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Whole-Blood Lymphocyte Stimulation Assay for Measurement of Cell-Mediated Immune Responses in Bovine Brucellosis

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A study was conducted to develop an in vitro whole-blood lymphocyte stimulation assay for measurement of cell-mediated immune response in bovine brucellosis. A soluble antigen (BASA) prepared from killed cells of *Brucella abortus* 1119-3 was used. Cattle infected with *B. abortus* field strains, *B. abortus* 19 calfhood- and adult-vaccinated cattle, and nonexposed cattle were tested. Blood was diluted 10-fold in RPMI-1640 medium (without added serum) and cultured with BASA (at a concentration of 2.2 μg per culture) at varying times of incubation. Results were assayed for [^{3}H]thymidine incorporation into deoxyribonucleic acid. A 6-day period was found to be optimal for incubating blood cultures to achieve maximum specific lymphocyte stimulation. Serological tests and bacteriological isolation attempts were conducted simultaneously with lymphocyte stimulation tests, and there was a significant correlation between cell-mediated immune response and bacteriological findings. There was a significant correlation between cell-mediated immune response and the level of serum antibodies on a group basis, but there was little correlation between the two systems on individual infected animals. Among vaccinated animals there was little or no correlation between cell-mediated immune and humoral responses. The whole-blood assay was found to be simple, fast, sensitive, and reproducible.

In vitro lymphocyte transformation using purified lymphocytes is gaining wide acceptance in the scientific community as a test for lymphocyte immune function, and it is applied in studies of immune responsiveness, delayed hypersensitivity, histocompatibility, and clinical allergy (2-4). Recently, studies have focused on the usefulness of lymphocyte stimulation in vitro as a diagnostic tool in infectious diseases, particularly those diseases where delayed hypersensitivity is an important component (3-6, 10, 12, 17, 18, 22).

In our laboratory, studies of brucellosis have focused on developing a suitable antigen that could be used in studies of in vitro lymphocyte stimulation. A suitable antigen, *Brucella abortus* soluble antigen (BASA), its protein and endotoxin content, and the kinetics of utilizing that antigen for in vitro purified lymphocyte stimulation have been reported (8). This antigen was utilized in later studies on bovine brucellosis (9).

The drawback of in vitro lymphocyte stimulation using pure lymphocytes is the limited number of cultures obtained from the small volumes of blood normally available; also, the time required to purify lymphocytes from peripheral blood is considerable, especially when testing large numbers of animals, as can be the case in herd testing.

Several lymphocyte transformation studies using whole blood (nonseparated) rather than lymphocyte-enriched preparations have been reported (2, 13-15). With the present program of eradication of bovine and swine brucellosis, large numbers of animals must be tested. A simplified in vitro lymphocyte stimulation test could prove very useful, especially in herd problems where serological diagnosis is questionable. The purpose of this study was to develop a whole-blood lymphocyte stimulation assay that could be used in bovine brucellosis studies. The specific objectives were: (i) to find the optimal period of incubating blood cultures; (ii) to correlate lymphocyte culture findings with serological results; (iii) to compare lymphocyte stimulation results

in lymphocytes from animals with different exposure experiences to *B. abortus*; and (iv) to correlate lymphocyte stimulation results with infection (infection being used here to mean animals from which brucellae were isolated).

MATERIALS AND METHODS

Animals and bleeding schedule. Dairy cattle were used, and information on the types and status of each animal is given (Tables 1 and 2). Animal no. 33, the whole of group 2 except no. 22, and the whole of group 4 were kept in Florida and were from one herd. Blood from these animals was collected and sent to the College of Veterinary Medicine, University of Minnesota, by air mail and received and tested the following morning. This was a single blind study, since the status of the animals from which samples were collected was not known by the technical staff at Minnesota. The remaining animals were kept at the College of Veterinary Medicine in St. Paul, Minn. The infected cattle (no. 21, 51, and 74) were kept in separate isolation units. The vaccinated (group 3) and control cattle (group 5) were kept in a brucella-free herd belonging to the Department of Animal Science, University of Minnesota, and remained clinically normal throughout the study. The same day the animals in Florida were bled, those at St. Paul were also bled, and blood was kept at room temperature and tested the next morning together with the samples from Florida. The study was conducted in two parts: the first part, conducted on 6 October 1976, was to find the optimal period of incubating blood samples, and the second part, conducted on 13 January 1977, was to use the optimal period of incubating blood cultures to test objectives (ii) through (iv).

