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Cell-Mediated and Humoral Immune Responses after Vaccination of Human Volunteers with the Live Vaccine Strain of *Francisella tularensis*

DAVID M. WAAG,[1]* KELLY T. McKEE, JR.,[2] GUNNAR SANDSTROM,[3] LAURA L. K. PRATT,[2] CHRISTOPHER R. BOLT,[1] MARILYN J. ENGLAND,[1] GENE O. NELSON,[4] AND JIM C. WILLIAMS[5]

Pathogenesis and Immunology Branch, Bacteriology Division,[1] Medical Division,[2] and Biometrics and Information Management Division,[4] U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702; Center for Biologics Evaluation and Research, Office of Vaccines Research and Review, Division of Vaccines and Related Product Application, Food and Drug Administration, Bethesda, Maryland 20892[5]; and Department of Microbiology, National Defence Research Establishment, S-901 82 Umea, and Department of Infectious Diseases, University of Umea, S-901 85 Umea, Sweden[3]

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The specific humoral and cell-mediated immune responses of human volunteers vaccinated with the *Francisella tularensis* live vaccine strain (LVS) were evaluated. In the search for an optimal antigen to measure the immunogenicity of the vaccine in an enzyme-linked immunosorbent assay, we tested irradiation-killed LVS, an aqueous ether extract of the LVS (EEx), lipopolysaccharide (LPS) from LVS, and a virulent strain (SCHU4). Volunteers were immunized with LVS by scarification. Immunoglobulin G (IgG) responses to LVS and LPS gave the highest background titers when tested with sera from unimmunized volunteers, whereas IgA, IgG, and IgM background titers to EEx and SCHU4 were low. Vaccination caused a significant rise ($P < 0.01$) in IgA, IgG, and IgM titers to all antigens tested, except for the IgG response to LPS. Eighty percent of vaccinated volunteers developed a positive IgG response to EEx 14 days postvaccination, while 50% were positive to LVS. By day 14 after vaccination, 70% of immunized volunteers exhibited a positive response to EEx in an in vitro peripheral blood lymphocyte proliferation assay. EEx, a specific and sensitive antigen for evaluating immune responses of vaccinated volunteers, may be a superior antigen for the diagnosis of tularemia.

Tularemia is a zoonotic disease which occurs in the Northern Hemisphere (7). Humans acquire the disease by directly contacting infected animals, by inhaling contaminated aerosols, by being bitten by insects, or by ingesting contaminated food or water (3). The clinical diagnosis of tularemia is aided by isolation of the microorganism, a procedure that involves the risk of laboratory exposure, and/or measurement of the presence of serum antibody. Immunity to reinfection by wild-type strains of *Francisella tularensis* seems to be due to a cell-mediated immune response (10). A concomitant increase in antibody to the microorganism provides a useful adjunct to patient history and symptomology to aid clinicians in the diagnosis of tularemia (8) and to evaluate the immunogenicities of tularemia vaccines. The heterogeneity in the immunogenic potentials of colony variants exhibited by different lots of the *F. tularensis* live vaccine strain (LVS) can result in a loss of the ability of the LVS to induce protection in animals (6). Because of this variability, all lots of LVS vaccine must be evaluated for immunogenicity in humans.

Several different antigen preparations have been used to assess the immune response to *F. tularensis* of persons sensitized by natural infection or vaccination, including a bacterial sonicate (9, 18, 22), lipopolysaccharide (LPS) (4, 22), outer membrane antigen (2), and LVS whole cells (8, 16). These antigens are used most frequently in the agglutination test and enzyme-linked immunosorbent assay (ELISA). Not all antigen preparations bind antibodies with high specificity. Antigens that bind antibodies in sera from nonimmune individuals can produce false-positive results in diagnostic assays. In order to overcome a lack of antigen specificity, the minimum positive criterion that defines a positive test can be arbitrarily raised, but this generally results in a decrease in test sensitivity. By developing test antigens that are highly specific and have low background levels of reactivity, the sensitivity of the assay is increased, leading to an improved ability to measure humoral immune responses after immunization or disease.

Antigens used to evaluate humoral immunity to *F. tularensis* vary in terms of specificity. Published reports have shown that the minimum criterion for a titer or absorbance to be considered positive is frequently higher for the immunoglobulin G (IgG) response than for the IgM or IgA response to *F. tularensis* antigen (9, 18). High-level nonspecific reactions with nonimmune sera at dilutions of 1:100 have also been reported (4). A previous study showed that vaccination with LVS induced significant rises in IgA, IgG, and IgM ELISA antibody responses ($P < 0.05$) and in vitro cell-mediated immune responses ($P < 0.01$) to irradiation-killed LVS (23). However, prevaccination and control (placebo) sera also exhibited high nonspecific IgG titers to the LVS antigen.

