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Stimulation of Subpopulations of Human Lymphocytes by a Vaccine Strain of *Francisella tularensis*

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When purified T lymphocytes from individuals vaccinated with a viable, attenuated strain of *Francisella tularensis* were incubated in vitro in the presence of heat-killed bacteria or a membrane preparation of the vaccine strain, they were stimulated to form blast cells and to synthesize deoxyribonucleic acid. The blast cells had the characteristics of T cells, being devoid of surface immunoglobulin and able to form rosettes with sheep erythrocytes. The stimulation occurred only when monocytes were present. A lymphocyte preparation enriched in B lymphocytes did not respond to the heat-killed bacteria or to the membrane preparation. In a stimulated mononuclear leukocyte preparation, about 70% of the blast cells formed rosettes with sheep erythrocytes, and 10 to 20% of them had surface immunoglobulin. The results show that there is an enlarged population of specifically committed T lymphocytes after tularemia vaccination. It is suggested that the lymphocyte stimulation test measures mainly T-lymphocyte reactivity when membranes or whole bacteria of *F. tularensis* LVS are used as antigen, and that the stimulation of human T lymphocytes by whole bacteria or bacterial membranes is completely monocyte or macrophage dependent. The present experimental procedure may provide a model for study of antigen-induced stimulation of human lymphocytes under controlled conditions. The technique used gave a reproducible, extremely purified preparation of T lymphocytes and a preparation of monocytes especially suitable for microcultures.

A specific antimicrobial resistance against tularemia is induced into various mammals by natural infection. It may also be induced by immunization with viable attenuated strains of *Francisella tularensis*. In both these situations, a cellular as well as a humoral immunity develops in the host. The cellular immunity seems to be more important to the resistance than humoral antibodies (1, 6, 21, 30). Thus, specific protection against tularemia can be conferred by passive transfer of thoracic duct lymphocytes from immunized to nonimmunized rats (18). Antiserum, on the other hand, provides no protecting effect (18). T lymphocytes have been found to mediate the specific resistance of mammals to other intracellular pathogens (4). There is a high proportion of T lymphocytes in the thoracic duct, and they may be responsible for protection against tularemia. However, the presence of an enlarged population of specifically committed T lymphocytes after immunization with *F. tularensis* has not yet been demonstrated, and our present knowledge on cellular immunity to *F. tularensis* has been obtained from studies in animals, whereas virtually no information is available from studies in humans.

By improvements in immunological techniques, it is now possible to look in humans for populations of T lymphocytes committed to specific antigens. Thus, T lymphocytes can be isolated from human blood, and they can be kept viable in in vitro cultures. Lymphocytes isolated from subjects that have been sensitized to an antigen respond when exposed to the antigen in vitro with blast transformation and increased synthesis of deoxyribonucleic acid (DNA) and other macromolecules. Because this response becomes overt only in cultures of lymphocytes from sensitized subjects (20), it indicates the presence in the donor of an enlarged population of specifically committed lymphocytes.

Recently, lymphocytes from tularemia-vaccinated individuals have been found to respond to antigen of the vaccine bacteria in vitro, whereas lymphocytes from nonvaccinated individuals responded poorly or did not respond at all (28). The purpose of the present study was to define the cell populations which are involved in the response. Special efforts were undertaken to obtain highly purified T lymphocytes.

MATERIALS AND METHODS

Blood donors. Blood donors had been vaccinated once or twice within the last 2 years with a lyophilized,

viable, attenuated tularemia vaccine, supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md.

Cell counts. Centrifuged (Shandon cytocentrifuge, Surrey, England) cell preparations and cultures were stained with Giemsa stain or for nonspecific esterase (23). Two hundred cells were examined, except for T-lymphocyte preparations, in which 2,000 cells were examined. The frequency of B lymphocytes was estimated (17) after staining with fluorescein-conjugated goat anti-human immunoglobulin antibodies (Hyland, Division of Travenol Laboratories, Brussels, Belgium). T lymphocytes were identified as rosette-forming cells (17). At least 200 cells were counted and, in T lymphocyte preparations, 2,000 cells.