TABLE 1. *Types and status of dairy cattle used*

Group	Animal no.	Age	Vaccinal status[a]	*B. abortus* culture results[b]
1	21	Adult	NV	Field strain +
	33	Adult	AdV	Field strain +
	51	Adult	NV	Field strain +
	74	Adult	NV	Field strain +
2	22	Adult	CV	Strain 19 +
	38	Adult	AdV	Strain 19 +
	337	Adult	AdV	Strain 19 +
	438	Adult	AdV	Strain 19 +
	561	Adult	AdV	Strain 19 +
	611	Adult	AdV	Strain 19 +
3	807	Heifer	CV	NA
	901	Heifer	CV	NA
	902	Heifer	CV	NA
4	324	Adult	AdV	−
	334	Adult	AdV	−
	450	Adult	AdV	−
	654	Adult	AdV	−
5	518	Calf	NV	NA
	581	Calf	NV	NA
	987	Calf	NV	NA
	988	Calf	NV	NA

[a] NV, Not vaccinated; AdV, adult vaccinated; CV, calfhood vaccinated.

+, Positive; −, negative; NA, not applicable.

Collection of blood. Approximately 30 ml of blood was collected by jugular venipuncture from each animal. A 20-ml portion of each blood sample was placed into a sterile tube containing heparin (50 U/ml; The 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 several serological tests (Table 2). Each time heparinized blood was received in the laboratory at St. Paul, it was subjected to the following procedures.

Preparation of cultures. The heparinized blood was diluted 10-fold using RPMI-1640 culture medium (Grand Island Biological Co., Grand Island, N.Y.) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). No other supplements were added.

Culture medium, mitogen, and antigen. Tissue culture medium was RPMI-1640. Concanavalin A (ConA; Miles-Yeda, Rehovot, Israel) at a concentration of 1.0 μg per culture was used. BASA (8) was used at a concentration of 2.2 μg of protein per culture.

Cell cultures. After blood dilutions were made, blood from each animal (100 μl per culture) was added in triplicate to wells of a microtitration culture plate by using an automatic dispenser. ConA and BASA were added to the appropriate wells. As negative controls, triplicate wells for each animal were set up with lymphocyte suspension, but neither ConA nor BASA was added. All cultures were set up at the same time and incubated for varied periods of 3, 4, 5, 6, and 7 days, and all were incubated at 37°C in an incubator with a 5% CO_2 humidified air atmosphere. Approximately 16 to 18 h before termination, 0.5 μCi of [*methyl*-^{3}H]thymidine (6.0 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) was added to each well. Processing of cultures was performed by the method of Hartzman et al. (7), and radioactivity was counted by liquid scintillation spectrometry.

Serological tests. The standard serum tube agglutination test, card test, Rivinol test, and compliment fixation test were conducted on sera from test animals according to U.S. Department of Agriculture procedures (19, 20) and interpretations (21).

***Brucella* culture attempts.** Isolation of brucellae from milk and tissues was conducted according to the methods recommended by Alton et al. (1). *B. abortus* field strains were isolated repeatedly from the milk of cows in group 1 and from their tissues after slaughter. *B. abortus* 19 was isolated repeatedly from the milk of cows in group 2. Attempts to isolate *Brucella* from the milk of cows in group 3 were unsuccessful.

RESULTS

Expression of lymphocyte stimulation results. Results of lymphocyte stimulation are expressed in two ways.

(i) Δcpm. Difference in counts per minute (Δcpm) is expressed as mean cpm of triplicate