In this study, we compared cellular and subcellular *F. tularensis* preparations with irradiation-killed LVS antigen to evaluate humoral and cell-mediated immune responses. Sera and peripheral blood lymphocytes from human volunteers immunized with *F. tularensis* LVS were tested.

MATERIALS AND METHODS

Vaccine. *F. tularensis* LVS (TSI-GSD 213, lot 1R) was produced at The Salk Institute, Swiftwater, Pa. The vaccine was distributed lyophilized, and each vial contained approximately 7.0×10^8 viable bacteria per ml of reconstituted vaccine. Sodium chloride (0.9%) was the placebo.

Vaccinees. Volunteers were recruited from a resident population of U.S. Army personnel stationed at the United States Army Medical Research Institute of

* Corresponding author. Phone: (301) 619-7453. Fax: (301) 619-4898.

Infectious Diseases, Fort Detrick, Frederick, Md. Both men and women participated in the study, which was conducted to assess the immunogenicity and safety of a new lot of *F. tularensis* LVS. Volunteers were between 18 and 25 years old, were in good health, and had no history of tularemia. Individuals were examined by a physician before enrolling in the study. Each volunteer gave written informed consent before participating. Before vaccination, individual baseline ELISA antibody titers and lymphocyte proliferative responses to *F. tularensis* antigens were determined. Volunteers were randomly assigned to vaccine and control groups before receiving a single dose of vaccine (7.0×10^7 bacteria per 0.1 ml) or placebo (saline) by scarification in a coded fashion so that neither vaccinees nor investigators knew the compositions of the immunizations. Vaccine or placebo was applied to the ventral forearm and pressed into the dermis 15 times with a bifurcated needle. Ten volunteers received vaccine, and 12 volunteers received placebo. Volunteers were sequestered on a clinical research ward for 7 days for observation. The code for the identities of the vaccinees and controls was not broken until all the data were analyzed. Volunteers were bled by venipuncture before vaccination (day 0) and 7, 14, 21, 28, and 63 days after vaccination.

Antigen preparations. Antigens used in the ELISA were LVS, LPS, SCHU4, and EEx (see below).

The attenuated *F. tularensis* LVS strain, provided by G. Sandstrom, was grown to log phase on modified Thayer-Martin (11) plates at 37°C in a 5% CO_2 atmosphere. Sterile saline was added to the plates, and the colonies were scraped off with a rubber policeman into a 50-ml centrifuge tube. The cell suspension was washed twice with sterile saline, killed with 2.1×10^6 rads of gamma irradiation (cobalt 60 source; GammaCell 40; Atomic Energy of Canada, Ltd., Kanata, Ontario, Canada) while frozen on dry ice, and then lyophilized.

F. tularensis LPS was prepared as previously described (12). Briefly, irradiated *F. tularensis* LVS whole cells were suspended at 10 mg (dry weight) per ml in water. An equal amount of 90% phenol was added, and the suspension was heated to 68°C for 15 min. The suspension was centrifuged at $10{,}000 \times g$ for 30 min, and the aqueous phase was collected. An equal amount of water was added to the phenol phase, and the cells were extracted twice. The preparation (phenol-water extract [PWE]) was dialyzed and lyophilized. For the removal of DNA and RNA, 50 mg of the PWE was rehydrated in 10 ml of Tris buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, and 0.2% NaN_3)–0.2 mg of DNase (Sigma Chemical Company, St. Louis, Mo.) per ml–0.35 mg of RNase (Sigma) per ml and incubated at 37°C overnight. The suspension was then treated with proteinase K (Sigma) at a concentration of 1 mg/ml and incubated overnight at 45°C. Approximately 10% (dry weight) of LVS was recovered as LPS. PWE was used for polyacrylamide gel electrophoresis and immunoblotting.

The virulent SCHU4 *F. tularensis* strain, obtained from the Rocky Mountain Laboratories, Hamilton, Mont., was grown on modified Thayer-Martin agar at 37°C in a 5% CO_2 atmosphere. Colonies were harvested in mid-log phase (2 days of incubation), and the cells were washed twice with sterile saline and suspended in 10 ml of saline containing 3% (final concentration) formalin for 18 h at 4°C to kill the microorganisms. The preparation was dialyzed (molecular mass cutoff, 3.5 kDa) against sterile water and lyophilized.

An aqueous ether extract of gamma irradiation-killed LVS (EEx) was prepared by suspending 100 mg of lyophilized LVS in 25 ml of phosphate-buffered saline (PBS) (pH 7.4) in a separatory funnel. Twenty-five milliliters of ether was added, the preparation was shaken, and the two phases were allowed to separate overnight at room temperature. The aqueous phase (fraction A) was collected, an additional 25 ml of PBS was added to the funnel, and the two phases were again allowed to separate overnight at room temperature. The aqueous phase, with the interface, was collected and pooled with fraction A. The aqueous phase was centrifuged at $12{,}000 \times g$ for 30 min. The supernatant was decanted and dialyzed (molecular mass cutoff, 3.5 kDa) against water for 3 days at 4°C with daily changes of water. The EEx was lyophilized before use. Approximately 40% (dry weight) of LVS was recovered as EEx.

