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Diversity of *Francisella tularensis* Antigens Recognized by Human T Lymphocytes

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The *Francisella tularensis* T-lymphocyte antigens, which may have a role in protection against tularemia, were investigated with vaccine-immunized subjects. Preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to fractionate the bacterial envelope preparation. The 23 fractions obtained represented membrane proteins of different apparent molecular masses ranging from 10 to 150 kilodaltons. Different fractions contained one to four separate protein bands stained with Coomassie blue. The lymphocyte blast transformation responses of five tularemia vaccine-immunized and three nonimmunized subjects were tested against bacterial material eluted out of SDS-PAGE fractions. Every fraction stimulated lymphocytes from at least one of the subjects. No clearly immunodominant or inhibitory antigens were detected among the envelope fractions. Expression of the HLA-DR antigen at the surface of CD4- and CD8-positive lymphocytes was also studied as a measure of cell activation. The numbers of $CD4^+$ DR^+ cells varied directly with the lymphocyte proliferation profiles, and very few $CD8^+$ cells were found in the preparations stimulated with the different fractions. The diversity of the antigens recognized by immune T lymphocytes was confirmed by using *F. tularensis*-specific T-lymphocyte clones obtained from vaccinated subjects. Most of the 36 T-lymphocyte clones tested were stimulated by one SDS-PAGE fraction only.

The antigen-specific T-cell proliferation detected by in vitro assays reflects cell-mediated immunity, which is crucial for protection against intracellular microbes such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Francisella tularensis*. Lymphocyte activation requires recognition of a specific antigen determinant presented in association with a major histocompatibility complex antigen at the surface of an antigen-presenting cell. In human tularemia, major histocompatibility complex class II-restricted antigen stimulation (16) is directed at bacterial proteins (13, 17).

Certain major membrane proteins have been found to stimulate immune lymphocytes into proliferation (13), but the cell envelope of *F. tularensis* is made up of a vast number of components whose antigenic significance is not known. The present work was aimed at acquiring more information on the diversity of the protein antigens involved in tularemia immunity. Cell envelope proteins of *F. tularensis* were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fractions representing polypeptides of different sizes were studied further.

The antigenicity of the fractions was characterized by analyzing the proliferation response of peripheral blood mononuclear cells (PBMC) and *F. tularensis*-specific $CD4^+$ T-lymphocyte clones (TLCs) obtained earlier from subjects immune to tularemia (16). The activation of different lymphocyte subclasses ($CD4^+$ and $CD8^+$) in the stimulated PBMC was also measured from their expression of an activation marker (HLA-DR antigen) by double immunofluorescence analysis.

MATERIALS AND METHODS

Bacteria and antigen preparations. *F. tularensis* LVS (BB IND 157.6111) was supplied by the U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Md., and was cultured and prepared as a whole-cell antigen as described earlier by Surcel et al. (16).

The cell envelope was isolated from the *F. tularensis* bacteria after the bacteria were harvested from the stationary culture by centrifugation at 7,000 × *g* for 15 min at 4°C. The bacteria were washed once with 30 mM cold HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer containing 1 mM mercaptoethanol (pH 7.3), resuspended in 30 ml of HEPES buffer, and disrupted by sonication.

After the intact cells were removed by centrifugation (7,000 × *g*, 5 min), the supernatant was ultracentrifuged at 100,000 × *g* (Beckman L8-80 with an 80Ti rotor) for 2 h at 4°C. The pellet was washed once with 40 ml of 5 mM EDTA–10 mM HEPES–1 mM mercaptoethanol (pH 7.3) and suspended in 5 mM EDTA containing 1 mM mercaptoethanol (pH 7.3). This suspension was termed the total envelope material.