TABLE 2. *Comparison of serological tests*

Group[a]	Animal no.	Vaccinated[b]			Tests[c]							
					6 October 1976				13 January 1977			
		Date	Dose (ml)	Route	STT	Card	RIV	CF	STT	Card	RIV	CF
1	21	Non-V	NA	NA	+200	Pos	+200	ND	+200	Pos	+200	ND
	33	2-5-76 & 6-10-76	1	S/C	+100	Pos	Neg 25	++++1:20	ND	ND	ND	ND
	51	Non-V	NA	NA	+400	Pos	+200	ND	+400	Pos	+200	ND
	74	Non-V	NA	NA	+400	Pos	+200	ND	+400	Pos	+200	ND
2	22	2-7-74	5	S/C	+400	Pos	+200	ND	+400	Pos	+200	ND
	38	2-5-76 &	0.1	I/C								
		6-10-76	0.1	I/C	+100	Pos	I50	++++1:40	+100	Pos	I100	++++1:80
	337	2-9-76	5	S/C	+200	Pos	+200	+1:80	+50	Pos	I100	++++1:40
	438	2-5-76	5	S/C	+200	Pos	+200	++++1:80	+200	Pos	I200	++++1:80
	561	2-5-76 &	0.1	S/C								
		6-10-76	0.1	S/C	+200	Pos	+200	++++1:80	+200	Pos	+200	++++1:80
	611	2-5-76 &		I/C								
		6-10-76		I/C	+50	Neg	Neg 25	−1:10	+200	Pos	+200	++++1:80
3	807	9-1-76	5	S/C	+400	Pos	+400	ND	I200	Pos	I100	ND
	901	9-1-76	5	S/C	+400	Pos	+400	ND	+200	Pos	+50	ND
	902	9-1-76	5	S/C	+400	Pos	+400	ND	+200	Pos	+50	ND
4	324	2-5-76	5	S/C	+200	Pos	+25	++++1:20	+50	Pos	Neg 25	++++1:20
	334	2-5-76	5	S/C	+25	Neg	Neg 25	−1:10	+25	Neg	Neg 25	−1:10
	450	2-5-76	5	S/C	+200	Pos	+25	++++1:20	+100	Pos	I50	++++1:10
	654	2-5-76	5	S/C	+200	Pos	I200	++++1:20	+25	Pos	I200	++++1:20
5	518	NA	NA	NA	Neg 25	Neg	Neg 25	ND	Neg 25	Neg	Neg 25	ND
	581	NA	NA	NA	Neg 25	Neg	Neg 25	ND	Neg 25	Neg	Neg 25	ND
	987	NA	NA	NA	Neg 25	Neg	Neg 25	ND	Neg 25	Neg	Neg 25	ND
	988	NA	NA	NA	Neg 25	Neg	Neg 25	ND	Neg 25	Neg	Neg 25	ND

[a] Group 1, *B. abortus* field strain-infected cattle; group 2, *B. abortus* 19 shedders cattle; group 3, *B. abortus* calfhood-vaccinated cattle; group 4, *B. abortus* adult-vaccinated cattle nonshedders; and group 5, control animals.

[b] Non-V, Nonvaccinated; NA, not applicable; S/C, subcutaneous; I/C, intraconjunctival.

[c] STT, Standard agglutination tube test (≥1:200 = positive); card, card test; RIV, Rivanol test (≥1:25 = positive); CF, complement fixation test (≥1:40 = positive). ND, Not done; I, incomplete; +, Pos, positive; −, Neg, negative. Titers are presented as reciprocal of dilution.

cultures without either ConA or BASA subtracted from mean cpm of triplicate cultures with ConA or BASA.

(ii) SI. Stimulation index (SI) is expressed as mean cpm of triplicate cultures with ConA or BASA divided by mean cpm of triplicate cultures without either ConA or BASA.

Results of lymphocyte cultures. Results of part 1 of the study (to find optimal incubation period of blood cultures) are shown (Fig. 1). SI greater than or equal to 3.0 was considered indicative of infection. The data from the first part of the study revealed that: (i) a 6-day incubation period was optimal for culturing blood to achieve highest specific blastogenesis in lymphocytes from brucella-infected cattle; (ii) lymphocytes from animals in groups 1 and 2 had positive responses throughout; (iii) except on day 3,