All preparations used in the ELISA were suspended in sterile deionized water at a concentration of 1 mg (dry weight) per ml.

Antigens used in the lymphocyte proliferation assay (LPA) were gamma-irradiated LVS, the rough strain of LVS (LVSR) (14), the residues after extraction of LVS and LVSR with chloroform-methanol (LVS-CMR and LVSR-CMR, respectively) (24), and EEx. All preparations used as recall antigens were prepared as stock suspensions of 55 μg/ml in RPMI 1640 cell culture medium.

Polyacrylamide gel electrophoresis and immunoblotting. The *F. tularensis* LVS, PWE, and EEx were compared by polyacrylamide gel electrophoresis and immunoblotting (23). Briefly, LVS, PWE, or EEx was suspended in Laemmli sample buffer at a concentration of 1 mg (dry weight) per ml and boiled for 5 min. Antigen (20 μg per 1-cm slot) was loaded onto a polyacrylamide gel (5% stacking gel and 12.5% separating gel), and bacterial components were separated electrophoretically. Banding patterns were visualized after silver staining (21).

Bacterial components in a sister gel were electrophoretically transferred to nitrocellulose at a 500-mA constant current for 16 h in Tris (25 mM)–Tricine (192 mM) buffer at pH 10.4 (by an adaptation of the method of Szewczyk and Kozloff [19]). The nitrocellulose was then blocked with 3% bovine serum albumin (immunoglobulin free) and cut into strips. Murine monoclonal anti-*F. tularensis* LPS (Fran4) ascites fluid, provided by C. A. Nacy (12), was diluted 1:500, incubated with the nitrocellulose strips containing *F. tularensis* antigens for 1 h, and washed. ^{125}I-protein A was added to individual nitrocellulose strips and incubated for 1 h. The nitrocellulose strips were washed six times in Tris-buffered saline (containing 0.02 M EDTA, 0.25% bovine serum albumin, and 0.05% Nonidet P-40) at pH 7.4 and dried, and bands that bound specific antibody were visualized after exposure to X-ray film (Kodak X-Omatic).

Humoral immunity. The appearance of antigen-specific antibodies after LVS vaccination was evaluated by ELISA. Levels of class-specific (IgA, IgG, and IgM) serum antibody to irradiation-killed LVS cells, LVS LPS, formalin-killed strain SCHU4, and EEx were determined by an ELISA similar to that described previously (23). Antigen preparations (1 mg/ml) were diluted 1:40 in sodium carbonate-bicarbonate buffer (pH 9.6), and 0.05 ml of the mixture was added into appropriate wells of 96-well Immunolon II microtiter plates (Flow Laboratories, McLean, Va.). The plates were dried overnight in a 37°C dry incubator. Wells of the entire plate were blocked with carbonate-bicarbonate buffer containing 0.25% gelatin (60 bloom) for 1 h at 37°C. Plates were then washed five times with PBS-Tween buffer at pH 7.4. Sera were diluted twofold from 1:16 to 1:32,768 in microtiter wells, and the plates were incubated in a humidified incubator at 37°C for 1 h. Microtiter plates were again washed five times with PBS-Tween buffer. Alkaline phosphatase–anti-immunoglobulin (anti-alpha chain, anti-gamma chain, or anti-mu chain [Kirkegaard and Perry, Gaithersburg, Md.]) conjugate in PBS-Tween–0.25% gelatin was added to appropriate wells, and the plates were incubated at 37°C for 1 h in a humidified incubator. After the plates were washed, enzyme substrate in diethanolamine buffer (pH 9.8) was added and the plates were incubated for an additional hour at 37°C. The conjugates were prepared by the manufacturer to be specific for a single antibody class. They also were titrated against 10 ng of homologous human mu, gamma, or alpha heavy chain per well. The highest dilution of conjugate giving an optical density reading of 1.0 (at 405 nm) after incubation for 1 h at 37°C was used as the working concentration. Optical densities of all wells were determined spectroscopically at 405 nm with a microplate reader. Assigned titers represent the highest dilution of serum giving a minimum difference of 0.05 optical density unit when the test and control wells were compared. For the purposes of statistical analysis, sera having an endpoint at a titer of <16 were assigned a titer of 8.