Preparative SDS-gel electrophoresis. The total envelope material was solubilized in SDS buffer at 25°C and fractionated by the preparative SDS-PAGE method (5) as described by Sarvas and Nurminen (14). A 5% (wt/vol) polyacrylamide stacking gel and a gradient of 10 to 20% (wt/vol) polyacrylamide in the separating gel were used for the fractionation. After electrophoresis (150-V constant voltage), the gel was cut horizontally into 23 strips from which the proteins were eluted by shaking overnight with 0.1% (wt/vol) SDS supplemented with 1 mM phenylmethylsulfate (Sigma Chemical Co., St. Louis, Mo.). After elution, the fractions were concentrated with Immersible CX-10TH ultrafiltration units (Millipore Corp., Bedford, Mass.) to a volume of about 500 μl. The amount of protein in the prepared fractions was estimated by comparing the SDS-PAGE staining intensity of the serially diluted fraction material with a Coomassie blue-stained low-molecular-mass standard protein kit containing phosphorylase *b* (94 kilodaltons [kDa]), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (Pharmacia Fine Chemicals, Uppsala, Sweden).

Lymphocyte blast transformation assay. Lymphocyte responses in adult laboratory staff members who had been

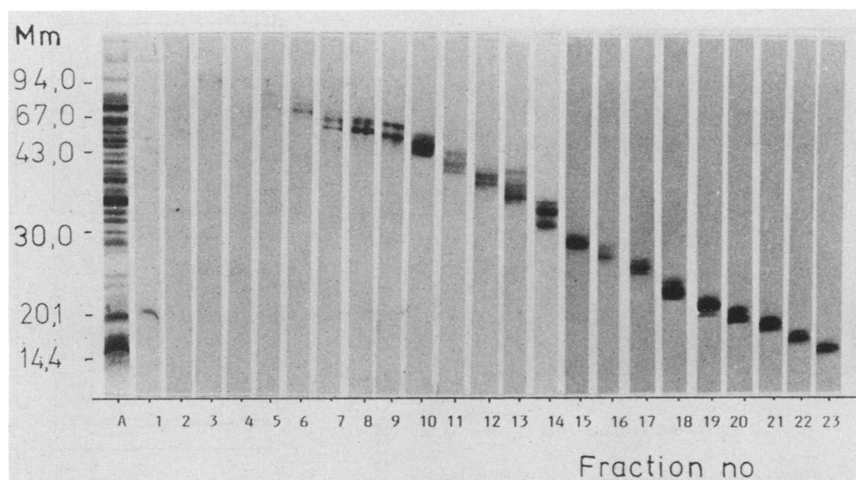

FIG. 1. Envelope proteins (20 μg per lane) of *F. tularensis* LVS before (lane A) and after (lanes 1 to 23) SDS-PAGE fractionation, stained with Coomassie blue. Molecular mass (Mm) standards in kilodaltons are indicated on the left.

immunized with the live vaccine strain *F. tularensis* LVS from 1 to 10 years earlier were studied. The controls were unvaccinated adults with no previous history of *F. tularensis* infection. PBMC were isolated with Lymphoprep (Nyegaard Co. a/s, Oslo, Norway) and suspended in RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with 10% human AB serum. The lymphocytes were cultured as described previously (3, 17) and stimulated with the whole-cell antigen (1.0 μg/ml), the unfractionated envelope preparation (3.5 μg/ml), and the SDS-PAGE fractions (2.0 μg/ml). Tetanus toxoid (10 μg/ml) or purified protein derivative (PPD) (10 μg/ml) was used as a control antigen in each experiment.

Culturing of TLCs. Separated PBMC (0.25×10^6/ml) that were suspended in RPMI 1640 (GIBCO) supplemented with 10% pooled human AB serum were stimulated with the whole-cell antigen (2.0 μg/ml) and cloned by the limiting-dilution method as described earlier (16). The proliferation responses of the clones were also analyzed as described previously (16).

Lymphocyte subpopulations. T-cell activation was characterized by analyzing the surface antigens of antigen-stimulated PBMC by flow cytometry (FACS analyzer; Becton Dickinson and Co., Mountain View, Calif.). HLA-DR antigen and CD4 (T helper) or CD8 (T suppressor, or cytotoxic) antigen were detected simultaneously by means of monoclonal antibodies conjugated with different fluorochromes (phycoerythrin- or fluorescein-conjugated anti-Leu-2, anti-Leu-3, and anti-HLA-DR antibodies; Becton Dickinson).

Statistical analysis. The Mann-Whitney test was used for statistical analysis.