Mononuclear leukocytes. Blood was defibrinated by gentle agitation with glass beads. Mononuclear leukocytes were separated by centrifugation on Ficoll-Isopaque (5), washed twice in Hanks balanced salt solution (Grand Island Biological Co., Madison, Wis.), and suspended in a culture medium containing 85% RPMI 1640–*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (RPMI-HEPES, Grand Island Biological Co.) and 15% pooled inactivated (56°C, 30 min) human serum. There was 100 μg of streptomycin sulfate per ml of medium. The density of mononuclear leukocytes was 5×10^6 cells per ml of medium, if not otherwise stated.

Mitomycin treatment. Mononuclear leukocytes were treated with mitomycin C as described by Bach and Voynow (3).

Purification of T and B lymphocytes. The purification procedure is shown in Fig. 1. To remove phagocytes, the mononuclear leukocytes were incubated with 0.02 g of iron particles (carbonyl iron powder, grade SF; GAF Corp., New York, N.Y.) per ml of suspension (19). After incubation for 30 min at 37°C with gentle agitation each 5 min, phagocytic cells were removed by a magnet (no. 939; James Neill Sheffield Ltd., Sheffield, England). The lymphocytes were washed twice in RPMI-HEPES containing 2% fetal bovine serum (Flow Laboratories, Irvine Ayrshire, Scotland) and suspended in fetal bovine serum at a density of 5×10^6 lymphocytes per ml. The suspension was divided in 1-ml volumes into round-bottomed glass tubes (16 by 110 mm).

The lymphocytes were separated into T and B cells by formation of E-rosettes (16) and differential centrifugation of rosetting and nonrosetting cells (11) according to the following procedure. Sheep erythrocytes were washed twice in phosphate-buffered saline (PBS; pH 7.4) containing 0.63 mM ethylenediaminetetraacetic acid and three times in Hanks balanced salt solution and thereafter were suspended in fetal bovine serum at a density of 150×10^6 cells per ml. A 1-ml volume of the suspension was added to each lymphocyte-containing tube by careful pipetting of the sheep erythrocytes on the top of the lymphocyte suspension. After incubation of the tubes at 37°C for 15 min, they were centrifuged at $200 \times g$ for 5 min and incubated overnight in an ice bath. Then the cells at the bottom of the tube were collected and centrifuged on Ficoll-Isopaque (5).

The cell suspension of the interface between serum and Ficoll-Isopaque was washed twice in PBS with

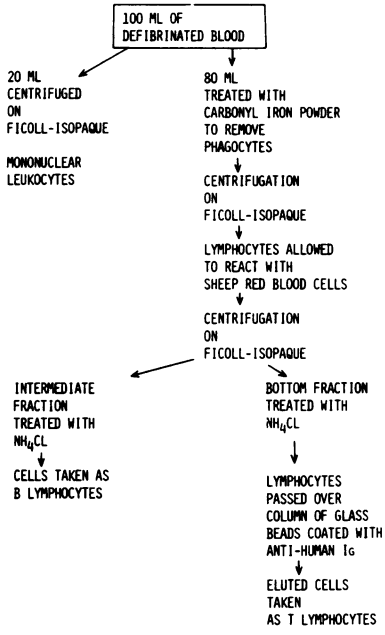

FIG. 1. *Purification of T and B lymphocytes from human blood.*

ethylenediaminetetraacetic acid, after which the cells were spun down and the supernatant was removed. The cells were treated with ammonium chloride (9), washed twice in PBS and once in RPMI-HEPES, and suspended in culture medium. These cells were taken as B lymphocytes. At least 70% of them had surface immunoglobulin.

The T lymphocytes that had formed rosettes with sheep erythrocytes were collected at the bottom of the tube under the Ficoll-Isopaque. They were washed, treated with ammonium chloride, washed again in the same way as the B cells, and suspended in RPMI-HEPES at a density of about 2×10^7 cells per ml.