Cell-mediated immunity. The LPA was previously described (23). Briefly, peripheral blood cells from 40 ml of human blood were separated into two 50-ml conical centrifuge tubes. Each tube received 20 ml of Hanks' balanced salt solution. Each cell suspension was layered on 15 ml of Histopaque (Sigma) and centrifuged at $500 \times g$ for 30 min. Leukocytes were collected from the central band, washed twice with Hanks' balanced salt solution containing 0.2% bovine serum albumin (immunoglobulin free), and suspended in RPMI 1640 medium containing 5% autologous or pooled human type AB serum (M.A. Bioproducts, Walkersville, Md.). Viable cells (360,000) in a volume of 0.180 ml were seeded into individual wells of a 96-well cell culture plate. LVS, LVSR, LVS-CMR, LVSR-CMR, or EEx was added to appropriate wells to give final concentrations of 5.5 μg/ml. Ninety-six hours after initiation of the culture, 1 μCi of [^{3}H]thymidine (specific activity, 5 Ci/mmol) was added per well. Radiolabeled cells were collected on glass fiber filters 20 h later. Radiolabel uptake was determined by standard scintillation techniques. Stimulation indices, representing counts per minute in antigen-stimulated cultures divided by counts per minute in unstimulated control wells, were calculated.

Sensitivity. The sensitivity of a diagnostic assay is the power to identify correctly persons who have had the disease (or who have developed an immune response to the etiologic agent or related antigens). The percentage of vaccinated volunteers having positive ELISA titers and LPA stimulation indices was determined from sera drawn and LPAs performed 7, 14, 21, 28, and 63 days after LVS vaccination.

Specificity. The specificity of a diagnostic assay is the ability to identify correctly persons who do not have the disease (or who have not developed an immune response to the etiologic agent or related antigens). We assumed that the 22 volunteers had never encountered tularemia antigens before vaccination. Therefore, we selected prevaccination ELISA titers and LPA stimulation indices to determine minimum positive ELISA titers and selected LPA stimulation indices to yield 5% or fewer false-positive volunteers, resulting in a test specificity of ≥95% (0.95 = number testing negative/total number tested).

Statistical analysis. Significant differences between vaccine and placebo groups were calculated by Student's *t* test.

RESULTS

Humoral antibody response. (i) mean ELISA titers. The geometric mean antibody titers to *F. tularensis* serodiagnostic antigens of volunteers vaccinated with LVS or a placebo are shown in Table 1. In vaccinees, the geometric mean IgA, IgG, and IgM titers to LPS, LVS, SCHU4, and EEx rose throughout the course of the study, whereas the mean antibody responses of the placebo group remained relatively constant. When the ELISA antigens were evaluated for nonspecific reactivity to nonimmune sera, various amounts of antigen binding were noted. Nonspecific reactivity was lowest when IgA antibodies were tested and highest when IgG antibodies were examined.

TABLE 1. IgA, IgG, and IgM antibody titers to *F. tularensis* ELISA antigens of 22 volunteers vaccinated with a placebo or LVS[a]

Ab/Ag and group	Ab titer (geometric mean ± geometric SD) on day after vaccination:					
	0	7	14	21	28	63
IgA/LPS						
P	12 ± 1	9 ± 1	10 ± 1	9 ± 1	10 ± 2	12 ± 2
V	10 ± 2	10 ± 1	23 ± 2	42 ± 2	111 ± 2	223 ± 3
IgG/LPS						
P	362 ± 7	287 ± 6	362 ± 6	342 ± 6	323 ± 7	362 ± 7
V	1,552 ± 6	1,448 ± 6	1,911 ± 5	1,911 ± 4	2,353 ± 4	3,327 ± 3
IgM/LPS						
P	15 ± 2	15 ± 2	15 ± 2	17 ± 2	19 ± 2	21 ± 2
V	16 ± 2	18 ± 2	32 ± 2	60 ± 3	256 ± 2	446 ± 2
IgA/LVS						
P	9 ± 1	9 ± 1	9 ± 1	10 ± 1	9 ± 1	9 ± 1
V	8 ± 1	9 ± 1	18 ± 2	79 ± 2	181 ± 3	724 ± 3
IgG/LVS						
P	85 ± 3	85 ± 2	96 ± 2	91 ± 3	76 ± 3	85 ± 3
V	111 ± 2	137 ± 2	194 ± 2	416 ± 2	832 ± 2	2,353 ± 3
IgM/LVS						
P	13 ± 2	14 ± 2	14 ± 2	14 ± 2	15 ± 2	17 ± 2
V	17 ± 2	17 ± 2	26 ± 2	74 ± 3	239 ± 3	446 ± 2
IgA/SCHU4						
P	9 ± 1	9 ± 1	9 ± 1	9 ± 1	9 ± 1	9 ± 1
V	9 ± 1	8 ± 1	21 ± 2	97 ± 3	239 ± 3	1,176 ± 3
IgG/SCHU4						
P	21 ± 3	16 ± 2	15 ± 2	14 ± 2	14 ± 2	15 ± 2
V	37 ± 3	37 ± 3	158 ± 2	446 ± 2	955 ± 2	3,566 ± 3
IgM/SCHU4						
P	8 ± 1	9 ± 1	9 ± 1	9 ± 1	9 ± 1	9 ± 1
V	8 ± 1	8 ± 1	14 ± 2	69 ± 3	223 ± 3	512 ± 3
IgA/EEx						
P	9 ± 1	9 ± 1	8 ± 1	9 ± 1	9 ± 1	8 ± 1
V	9 ± 1	9 ± 1	23 ± 2	85 ± 3	239 ± 3	549 ± 3
IgG/EEx						
P	12 ± 2	20 ± 3	13 ± 2	24 ± 2	14 ± 2	23 ± 2
V	15 ± 2	23 ± 2	91 ± 2	362 ± 2	588 ± 2	2,195 ± 3
IgM/EEx						
P	10 ± 1	14 ± 2	10 ± 2	14 ± 2	13 ± 2	14 ± 2
V	10 ± 2	14 ± 2	23 ± 2	119 ± 3	158 ± 3	588 ± 3