RESULTS

SDS-PAGE fractions of the *F. tularensis* envelope preparation. The SDS-PAGE fractionation gel of the *F. tularensis* envelope preparation was cut into 23 fractions with apparent molecular masses ranging from 150 (fraction 1) to 10 kDa (fraction 23). Proteins were eluted from the fractions and adjusted to the same concentration. One to four separate protein bands were seen in different fractions when the fractions were analyzed by Coomassie blue staining and SDS-PAGE. Slight overlapping of the proteins with a neighboring fraction was seen (Fig. 1).

Lymphocyte responses to the SDS-PAGE fractions. The PBMC responses of five subjects immune to tularemia (stimulation index > 4) and three nonimmune subjects (stimulation index < 4) to the 23 SDS-PAGE fractions were studied. The proliferation profiles of the lymphocytes from subjects immune to tularemia revealed distinct peaks and valleys (Fig. 2) which were different for each subject. The strongest responses detected as a peak in proliferation were not responses to the same SDS-PAGE fractions. This result was confirmed in a repeated lymphocyte blast transformation assay performed for two subjects (data not shown).

Despite the individual differences, an increased proliferation response common to all the immune lymphocytes was

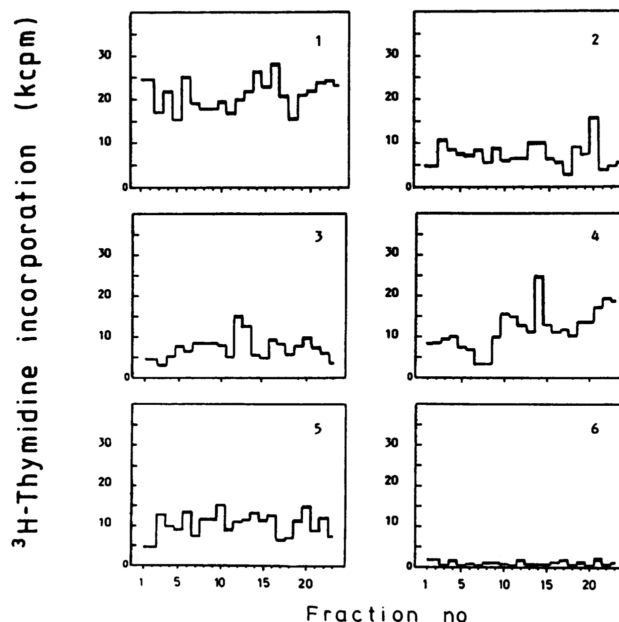

FIG. 2. Proliferative responses of immune (subjects 1 to 5) and nonimmune (subject 6) lymphocytes induced by *F. tularensis* SDS-PAGE protein fractions 1 to 23. Data are expressed as means in thousands of counts per minute (kcpm) minus background of triplicate cultures after 6 days of incubation with antigen (2 μg/ml). The standard error of the mean was 2,436 ± 390 cpm (mean ± standard error of the mean) for subject 1, 1,575 ± 211 cpm for subject 2, 1,575 ± 186 cpm for subject 3, 2,250 ± 260 cpm for subject 4, 2,030 ± 153 cpm for subject 5, and 538 ± 76 cpm for subject 6.

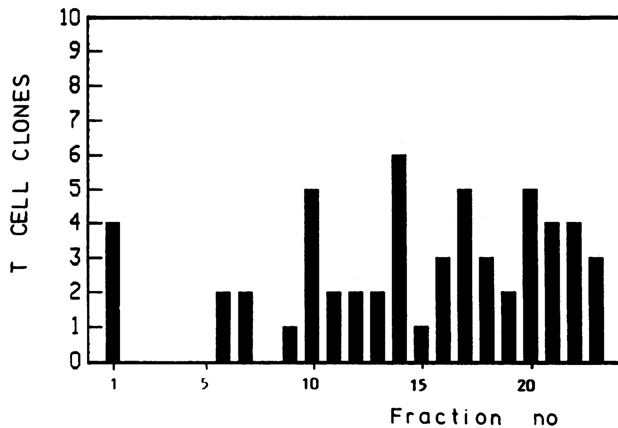

FIG. 3. Frequency of *F. tularensis*-specific TLCs (36 clones) recognizing proteins in different SDS-PAGE fractions.