The T lymphocytes were further purified from immunoglobulin-bearing lymphocytes on a column of anti-immunoglobulin G-coated glass beads (33). Glass beads (12 ml; Superbrite, 100-5005; 3M Co., St. Paul, Minn.) were packed in a column (1.5 by 30 cm). The column was treated overnight with 70% ethanol and washed thoroughly in PBS. It was then filled with a solution containing 1 mg of human immunoglobulin G (KABI AB, Stockholm, Sweden) per ml in PBS and left overnight at 4°C. After thorough washing in PBS, the column was filled with a solution of polyvalent rabbit anti-human immunoglobulin antibodies of a concentration high enough to immediately precipitate immunoglobulin G at a concentration of 1 mg/ml. After incubation at room temperature for 1 h, the excess of antibodies was eluted with 20 ml of PBS. The T lymphocytes were applied to the column and eluted with 1 to 2 ml of RPMI-HEPES per min. Cells passing the column were centrifuged and suspended in culture medium. They were taken as T lymphocytes. All preparations contained less than one cell other than T lymphocytes per 100 T lymphocytes.

Purification of monocytes. Monocytes were separated from the mitomycin-treated mononuclear leukocyte suspension using their adhering ability (25). Plastic disks (diameter, 5.5 mm) were punched out by an ordinary perforator (art. no. 9386; Ossvald Johnsson, Eskilstuna, Sweden) from a polyester film (projection film for plane paper copier 400, art. no. 6290, 0.10 mm; Rosendahls Fabriker AB, Filipstad, Sweden), heat-disinfected (70°C, 1 h), and placed at the bottom of each well of a Microtest II tissue culture plate (Falcon Plastics, Los Angeles, Calif.). A 100-μl volume of the suspension was allowed to settle in the well. The plate was closed with a film (Falcon Plastics) and incubated overnight at 37°C. To remove nonadhering cells, the plastic disks were gently rinsed at room temperature in RPMI-HEPES and placed in other wells containing 100 μl of culture medium. Disks were also mounted on glass slides and stained in Giemsa solution as well as for nonspecific esterase (23). At least 95% of the cells had the characteristics of monocytes, i.e., were mononuclear and showed a high esterase activity. The number of cells that had adhered to the plastic disks was estimated by examination under microscope of one-hundredth of the total surface of the disk.

Lymphocyte stimulating agents. Heat-killed bacteria of *F. tularensis* LVS were prepared as previously described (28). Membranes of the bacteria were prepared following a modification used on *Neisseria gonorrhoeae* (34) which had originally been worked out for *Escherichia coli* (35). Bacteria were grown in a blood-free medium (31), which had been kept frozen at −20°C and thawed at 37°C 24 h before use. After 16 h of incubation in 300-ml batches, the bacteria were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C. After incubation with lysozyme and ethylenediaminetetraacetic acid in 30% sucrose and with ribonuclease and deoxyribonuclease, a soluble fraction was obtained by centrifugation at 12,000 × *g* for 15 min. This fraction was brought to pH 5.0 by addition of HCl, after which visible aggregation occurred. The precipitated material was washed four times in 5 ml of ice-cold 5 mM ethylenediaminetetraacetic acid (pH 7.5) and three times in Hanks balanced salt solution. It was then suspended in culture medium and referred to as the membrane preparation.

Isopycnic sucrose density gradient centrifugation of the membrane preparation yielded a major band with a buoyant density of 1.25 g/cm^3, which has been reported to be the density of outer membranes of *E. coli* (35) and *N. gonorrhoeae* (34).

Lymphocyte cultures. Three types of experiment were performed. In the first, the stimulation of lymphocytes was assayed in microcultures (12). To each well of a Microtest II tissue culture plate was added 100 μl of a suspension containing mitomycin-treated mononuclear leukocytes. Plates were closed by a film and stored overnight at room temperature waiting for the lymphocyte purification to be completed. Then 50 μl of a suspension of T or B lymphocytes and 50 μl of a suspension of heat-killed bacteria or membrane preparation were added to each well of the plate. The final density of T or B lymphocytes was 1.5 × 10^6 cells per ml of culture. The final density of heat-killed bacteria was 15 μg/ml (dry weight) and of membrane preparation, 8 μg of protein per ml. The plates were closed and incubated at 37°C for 6 days. To estimate DNA synthesis, the cultures were then pulsed with [^{14}C]thymidine and harvested as previously described (28).

In another type of experiment, purified monocytes on a plastic disk were added to the culture instead of the mitomycin-treated mononuclear leukocytes, while other conditions were kept identical.