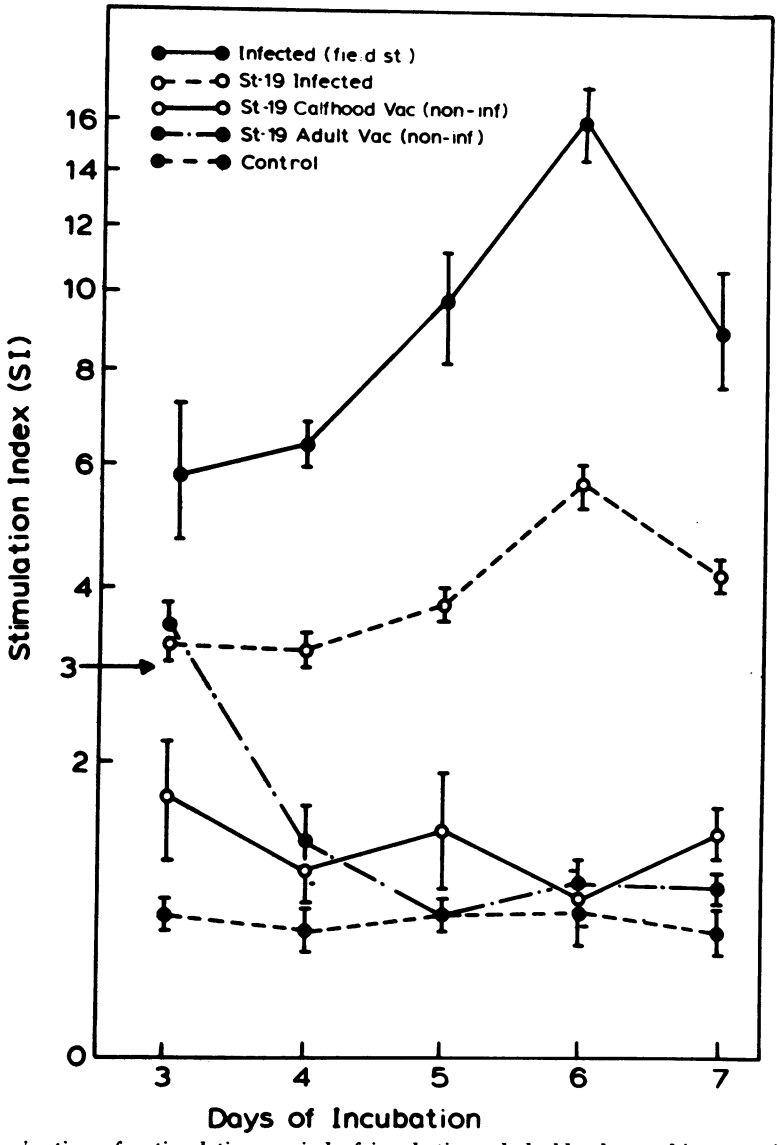

FIG. 1. *Determination of optimal time period of incubating whole blood to achieve maximum specific lymphocyte transformation with BASA. Blood was from B. abortus field strain-infected cattle (●——●); B. abortus 19 shedders (○- - -○), B. abortus calfhood-vaccinated, nonshedders (○——○); B. abortus 19 adult-vaccinated cattle (nonshedders) (●-·-●); and brucella nonexposed control cattle (●····●). Bars represent one standard error of the means. SI of ≥3 = positive (infection), shown by arrow (→); SI of ≤1 = negative response (not infected).*

group 1 had a significantly higher response than group 2 ($P < 0.05$); groups 3 and 4 were basically the same, except on day 3 when group 4 reacted above the positive (infection) mark; and (iv) there was no specific lymphocyte stimulation (SI = 1 or less) induced by BASA in lymphocytes from control animals.

Serological tests results. The serological tests on cattle in 5 groups are shown in Table 2. Group 3 had the highest serum agglutination antibody titers, followed by group 1, whereas group 4 had the lowest, followed by group 2. On a group basis, the level of serum agglutination titers did not correlate with specific lymphocyte stimulation response (LSR).

From the second part of the study (Fig. 2 and 3 and Table 3), the following findings could be stated: (i) on a group basis, lymphocytes from group 1 animals underwent a significantly higher specific LSR than those from group 2 ($P < 0.005$); (ii) lymphocytes from group 2 animals underwent a significantly higher specific LSR than those from groups 3 and 4 ($P < 0.01$); and (iii) no LSR was elicited by BASA in lymphocytes from control animals. Results of serological tests (Table 2) show that on a group basis the