[a] Ab, antibody; Ag, antigen; P, placebo; V, live LVS vaccine (TSI-GSD 213). The IgA, IgG, and IgM antibody titers to the *F. tularensis* LPS, gamma-irradiated LVS, formalin-inactivated SCHU4, and EEx antigens were determined by ELISA. Sera were collected from 22 volunteers before (day 0) and 7, 14, 21, 28, and 63 days after administration of placebo or live LVS vaccine and were titrated at dilutions from 1:16 through 1:32,768. Titers determined to be <16 were assigned a titer of 8.

The nonspecific binding of IgG to LPS and LVS was the highest. In nonimmune sera, the mean IgA and IgM titers to LPS, LVS, SCHU4, and EEx and the mean IgG titer to EEx were ≤16. We did not see any early rise in the IgM response to any antigen tested.

(ii) Specificity and sensitivity of the ELISA. Antibody titers were measured by using sera drawn before vaccination from persons with no history of exposure to *F. tularensis* antigens. Serological titers that were exceeded by no more than 5% of prevaccination sera were selected as minimum positive titers. Those titers ranged from 16 (IgM with SCHU4) to 16,384 (IgG with LPS) (Table 2).

The sera collected after vaccination were used to evaluate the sensitivities of the test antigens (Table 3). At 14 and 21 days after vaccination, the IgG response to EEx was the most sensitive. Eighty percent of vaccinees had a positive IgG anti-EEx titer 14 days after vaccination, and by 21 days, 100% of vaccinated volunteers had ELISA titers meeting or exceeding the minimum positive titer. SCHU4 and LVS each exhibited maximum antibody sensitivities of 50 and 90% on days 14 and 21, respectively.

(iii) Humoral antibody response: significant differences between vaccine and placebo groups. By day 14 after immunization, IgA responses to all tested antigens and IgG responses to all antigens except LPS and LVS increased significantly ($P < 0.01$) compared with control responses (Table 4). The IgM responses to LPS, LVS, and EEx by day 14 also increased significantly ($P < 0.05$). The IgA, IgG, and IgM responses to all tested antigens (except for the IgG response to LPS) increased significantly ($P < 0.01$) compared with the control responses by 28 days after vaccination.

(iv) Polyacrylamide gel electrophoresis and immunoblotting of LVS, LPS, and EEx. To gain information on primary constituents, EEx was separated by polyacrylamide electrophore-

TABLE 2. Minimum positive ELISA titers[a]

Antibody class	ELISA titer[b] with antigen:			
	LPS	LVS	SCHU4	EEx
IgA	32	32	32	32
IgG	16,384	512	256	64
IgM	128	128	16	32

[a] Antibody class-specific (IgA, IgG, and IgM) responses to *F. tularensis* LPS, LVS, SCHU4 strain, and EEx antigens were measured.

[b] ELISA titers giving a specificity of ≥95% as determined by empirical analysis of prevaccination sera.

TABLE 3. Percentages of volunteers responding with a positive ELISA titer after vaccination[a]

Day after vaccination and antibody class	% with positive titer[b] against ELISA antigen:			
	LPS	LVS	SCHU4	EEx
7				
IgA	0	0	0	0
IgG	10	0	0	10
IgM	0	0	0	10
14				
IgA	50	50	50	50
IgG	10	10	50	80
IgM	0	0	50	60
21				
IgA	80	90	80	80
IgG	10	60	80	100
IgM	30	40	90	100
28				
IgA	100	90	100	100
IgG	10	90	100	100
IgM	80	90	100	90
63				
IgA	100	100	100	100
IgG	10	90	100	100
IgM	100	90	100	100

[a] Sera from 10 volunteers vaccinated with live *F. tularensis* LVS (TSI-GSD 213) were tested 7, 14, 21, 28, and 63 days after vaccination; antibody class-specific (IgA, IgG, and IgM) responses to *F. tularensis* LPS, LVS, SCHU4 strain, and EEx antigens were measured.

[b] Percentage of vaccinated volunteers with minimum positive titers.