In experiments designed to determine the surface characteristics of blast cells, 3 × 10^6 mononuclear leukocytes in 1 ml of culture medium were mixed in round-bottomed glass tubes (16 by 110 mm) with 1 ml of a suspension of heat-killed bacteria or membrane preparation. Tubes were firmly closed and incubated for 3 to 7 days at 37°C. To estimate DNA synthesis, 0.2 ml of suspension from each tube was then added to a tissue culture plate. Blast cells were examined with regard to surface immunoglobulin (15), using the fluorescein-conjugated goat anti-human immunoglobulin antiserum. Cells were also allowed to form rosettes with sheep erythrocytes (15). They were washed once and suspended in fetal bovine serum before addition of sheep erythrocytes. Conditions of rosette formation were the same as those described in the purification of T and B lymphocytes. After incubation overnight in an ice bath, the mixture of sheep erythrocytes and cultured cells was carefully suspended in 0.5 ml of fetal bovine serum, and various amounts of the suspension were centrifuged in a cytocentrifuge. Slides were fixed in methanol and stained in Giemsa solution. Cytocentrifugation was found necessary to determine whether the rosette-forming cells were blast cells or not.

RESULTS

When T lymphocytes were incubated with heat-killed bacteria of *F. tularensis* LVS but in the absence of other mononuclear leukocytes, the incorporation of [^{14}C]thymidine into DNA was about as low as in the absence of stimulating agent (Table 1). In the presence of mitomycin-treated mononuclear leukocytes, on the other hand, the bacteria induced a high incorporation of [^{14}C]thymidine into the T lymphocytes. The bacteria did not induce any incorporation into the mitomycin-treated mononuclear leukocytes (Table 1). The results indicate that T lymphocytes can be stimulated by heat-killed bacteria of *F. tularensis* LVS in the presence of mononuclear leukocytes.

B lymphocytes (Table 1) did not incorporate more radioactivity in the presence than in the absence of the bacteria, whether mitomycin-treated mononuclear leukocytes were present or not.

Similar results were obtained when the membrane preparation of *F. tularensis* LVS was used as antigen. When lymphocytes from nonimmunized individuals were used, the incorporation of radioactivity was about as low in the presence as in the absence of stimulating agent.

TABLE 1. *Response of T and B lymphocytes from vaccinated individuals to heat-killed F. tularensis LVS*[a]

Presence in culture of:				Mean cpm ± SEM of 4 cultures
T lymphocytes	B lymphocytes	Mitomycin-treated mononuclear leukocytes	Heat-killed bacteria	
+	−	+	+	4,500 ± 947
+	−	−	+	132 ± 6
+	−	+	−	62 ± 9
−	+	+	+	310 ± 47
−	+	−	+	121 ± 32
−	+	+	−	412 ± 50
−	−	+	+	65 ± 15

[a] Lymphocytes were incubated for 6 days, and [^{14}C]thymidine incorporation into DNA (counts per minute [cpm] ± standard error of the mean [SEM]) was estimated.

In the presence of a plastic disk with monocytes, the membrane preparation induced a high incorporation of [^{14}C]thymidine into the T lymphocytes (Table 2). No incorporation was induced in cultures supplemented with a plastic disk without monocytes. Thus, monocytes facilitated the response of T lymphocytes to membrane antigens of *F. tularensis* LVS.

Various numbers of mitomycin-treated mononuclear leukocytes were added to cultures of T lymphocytes, and the lymphocyte response to heat-killed bacteria was estimated. When the monocyte-T lymphocyte ratio was as low as 0.1:100, the incorporation of [^{14}C]thymidine into DNA was low (Fig. 2). When the ratio was increased from 0.3:100 to 1:100, there was an increasing incorporation. One to 3 monocytes per 100 T lymphocytes seemed to be enough to reach an optimal incorporation.

The expression of surface markers on blast cells was studied after incubation of mononuclear leukocytes in the presence of heat-killed bacteria or the membrane preparation. From 10 to 20% of the blast cells were stained by fluorescein-conjugated goat anti-human immunoglobulin antibodies, whereas about 70% of them formed rosettes with sheep erythrocytes (Table 3). The B-cell frequencies, when estimated as frequencies of stained cells, are probably maximal, since nonspecific staining cannot be excluded. The frequencies of rosette-forming cells, on the other hand, are probably minimal, since rosettes may have broken during the centrifugation procedure.