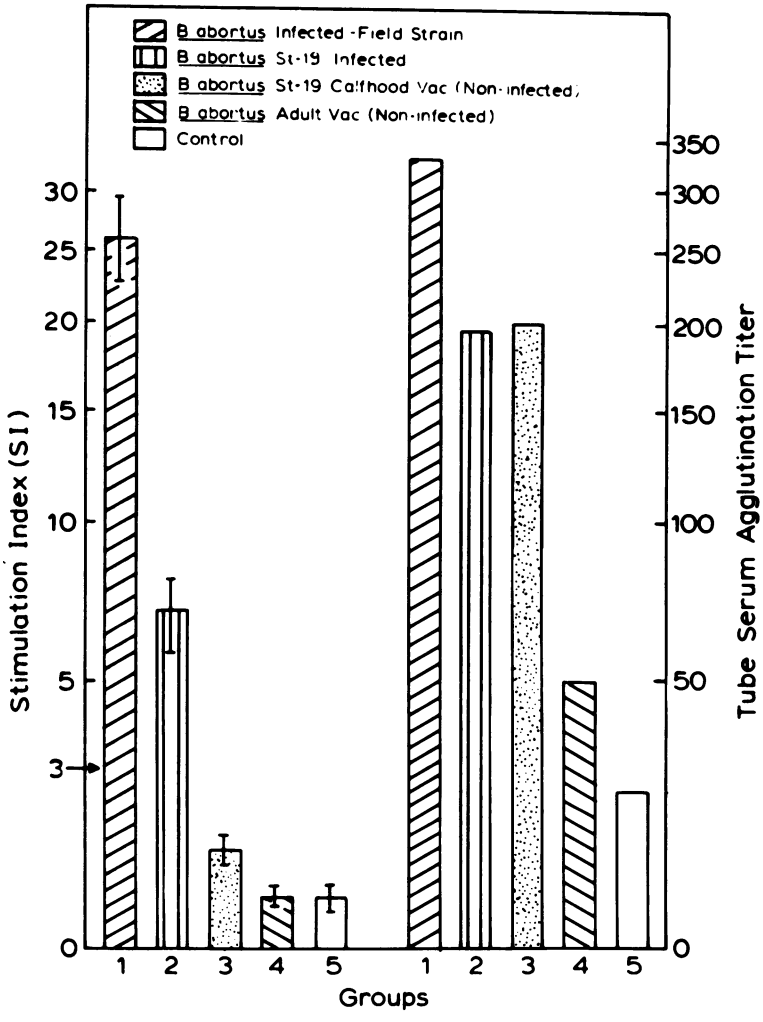

FIG. 2. *Comparison of lymphocyte stimulation results elicited by BASA in lymphocytes from cattle of different B. abortus exposure experience and nonexposed control animals with the serum tube agglutination test on an animal group basis. Lymphocytes were incubated for 6 days. Bars represent 1 standard error of the mean. SI of ≥3 = positive response (infection), represented by arrow (→); SI of ≤1 = negative response (not infected).*

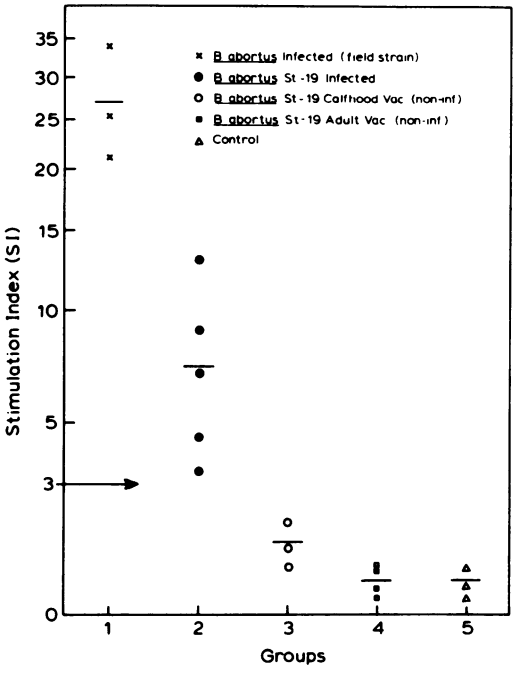

FIG. 3. *Comparison of specific lymphocyte stimulation, on a single-animal basis, elicited by BASA in lymphocytes from animals with different B. abortus exposure experience and from control animals. Lymphocytes were incubated for 6 days. Bars indicate mean values for each group of animals, and each mark represents SI of a single animal. SI of ≥3 = positive response (infection), represented by arrow (→); SI of ≤1 = negative response (not infected).*

level of antibody titers went down in all groups, except in group 1, where the titers remained the same.

