeling, harvesting, and liquid scintillation counting were conducted as reported earlier (10, 11).

Serological tests. Serum samples from each animal were subjected to the following serological tests: (i) plate and tube standard seroagglutination tests, and (ii) brucella buffered antigen test (card), Rivanol, and 2-mercaptoethanol supplemental tests. These tests were conducted according to the U.S. Department of Agriculture procedures (22, 23).

RESULTS

LST results. LST results are expressed in two ways: (i) mean counts per minute of triplicate cultures with mitogen or antigen or without either mitogen or antigen; (ii) stimulation index = mean counts per minute of triplicate cultures without either mitogen or antigen divided by mean counts per minute of triplicate cultures without mitogen or antigen.

Table 2 shows the results (counts per minute) for each animal. The stimulation index values are given in Table 1.

The data show the following points. (i) The lymphocytes from these animals were immunocompetent, as judged by the high responses induced by concanavalin A. (ii) *B. abortus* soluble antigen induced significant positive LST in lymphocytes from two animals only (no. 701 and 723, Table 1). (iii) Using the criterion that a stimulation index ≥3.0 was indicative of infec-

TABLE 2. *Counts per minute of in vitro lymphocyte stimulation induced by concanavalin A (ConA) or B. abortus soluble antigen (BASA) in lymphocytes from tested cattle*

Animal no.	ConA	BASA	Control cultures
701	193,143	3,809	173
702	122,580	165	153
704	29,138	163	126
706	66,110	143	102
707	36,097	132	192
708	10,135	191	195
709	91,147	126	187
710	21,473	133	229
711	72,026	103	158
712	17,167	278	547
713	97,123	109	165
714	11,538	98	78
715	26,258	258	221
716	31,604	175	104
717	55,660	158	150
718	3,970	162	97
719	77,565	126	131
723	23,224	2,212	222
724	30,662	205	350
725	60,351	109	111
726	143,213	955	779

TABLE 1. *Results of serological tests, LST (presented as stimulation indexes), and B. abortus culture and typing results*[a]

Animal no.	BBA (card)	Plate test	STT	RIV	2-ME	Stimulation index	*B. abortus* culture results
701	Negative	− 25	− 25	− 25	− 25	23.4	Positive[b]
702	Negative	− 25	+ 25	− 25	− 25	1.0	Negative
704	Negative	− 25	− 25	− 25	− 25	0.7	Negative
706	Negative	− 25	− 25	− 25	− 25	0.3	Negative
707	Negative	− 25	+ 25	− 25	− 25	1.7	Negative
708	Negative	− 25	− 25	− 25	− 25	1.0	Negative
709	Negative	− 25	− 25	− 25	− 25	0.6	Negative
710	Negative	− 25	− 25	− 25	− 25	0.4	Negative
711	Negative	− 25	− 25	− 25	− 25	0.7	Negative
712	Negative	− 25	+ 25	− 25	− 25	1.5	Negative
713	Negative	− 25	− 25	− 25	− 25	0.6	Negative
714	Negative	− 25	− 25	− 25	− 25	0.9	Negative
715	Negative	− 25	+ 25	− 25	− 25	1.9	Negative
716	Negative	− 25	− 25	− 25	− 25	0.7	Negative
717	Negative	− 25	− 25	− 25	− 25	1.1	Negative
718	Negative	− 25	− 25	− 25	− 25	1.1	Negative
719	Negative	− 25	− 25	− 25	− 25	0.8	Negative
723	Negative	− 25	− 25	− 25	− 25	12.5	Positive[b]
724	Negative	− 25	− 25	− 25	− 25	0.6	Negative
725	Negative	− 25	− 25	− 25	− 25	0.9	Negative
726	Negative	− 25	− 25	− 25	− 25	0.3	Negative

[a] BBA, brucella buffered antigen test; STT, standard tube test (a complete agglutination at 1:100 or higher dilution is considered positive [21]); RIV, Rivanol precipitation test; 2-ME, mercaptoethanol agglutination test. A stimulation index ≥ 3.0 is considered positive (infection). Titers are presented as reciprocals of dilution.

[b] *B. abortus* biotype 1 (not strain 19) isolated.

tion, animals 701 and 723 were classified as infected by the LST and the rest were classified as not infected.

Serological test results. The results of the various serological tests used are presented (Table 1). Using the U.S. Department of Agriculture criterion that nonvaccinated adult cattle are classified as brucellosis "reactors" if blood serum shows complete agglutination at the 1:100 dilution on either the tube or plate standard agglutination test (3), none of the 21 animals tested was a "reactor."

Bacteriological culture results. *B. abortus* biotype 1 was isolated from the tissues of animals 701 and 723. Brucellae were not isolated from the tissues taken from the other animals.

DISCUSSION

The in vitro LST has been utilized as an aid in the diagnosis of several infections (1, 2, 4–9, 13, 14, 17–19, 24). In brucellosis the LST has been reported in a few cases (3, 10–12, 15, 16, 20).

Bovine brucellosis presents a real diagnostic problem in that the agent may be excreted intermittently from the animal body via the mammary gland, genital systems, and other portals of exit. Associated with the problem of shedding is the question of how these reservoirs of infection may be detected. Abortion is the only dramatic sign of bovine brucellosis, yet this is useful only in pregnant animals. Culturing of brucella agent is quite a problem and cannot be used for a routine diagnostic laboratory, especially where a lot of animals must be tested, as in eradication programs. Serological tests have been used for decades as aids in the diagnosis of brucellosis. However, these tests may not detect all the animals in their incubation periods of the disease. These undetected animals create a very serious epidemiological problem, because they would move and may mix freely with uninfected animals. A test that would have high sensitivity and specificity (higher than the serological tests) would be extremely useful. The central purpose of this report is to evaluate the LST as an aid in the diagnosis of bovine brucellosis.

The data presented in this study show that the LST exhibited significant sensitivity and specificity. The animals that were designated as infected by the LST were negative on the serological tests, which represents a low sensitivity and shows that these results might have been missed if only serological tests were conducted. The observation in this experiment demonstrates the difficulty of determining the incubation period in bovine brucellosis. If milk or seroagglutination antibodies are not detected, it would normally be assumed that these animals are not infected. Epidemiologically, it would be useful if we could detect the potentially infected animals very early in their incubation period. In this study, the LST detected, very early in the incubation period, two cows which proved to be infected, as demonstrated by isolation of *B. abortus*. More observations of this nature might strengthen the use of this test as an aid in the diagnosis of bovine brucellosis, in particular, and encourage the development of similar procedures for other species of brucella involving other animals, including humans.

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