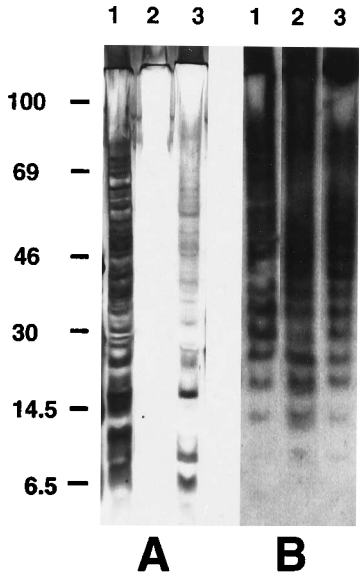

FIG. 1. Silver stain (A) and immunoblot (B) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated *F. tularensis* LVS (lanes 1), *F. tularensis* PWE (lanes 2), and EEx (lanes 3). The blot was probed with a murine monoclonal antibody (Fran4) (1:500) against *F. tularensis* LPS. Bound antibodies were detected with ^{125}I-protein A. Molecular mass markers in kilodaltons are shown on the left.

sis and silver stained, and the resulting profile was compared with gel profiles of LVS and *F. tularensis* PWE (Fig. 1A). The EEx contained bands with molecular masses similar to those observed with LVS, but the numbers of bands were reduced. *F. tularensis* PWE did not stain with silver stain.

When electrophoretically separated constituents of LVS, PWE, and EEx were transferred to nitrocellulose and probed with an anti-*F. tularensis* LPS monoclonal antibody, all lanes were stained (Fig. 1B).

Cell-mediated response. (i) mean lymphocyte responses to antigens. Within 7 days of LVS vaccination, the differences in responses of vaccinees and controls to EEx were significant ($P < 0.01$) (Table 5). By day 14 after vaccination and throughout the remainder of the study, differences in responses of vaccinees and the placebo group to all recall antigens were significant ($P < 0.01$ or $P < 0.05$).

(ii) Specificity and sensitivity of the LPA. Minimum positive LPA stimulation indices that were exceeded by ≤5% of tested prevaccination specimens were chosen (i.e., a specificity of ≥95%). Those stimulation indices were 32, 42, 31, 27, and 12 for LVS, LVSR, LVS-CMR, LVSR-CMR, and EEx, respectively. Only the sensitivity of EEx exceeded 30% (Table 6). For EEx, the responses of 40% of vaccinated volunteers met or exceeded the minimum positive response to EEx 7 days after vaccination, while 80% of vaccinees were positive 63 days after vaccination.

TABLE 4. Student's *t* test comparison of antibody responses to *F. tularensis* antigens in volunteers vaccinated with live LVS or a placebo[a]

Ab/Ag	Prob > |*t*|[b] on day after vaccination:					
	0	7	14	21	28	63
IgA/LPS	0.5755	0.4914	**0.0128**	**0.0063**	**0.0109**	**0.0067**
IgG/LPS	0.2581	0.2281	0.2063	0.2357	0.2705	0.1043
IgM/LPS	0.7646	0.5345	0.0208	0.1034	**0.0009**	**0.0099**
IgA/LVS	0.1926	0.8985	**0.0057**	**0.0025**	**0.0020**	**0.0011**
IgG/LVS	0.7456	0.2066	0.0456	**0.0065**	**0.0029**	**0.0019**
IgM/LVS	0.3059	0.3208	0.0529	0.0409	**0.0066**	**0.0080**
IgA/SCHU4	0.8985	0.3714	**0.0009**	0.0207	**0.0008**	**0.0033**
IgG/SCHU4	0.3511	0.0488	**0.0002**	**0.0089**	**0.0008**	**0.0006**
IgM/SCHU4	NA[c]	0.3741	0.1176	0.0400	**0.0060**	**0.0081**
IgA/EEx	0.8995	0.8985	**0.0113**	0.0183	**0.0057**	**0.0049**
IgG/EEx	0.7938	0.9231	**0.0017**	**0.0042**	**0.0055**	**0.0024**
IgM/EEx	0.8463	0.9650	0.0156	0.0690	**0.0041**	**0.0043**

[a] Sera were collected from 10 immunized volunteers and 12 placebo controls before (day 0) and 7, 14, 21, 28, and 63 days after vaccination. The IgA, IgG, and IgM antibody (Ab) titers to the *F. tularensis* LPS, gamma-irradiated LVS, formalin-inactivated SCHU4, and EEx antigens (Ag) were determined by ELISA.

[b] Prob > |*t*|, probability that the differences between the vaccine and placebo groups were not statistically significant. Boldface indicates $P \leq 0.01$; underlining indicates $0.01 < P \leq 0.05$.

[c] NA, no estimate of variability was possible; titers were 8 for all volunteers.