seen for fractions 20 to 23 (molecular mass range, 10 to 20 kDa). The other SDS-PAGE fractions having the capacity to induce a proliferation peak for at least one preparation of the immune lymphocytes were fractions 14 to 18 (molecular mass range, 25 to 30 kDa), fractions 8 to 12 (molecular mass range, 35 to 50 kDa), and fractions 5 to 7 (molecular mass range, 65 to 70 kDa). No fraction was totally nonstimulative, but all of the fractions induced immune lymphocytes to proliferate four to five times more strongly (indicated in counts per minute) than did any of the nonimmune lymphocytes (Fig. 2).

Recognition of SDS-PAGE fractions by *F. tularensis*-induced T-cell clones. Since each TLC recognizes one specific epitope, these clones were used to confirm the diversity of the stimulating antigens. Growth of the TLCs was induced with *F. tularensis* whole-cell antigen from lymphocytes of a tularemia-vaccinated subject. Figure 3 shows the reactivity of 36 TLCs to the SDS-PAGE fractions. Twenty-three of thirty-six TLCs recognized a single SDS-PAGE fraction each, as determined by the presence of a proliferation peak, and in 18 cases the peak was in fractions 18 to 23, which had low molecular masses (less than 25 kDa). None of the 36 TLCs tested recognized antigen in fractions 2 to 5 (molecular mass range, 60 to 100 kDa). Thirteen of the TLCs responded to more than one SDS-PAGE fraction (Figure 4; for example, TLC 3). Some of the TLCs were stimulated with the SDS-PAGE fractions three times. Each time, the TLCs responded to the same fractions, although the level of reactivity changed (range of stimulation indices, 10 to 35). Figure 4 shows representative proliferation patterns for the TLCs tested.

The possible cross-reactivity of *F. tularensis*-specific TLCs was investigated by stimulating them with PPD and with a selected panel of other gram-negative bacteria prepared in a similar way to the *F. tularensis* whole bacteria (see Materials and Methods). The TLCs responded to *F. tularensis* whole-cell antigen more effectively than to any other bacteria ($P < 0.005$) or to PPD antigen ($P < 0.005$). The only cross-reaction found was by clone 16, which was stimulated by PPD (1,333 cpm) almost as effectively as by the original antigen (1,792 cpm) (Table 1).

Activation of T-lymphocyte subsets by SDS-PAGE fractions. Lymphocyte activation was further characterized by measuring the expression of HLA-DR antigen as a T-cell activation marker on the surface of CD4 (T helper)- and CD8 (T suppressor, or cytotoxic)-positive T cells. Double immunofluorescence analysis showed that the SDS-PAGE fractions preferentially stimulated T-helper cells and that none of the fractions were especially specific to T-suppressor cells. The number of activated T-helper ($CD4^+$ $HLA\text{-}DR^+$) cells was 16.0 to 26.6% of the number of live lymphocytes in the stimulated cultures. The number of activated T-suppressor, or cytotoxic ($CD8^+$ $HLA\text{-}DR^+$), cells did not differ much between the stimulated (0.6 to 1.3%) and unstimulated cultures. The curve representing the number of activated T cells ran parallel to the peaks and valleys of the simultaneously analyzed lymphocyte proliferation profile (Fig. 5).