When purified T lymphocytes were incubated in the presence of mitomycin-treated leukocytes, heat-killed bacteria induced blast cells, more than 80% of which formed rosettes with sheep erythrocytes. No immunoglobulin-positive cell was found out of 100 blast cells.

DISCUSSION

This seems to be the first study of the direct interaction between antigens of *F. tularensis* and T lymphocytes. It shows that peripheral blood of individuals vaccinated with a viable,

TABLE 2. *Effect of autologous monocytes on the response of T lymphocytes from vaccinated individuals to the membrane preparation of F. tularensis LVS*[a]

Presence in culture of:			cpm ± SEM of 4 cultures
T lymphocytes	Membrane preparation	Monocytes	
+	+	+	3,004 ± 578
+	+	−	57 ± 4
+	−	+	90 ± 17
−	+	+	88 ± 17

[a] Each culture contained one or more of the following components in a total volume of 0.2 ml: 0.3×10^6 T lymphocytes, a plastic disk with or without 0.1×10^6 monocytes, and 1.6 μg (protein) of a membrane preparation of *F. tularensis* LVS. The lymphocytes were incubated for 6 days, and the incorporation of [^{14}C]thymidine into DNA (counts per minute [cpm] ± standard error of the mean [SEM]) was estimated.

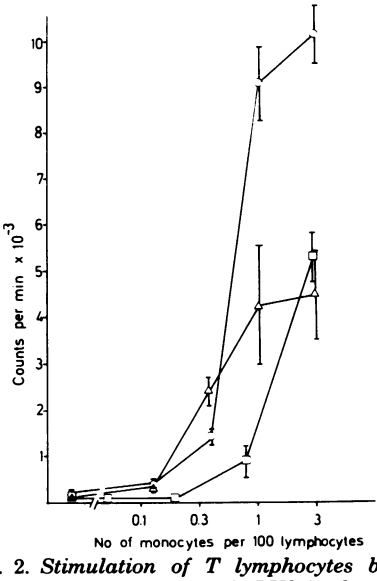

FIG. 2. *Stimulation of T lymphocytes by heat-killed bacteria of F. tularensis LVS in the presence of various numbers of mitomycin-treated mononuclear leukocytes. The incorporation of [^{14}C]thymidine into DNA of the lymphocytes was measured, and counts per minute × 10^{-3} ± the standard error of the mean of four to five cultures was plotted against the number of monocytes of the mononuclear leukocyte preparation added per 100 T lymphocytes.*

TABLE 3. *Expression of surface markers on blast cells*[a]

Experiment no.	Stimulating agent	Days of incubation	Cells with surface immunoglobulin	Cells with SRBC[b] receptors	Incorporation of [^{14}C]thymidine[c]
1	Heat-killed bacteria	4	17	72	0.8
		5	15	65	1.3
		6	10	69	1.0
		7	13	76	0.6
	None	4–7	ND[d]	ND	0.0–0.1
2	Heat-killed bacteria	4	11	63	2.3
		6	14	67	1.3
	Membrane preparation	4	7	58	0.9
		6	6	63	1.3
	None	4–6	ND	ND	0.2–0.3
3	Heat-killed bacteria	7	5	83	3.3
	Membrane preparation	7	7	78	1.6
	None	7	ND	ND	0.5
4	Heat-killed bacteria	4	15	74	4.0
		5	12	77	3.9
		6	16	81	4.9
	Membrane preparation	4	12	67	1.8
		5	13	57	2.9
		6	12	75	4.0
	None	4–6			0.2–0.4

[a] Lymphocytes from vaccinated individuals were stimulated by heat-killed bacteria or a membrane preparation of *F. tularensis* LVS.

[b] SRBC, Sheep erythrocytes.

[c] Mean counts per minute $\times 10^{-3}$.

[d] ND, Cell counts were not done since nonstimulated cultures contained very few blast cells.

attenuated strain of *F. tularensis* contains a proportion of specifically committed T lymphocytes that is high enough to be demonstrated in the lymphocyte stimulation test. We have previously found the degree of stimulation to approximate the purified protein derivative-induced stimulation of lymphocytes from purified protein derivative-sensitive individuals (28). It has been calculated that about 1 to 2% of blood lymphocytes may respond to purified protein derivative under similar conditions (8, 20). In conclusion, the present results together with those recently reported (28) demonstrate the presence after vaccination of an enlarged population of T lymphocytes interacting with antigens of *F. tularensis* LVS.