Correlation between serological response and lymphocyte stimulation test. On a group basis, there was good correlation between antibody levels and LSR results in both groups 1 and 2; both reached infection level. In group 3 there was no correlation (Fig. 2) between the two systems. There was correlation between groups 4 and 5 between antibody titers and LSR (Fig. 2). On an individual basis, there was little correlation between level of antibody titer and LSR. Animals no. 21 and 38, for example, had the highest LSR in groups 1 and 2, respectively, but their titers were not the highest. Similarly, animals no. 74 and 438 of groups 1 and 2 had the highest antibody titers, but their specific LSR was the lowest for their respective groups. A similar trend was observed in animals of groups 3 and 4.

Correlation between lymphocyte stimulation and bacterial culture. Considering an SI for indication of infection as 3 or greater, all animals in groups 1 and 2 were called infected by LSR test. *B. abortus* was isolated from all those animals. Thus, there was 100% correlation between LSR and recovery of *B. abortus*.

DISCUSSION

The data generated in this study indicate that the whole-blood lymphocyte culture procedure

TABLE 3. *Lymphocyte stimulation induced by ConA and BASA in lymphocytes from cattle of different B. abortus exposure experience and in control animals*[a]

Group	Animal no.	Treatment	Δcpm	SI[b]
1	21	ConA	934,787	563.4
		BASA	10,845	33.8
	51	ConA	69,473	97.6
		BASA	9,074	25.4
	74	ConA	732,911	107.3
		BASA	9,973	21.4
2	22	ConA	912,331	390.3
		BASA	3,774	6.9
	38	ConA	704,775	466.2
		BASA	4,296	13.4
	337	ConA	85,933	204.1
		BASA	1,889	4.5
	438	ConA	100,919	270.6
		BASA	1,269	3.4
	611	ConA	87,044	344.0
		BASA	2,270	9.0
3	807	ConA	75,859	23.5
		BASA	936	0.9
	901	ConA	121,193	234.9
		BASA	1,149	1.3
	910	ConA	89,704	98.4
		BASA	1,168	2.0
4	324	ConA	96,144	61.5
		BASA	1,203	0.8
	334	ConA	73,051	107.3
		BASA	337	0.5
	450	ConA	107,996	44.8
		BASA	1,048	0.4
	654	ConA	51,129	97.9
		BASA	756	0.9
5	518	ConA	120,169	102.0
		BASA	447	0.2
	987	ConA	934,173	96.8
		BASA	912	0.6
	988	ConA	811,330	102.3
		BASA	471	0.9

[a] Group 1, *B. abortus* field strain-infected cattle; group 2, *B. abortus* 19 shedder cattle; group 3, *B. abortus* calfhood-vaccinated cattle; group 4, *B. abortus* adult-vaccinated nonshedder cattle; group 5, control animals.

[b] SI: ≥3, positive response; ≤1, negative response.

can be adapted to study cell-mediated immunity in bovine brucellosis. The observation that animals infected with *B. abortus* field strains (group 1) had a significantly higher cell-mediated immune response than cattle with *B. abortus* 19 infection (group 2) may be due to a difference in virulence. *B. abortus* 19 is a far less virulent organism than the field strain. It is, therefore, possible that the field strain multiplies and sensitizes a large proportion of lymphocytes. The difference in cell-mediated immune response in these groups of animals could have very important diagnostic value. The general lack of correlation between the cell-mediated immune response of calfhood-vaccinated animals (group 3) and the level of antibody titers agrees with our earlier studies in bovine brucellosis (9) where pure lymphocytes were used. The high level of correlation between infection and cell-mediated immune response in this study shows promise in brucellosis diagnosis.

The results of this study clearly indicate that this method has numerous advantages over more conventional techniques. In our experience, we found it simple, sensitive, fast, and reproducible. In the experiment reported here, there was no additional serum supplement added to the RPMI-1640 culture medium (serum supplements are needed when using purified lymphocytes). Additionally, the results demonstrate that there is no need to remove erythrocytes, polymorphonuclear leukocytes, platelets, or residual autologous plasma to obtain a significant mitogenic or antigen-induced blastogenesis. The use of nonseparated blood thus retains blood cells, especially monocytes, in natural proportions, allowing maximum enhancement of immune response through cell cooperation. The whole-blood lymphocyte culture procedure eliminates many of the steps involved in cell separation, thus avoiding many possible sources of technical errors, and it requires only small volumes of blood. Large numbers of samples can be set up, which could offer some savings in effort and real costs. With programs for the eradication of brucellosis in cattle and swine, large numbers of animals must be tested, and many of them cannot be properly identified serologically without bacterial culturing. A simplified test like the one described here could be a valuable aid in these programs.

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