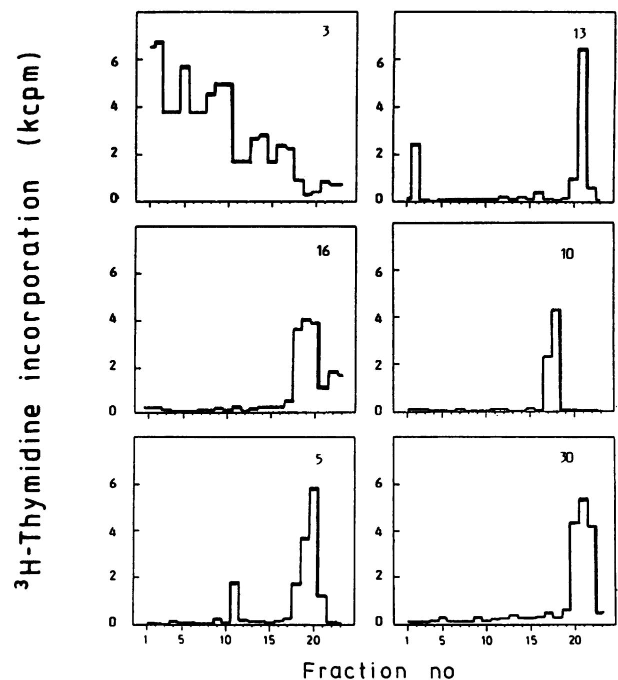

FIG. 4. Representative proliferation patterns for *F. tularensis*-specific TLCs in response to bacterial proteins fractionated by SDS-PAGE. Data are expressed as means of [^{3}H]thymidine incorporation in thousands of counts per minute (kcpm) minus background after 48 h of incubation with antigen (2 μg/ml) and antigen-presenting cells. The standard error of the mean was 111 ± 13 cpm for TLC 3 (mean ± standard error of the mean), 33 ± 3 cpm for TLC 13, 94 ± 22 cpm for TLC 16, 95 ± 43 cpm for TLC 10, 29 ± 6 cpm for TLC 5, and 52 ± 22 cpm for TLC 30.

DISCUSSION

The purpose of the present work was to screen and characterize T-cell antigens of *F. tularensis*. This was done by stimulating lymphocytes of vaccine-immunized subjects with SDS-PAGE-fractionated antigens after they had been eluted from the gel and concentrated. This technique allows the protein levels in the different fractions to be adjusted to the same concentration, thus differing from the Western blot (immunoblot) method first described by Young and Lamb (20). The lymphocyte responses to the SDS-PAGE fractions were detected as distinct peaks and valleys in the proliferation profiles. This may reflect the number of antigen epitopes in each fraction recognized by immune T lymphocytes or the immunodominance of some epitopes with large clones of specific cells. A fraction inducing strong lymphocyte responsiveness could include an important epitope that has a role in protective cell-mediated immunity. A low proliferation may,

TABLE 1. Responses of triplicate TLC cultures to a panel of whole-cell antigens of gram-negative bacteria (1 μg/ml) and to PPD (10 μg/ml)

TLC no.	TLC responses (cpm [mean ± SEM]) induced by:							
	No antigen	*Francisella tularensis*	*Yersinia enterocolitica*		*Salmonella typhimurium*		*Escherichia coli*	PPD
			O9	Not O9, not O3	SL696	SH9013		
3	135 ± 21	2,895 ± 213	131 ± 17	121 ± 2	125 ± 20	138 ± 13	87 ± 18	199 ± 67
5	67 ± 15	6,560 ± 257	61 ± 44	73 ± 12	43 ± 5	67 ± 15	44 ± 4	165 ± 28
13	42 ± 17	3,849 ± 617	160 ± 36	120 ± 7	101 ± 3	137 ± 15	111 ± 10	222 ± 40
16	40 ± 9	1,792 ± 141	79 ± 23	63 ± 5	51 ± 4	81 ± 10	50 ± 11	1,339 ± 206
29	58 ± 32	1,982 ± 132	52 ± 10	85 ± 8	110 ± 12	122 ± 29	132 ± 10	118 ± 50
30	65 ± 14	2,354 ± 84	261 ± 74	208 ± 25	124 ± 2	182 ± 31	111 ± 35	316 ± 59
Mean	67.8	3,233.7	124.0	111.7	92.3	121.2	89.2	393.2
SEM	14.2	729.8	32.3	21.6	14.8	17.1	14.6	191.1

on the other hand, reflect either a lack of epitopes which can be recognized in the context of the given set of major histocompatibility complex products or a T-suppressor cell-specific antigen determinant.