TABLE 5. In vitro lymphoproliferative responses of volunteers vaccinated with placebo or *F. tularensis* LVS[a]

Group	Day	SI (mean ± SD)[b] with recall antigen:				
		LVS	LVSR	LVS-CMR	LVSR-CMR	EEx
P	0	14.1 ± 9.0	16.5 ± 9.3	14.7 ± 8.8	8.4 ± 7.0	4.5 ± 3.1
V	0	10.3 ± 8.1	12.8 ± 12.0	10.0 ± 7.7	11.7 ± 7.4	4.2 ± 4.1
P	7	10.6 ± 2.8	10.2 ± 3.3	8.4 ± 2.4	6.5 ± 2.0	3.8 ± 1.9
V	7	19.2 ± 12.6	19.2 ± 11.2#	14.4 ± 8.3	12.8 ± 8.0#	9.6 ± 7.0*
P	14	7.3 ± 3.7	7.8 ± 4.5	5.7 ± 2.8	4.0 ± 1.5	2.1 ± 1.6
V	14	23.2 ± 10.5*	21.2 ± 10.1*	17.1 ± 9.8*	17.7 ± 9.4#	16.6 ± 10.2*
P	21	12.2 ± 5.5	11.4 ± 4.4	9.2 ± 3.5	7.7 ± 3.2	2.9 ± 1.0
V	21	23.1 ± 12.3#	21.0 ± 10.3#	17.7 ± 9.3#	19.7 ± 9.4#	16.6 ± 9.8*
P	28	9.2 ± 3.2	9.5 ± 3.5	7.6 ± 2.2	6.6 ± 2.4	2.0 ± 1.2
V	28	21.8 ± 12.7#	21.6 ± 13.9#	18.9 ± 9.4*	20.1 ± 10.6*	16.6 ± 8.0*
P	63	9.5 ± 4.0	10.4 ± 4.3	7.6 ± 2.7	7.2 ± 4.0	3.1 ± 1.3
V	63	24.2 ± 17.5#	24.6 ± 17.7#	22.5 ± 18.6	25.2 ± 20.4#	23.0 ± 18.3*

[a] Peripheral blood cells were obtained from volunteers before vaccination (day 0) or 7, 14, 21, 28, or 63 days after vaccination with placebo (P) or *F. tularensis* live LVS (TSI-GSD 213) vaccine (V). Peripheral blood cells were cultured in the presence of 5% pooled human type AB serum and irradiation-killed *F. tularensis* LVS, LVSR, LVS-CMR, or LVSR-CMR, or EEx (5.5 μg/ml).

[b] SI, stimulation index; standard deviations are unbiased. #, significant differences between the placebo and vaccine groups at the $P < 0.05$ level (by Student's *t* test); *, significant differences between the two groups at the $P < 0.01$ level.

DISCUSSION

We used two methods to evaluate specific antigens to measure the immune response to *F. tularensis* after vaccination. First, we empirically determined the diagnostic sensitivities of the antigens by using sera and peripheral blood cells from volunteers vaccinated with LVS after setting the minimum acceptable specificity at 95% by using sera and peripheral blood cells obtained before vaccination. Also, we attempted to measure a statistically significant immune response to these preparations by using sera and peripheral blood cells from vaccinated volunteers.

While the kinetics, antibody specificity, and humoral phenotypes of the immune response following natural tularemia infection are likely different from those observed in individuals vaccinated with LVS, we compared the specificity and sensitivity of our test with those of tests used by others in the diagnosis of tularemia. If our antigens exhibit a similar level of specificity and sensitivity, we will then test our antigens with sera from clinically confirmed cases of tularemia and suitable control sera.

We identified two antigen preparations, LVS and EEx, both of which are easily prepared in the laboratory, which exhibited low levels of reactivity when tested with nonimmune sera or peripheral blood cells. However, when tested with immune sera or immune peripheral blood cells, these antigens generated statistically significant responses ($P < 0.05$) as early as 7 to 14 days after LVS vaccination. In addition, 80% of volunteers responded with a positive IgG response to EEx 14 days after vaccination. This is the earliest reported rise in antibody titers after natural infection or vaccination with LVS. Pioneering studies by Francis and Evans (5) and Ransmeier and Ewing (13) determined that agglutinating antibodies appear during the second week of illness and are detectable more than 10 years later. Since the time of those studies, diagnostic rises in antibody titer earlier than 2 weeks after disease onset, or approximately 3 weeks after infection, have not been noted (4, 9, 22). A competition ELISA with outer membrane *F. tularensis* antigens was used to test paired serum specimens from 23 tularemia patients matched with 25 sera from patients with infectious diseases other than tularemia (2). With a dilution of 1:64, this test had a sensitivity of 95.7% and a specificity of 96%. However, sera were collected from ill patients, probably later than 2 weeks after infection.