The evidence was strong that T lymphocytes were stimulated in the present experiments. The T-lymphocyte preparations had been highly purified from other blood cells. Furthermore, most blast cells of the stimulated T-lymphocyte cultures had the surface characteristics of T cells, whereas no blast cells had the characteristics of B cells. However, the results also indicate that B lymphocytes in cultures of mononuclear leukocyte preparations respond to antigens of *F. tularensis* LVS. Partly purified B lymphocytes, on the other hand, were not able to respond. These results are in accordance with results (10) indicating that human T lymphocytes may be induced by tetanus toxoid to produce a soluble factor that stimulates autologous B lymphocytes. However, there was no relative increase with time of blast cells having the surface characteristics of B lymphocytes. Such an increase has been reported to occur during phytohemagglutinin-induced stimulation of mouse lymphocytes (24).

The stimulation of human lymphocytes by soluble antigen and allogeneic cells has been found to be diminished by removal of monocytes (2, 13, 27). Stimulation of purified T lymphocytes from guinea pigs by soluble antigens, allogeneic cells, and mitogens has been found to be completely monocyte or macrophage dependent (26, 32). The high degree of stimulation reported in cultures of purified human T lymphocytes in the presence of soluble antigen (7, 10) should not be taken as contradicting, as these T-lymphocyte preparations were purified mainly with regard to B lymphocytes and may have contained some monocytes. The possible requirement of monocytes in stimulation of T lymphocytes by bacteria or bacterial membranes seems

not to have been studied in humans or in other mammals. According to the present results, such stimulation is completely monocyte dependent. This conclusion seems valid, since the stimulation of T lymphocytes was completely abolished and restored by addition of mitocycin-treated monocytes. The abolishment was due to the excessive purity of the T-lymphocyte preparations. The T lymphocytes were purified in a first step by rosette formation with sheep erythrocytes followed by isopycnic centrifugation of rosetting from nonrosetting lymphocytes and, in a second step, by chromatography of the lymphocytes on a column of anti-immunoglobulin G-coated glass beads. Thus, a combination of two procedures based on different principles was chosen rather than a repeated separation. This may favor the removal of subpopulations of non-T lymphocytes with various properties. It should be remarked that omission of either of the two steps resulted in cell preparations with an ability to respond that was only partly reduced. A similar combination of separation procedures has recently been found by others to yield T lymphocytes of high purity (14). The addition of as few as 0.3% monocytes facilitated the response of T lymphocytes to the membrane preparation of *F. tularensis* LVS, and the presence of 1 to 3% seemed to be enough to reach an optimal stimulation. This is in fair agreement with studies on mitogen-induced stimulation of guinea pig lymphocytes (26).

The present experimental procedure may be useful in further studies on the mechanism of specific stimulation of T lymphocytes. Vaccination with *F. tularensis* LVS gives an immune response which is not ubiquitous in the population. Therefore, possible nonspecific stimulating effects of an antigen preparation can be controlled by the use of lymphocytes from nonexposed individuals. The cell fractionation procedure yielded highly purified T lymphocytes. A new simple technical modification was used to isolate monocytes and was found suitable for microcultures. By the present model, the interaction between antigen, monocytes, and T lymphocytes may be studied under standardized conditions and with the use of reasonable amounts of human blood.

It is probable that the T-lymphocyte proliferation induced by immunization with *F. tularensis* LVS is important to the antimicrobial defence of the host. Thus, thoracic duct lymphocytes from immunized rats have been shown to confer specific antimicrobial resistance on normal recipients, whereas antiserum afforded no protection (18). A lack of protection by humoral antibodies has also been found after vaccination with killed bacteria (22). Furthermore, antibodies have not affected the survival of *F. tularensis* in in vitro experiments, whereas mononuclear cells from specifically immunized animals have killed the bacteria efficiently (21, 29). The present results suggest that the lymphocyte stimulation test estimated mainly T-lymphocyte reactivity when membranes or whole bacteria of *F. tularensis* are used as antigen. As an indicator of protection, the test might therefore be better than antibody assays.

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