F. tularensis membrane proteins with molecular masses of 61, 37, 32, and 17 kDa were known previously to stimulate immune lymphocytes into proliferation (13, 15, 17), and high proliferation responses were also seen in this study at molecular mass ranges correlating to the proteins mentioned above. A strong immune lymphocyte blast transformation was also induced by other *F. tularensis* SDS-PAGE fractions, however. Actually, none of the fractions were totally nonstimulative. A regular distribution among the antigen determinants to the different molecular mass ranges is also found in *M. tuberculosis* (6), *M. leprae* (1, 7), and *Mycobacterium bovis* (1); in those species, several SDS-PAGE-fractionated bacterial antigens bound to a nitrocellulose filter stimulate immune T lymphocytes to respond. As with *F. tularensis*, no immunodominantly stimulating antigen was found.

It cannot be determined by studying PBMC responses whether the stimulating antigen determinants in each fraction are different or shared. The reactivity of each *F. tularensis*-specific TLC to one or more of the SDS-PAGE fractions confirmed the diversity of the stimulating antigen determinants. Most of the clones recognized a single antigen

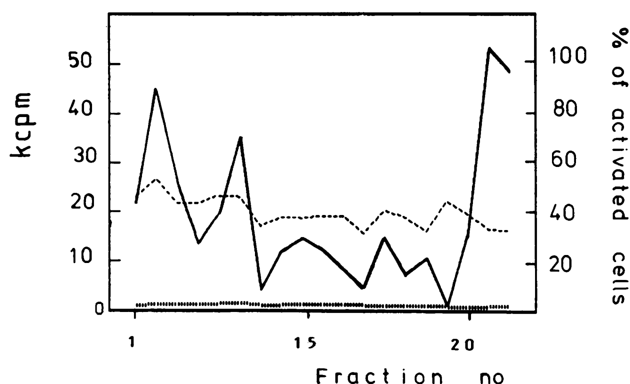

FIG. 5. Activation of immune lymphocytes induced by SDS-PAGE-fractionated *F. tularensis* proteins analyzed in terms of proliferative response, in thousands of counts per minute (kcpm) (——), and percentage of lymphocytes expressing HLA-DR antigen at the surfaces of CD4-positive (– – –) or CD8-positive (·····) lymphocytes.

fraction, and in 18 of the 36 cases this fraction was in the low-molecular-mass range (molecular mass less than 25 kDa). Thirteen of the thirty-six TLCs responded to several antigen fractions, suggesting distribution of the same antigen epitope among different SDS-PAGE fractions.

It is significant, however, that irrespective of the strong reactivity of PBMC to all the *F. tularensis* SDS-PAGE fractions, the TLCs obtained by stimulating PBMC immune to tularemia with the whole-cell antigen did not recognize the antigen in all the fractions. The inability of TLCs to respond to the same fractions could be caused by suppressing antigen determinants. Assuming that such determinants induce HLA-DR antigen expression at the cell surface of CD8-positive cells, the surface phenotype of activated PBMC was characterized with monoclonal antibodies after stimulation with *F. tularensis* SDS-PAGE fractions. The activated lymphocytes were mostly $CD4^+$ T-helper cells, and the number of activated T-suppressor, or cytotoxic, cells against any SDS-PAGE fraction was not greatly increased. Thus, the result is in accordance with our recent analysis of *F. tularensis*-specific TLCs. All the TLCs were $CD4^+$, and it was concluded that they must reflect an important role for $CD4^+$ lymphocytes in tularemia immunity (16).

On the other hand, the observation described above may indicate that the TLCs obtained for continuous growth do not represent all the immune T cells that proliferate against *F. tularensis* whole-cell antigen in PBMC culture. Interactions of functionally different lymphocytes secreting different lymphokines may be important, and experiments in which continuously growing TLCs are used as tools to screen bacterial T-cell target antigens (2, 9–11) may thus reveal only one facet of reactivity.

The protective cell-mediated immunity that develops after vaccination (4) or natural *F. tularensis* infection (18) lasts for decades and resembles mycobacterial immunity in many respects. However, chronic or recurring infections are unusual in tularemia (19). As stated here, *F. tularensis* target antigens are remarkably specific, and cross-reactivity with other gram-negative bacteria seems unlikely. The heterogeneity of the stimulating antigen determinants and, in contrast to the situation with mycobacterial antigens (8, 10, 12), the lack of suppressing determinants are regarded as a possible explanation for the specificity and long-lasting nature of tularemia immunity.

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