Comparisons between alternative serodiagnostic procedures are complicated by differences in assay sensitivities, in methods used to determine antibody titers, and in antigen preparations used in serodiagnostic tests. While some investigators use an *F. tularensis* cellular antigen (8, 16), others use a bacterial sonicate (8, 9, 18, 22) or outer membrane antigens (2). Sources of antigen and/or methods of antigen preparation may influence test results. Some studies have found that a bacterial sonicate and LPS are useful diagnostically and gave identical results in an ELISA (22). In our test, the IgG response to LPS gave unacceptably high background readings and no more than 10% of vaccinated volunteers had a positive IgG response to LPS. Also, antibody responses to an ether-extracted antigen have been evaluated by using sera from volunteers vaccinated with *F. tularensis* LVS (15). The preparation used in that study was very reactive with sera from nonimmune individuals, while our

TABLE 6. Percentages of volunteers responding with a positive diagnostic LPA stimulation index after *F. tularensis* LVS vaccination[a]

Day after vaccination	% with positive response[b] to LPA recall antigen:				
	LVS	LVSR	LVS-CMR	LVSR-CMR	EEx
7	10	0	0	10	40
14	20	0	10	10	70
21	20	10	10	10	60
28	20	10	20	30	70
63	10	10	20	30	80

[a] *F. tularensis* LVS, LVSR, LVS-CMR, LVSR-CMR, and EEx were used as recall antigens (5 μg/ml) in an LPA. Sera were collected from immunized volunteers 7, 14, 21, 28, and 63 days after vaccination.

[b] Percentage of immunized volunteers with responses greater than or equal to minimum positive stimulation indices.

preparation had no background reactivity. Subtle differences in the preparation of this antigen may account for observed differences in specificity.

The presence of long-lasting serum antibodies may make it difficult to distinguish current and past infections by serology. A negative test may be sufficient to rule out a diagnosis of tularemia, but a positive initial test may require another serum sample drawn during disease convalescence to check for a fourfold rise in titer. The use of antigens that are more sensitive in diagnostic testing may decrease the time required to identify that fourfold rise. Another barrier to accurate testing is the possibility of serological cross-reactions. *F. tularensis* antigens may cross-react with *Brucella abortus* antigens (1). We plan to analyze sera from patients with clinically confirmed tularemia and other clinical diagnoses to verify and confirm our findings.

While some researchers have noted a concurrent rise in IgA, IgG, and IgM antibody responses to *F. tularensis* antigens after vaccination or natural infection (2, 8, 9, 18, 22, 23), others have noted an early onset of IgM antibody responses (8). These differences may reflect different diagnostic antigens used, different serological techniques, or differences between natural disease and immunization. We noted a simultaneous rise in IgA, IgG, and IgM antibodies to all antigens we tested.

On the basis of the immunoblot and polyacrylamide gel profiles, EEx and LVS contained *F. tularensis* LPS, but the number of protein constituents in EEx appeared to be markedly reduced. However, specific antigens critical for the evaluation of the immune response after LVS vaccination were not removed by the ether extraction. We do not know why EEx and LVS were more specific diagnostic antigens than LPS in our serological test. Perhaps IgG-binding LPS epitopes in EEx and LVS were masked by other constituents.

Significant ($P < 0.01$) cell-mediated immune responses to the EEx occurred earlier (7 days after vaccination) than those to other antigens tested. However, at that time, only 40% of vaccinated volunteers exhibited positive responses. Yet at all time points the EEx was a more sensitive antigen in the LPA than other preparations evaluated. The sensitivity increased to 70% by day 14 after vaccination. Lymphocyte proliferative responses to LVS outer membranes have been found by other researchers to have diagnostic value 2 weeks after the onset of disease (17, 20) (or approximately 3 weeks after infection). Investigators found that an LPA with *F. tularensis* whole cells had a sensitivity of 96.8% during the second week of illness but a sensitivity of only 21.3% in the first week (17). In another study, only 60% of volunteers responded with a positive LPA result to LVS membranes within 4 weeks of LVS vaccination (20). Because of having a greater sensitivity than other antigen preparations tested, EEx may be useful in aiding the diagnosis of tularemia early in infection, particularly if the initial serological results are inconclusive.

In summary, the most useful combination of antibody class and antigen for the diagnosis of tularemia and evaluation of vaccine immunogenicity should (i) have a low background antigen reactivity with nonimmune sera, (ii) exhibit a rise to significant levels after patient exposure to the native microorganism or *F. tularensis* vaccine, and (iii) be as specific and sensitive as possible. We identified two antigen preparations, gamma-irradiated LVS and EEx, which we easily prepared in the laboratory and which exhibited low levels of reactivity when nonimmune sera or peripheral blood monocytes were tested. While a greater number of participants in the study would allow us to refine the specificity and sensitivity of the assay, our current evaluation suggests that the IgG response to EEx is the superior test for evaluating the immune response to *F. tularensis*. Preparation of the antigen was not complex, background antibody levels in sera from nonimmune individuals were low, and sera from a majority of individuals were positive within 14 days of LVS vaccination. The second most sensitive antigen was SCHU4. However, the special containment required for its growth and purification would probably override any perceived advantages over other preparations. These preliminary results suggest that EEx may be a superior, highly sensitive and specific antigen for the early diagnosis of tularemia by ELISA or LPA.

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