INFECTION AND IMMUNITY, July 1984, p. 101–106
0019-9567/84/070101-06$02.00/0

Vol. 45, No. 1

Antigen from *Francisella tularensis*: Nonidentity Between Determinants Participating in Cell-Mediated and Humoral Reactions

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Received 20 December 1983/Accepted 28 March 1984

After tularemia vaccinations, most individuals respond with cell-mediated and humoral immunity as disclosed by the lymphocyte stimulation test and enzyme-linked immunosorbent assay (ELISA), respectively. There is, however, no correlation between the magnitudes of the two responses, and some individuals show one of the responses only. We now report that the two responses are directed towards different antigenic determinants of the bacterium. Ether-water extraction of the live vaccine strain of *Francisella tularensis* gave a high yield of material reacting in ELISA as well as in the lymphocyte stimulation test. However, the specificity of the extract was low insofar as it reacted not only with lymphocytes and antibodies of tularemia-vaccinated individuals but also to a fairly high extent with those of nonvaccinated individuals. By using the extract as a starting material, a preparatory procedure was developed, resulting in antigen of high specificity in the two tests. The antigen prepared was a high-molecular-weight, carbohydrate-protein complex. Proteinase K treatment of the antigen abolished the lymphocyte-stimulating activity but did not decrease ELISA activity at all. Periodate treatment, on the other hand, greatly reduced ELISA activity but did not decrease the lymphocyte-stimulating activity. Thus, determinants of *F. tularensis* responsible for immunospecific lymphocyte stimulation seem to reside in protein, whereas ELISA activity seems to be due mostly to carbohydrate determinants.

Various strains of *Francisella tularensis* seem to have a similar antigenic composition (6). Therefore, the live vaccine strain (LVS) may be used when preparing an antigen for the assay of immune responses towards wild strains as well. For the assay of humoral immunity, formalinized LVS organisms can be used in the agglutination test (9), and phenol-water extract (7) or sonicate (16) of the organisms can be used in enzyme-linked immunosorbent assay (ELISA). Cell-mediated immunity against *F. tularensis* can be assayed with heat- or Formalin-killed bacteria (29, 16) or bacterial membranes (30) of LVS as the antigen in the lymphocyte stimulation test.

In tularemia-vaccinated individuals, there seems to be no correlation between the magnitude of lymphocyte response and the titer of serum antibodies towards *F. tularensis* (29, 30). The nature of antigenic determinants and the relation between determinants involved in the different immune reactions are unknown.

F. tularensis seems unique among clinically important gram-negative bacteria insofar as more than 50% (wt/vol) of its outer structures are lipids (11). Accordingly, ether-water extraction is known to be an efficient way to release antigens (21, 22, 25, 26). When compared by immunoprecipitation, this extraction procedure is better than several others, including phenol-water extraction (20). Ether-water extracts, however, have not previously been utilized in ELISA or the lymphocyte stimulation test.

The aim of this study was to try to discriminate between determinants of *F. tularensis* which stimulate lymphocytes and those involved in ELISA with cells and serum from tularemia-vaccinated individuals. For this purpose, an antigen of high specificity in the two tests was prepared from an ether-water extract of LVS organisms.

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MATERIALS AND METHODS

Bacteria. *F. tularensis* LVS was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. The bacteria were cultivated on modified Thayer-Martin agar containing Gc-medium base (36 g/liter; Difco Laboratories, Detroit, Mich.), hemoglobin (10 g/liter; Difco) and IsoVitaleX (10 mg/liter; BBL Microbiology Systems, Cockeysville, Md.) at 37°C in 5% CO_2 in air. After 3 days, bacteria were harvested from the surface of the agar and suspended in phosphate-buffered saline at a density of 1.5×10^{11} bacteria per ml.

Analytic procedures. Protein content was determined by using the Folin phenol reaction (19). Bovine serum albumin was used as a standard. Carbohydrate content was measured with a modified Elson-Morgan method (3) and an anthrone method with increased sensitivity (12). Lipid content was measured as fatty acids (11).

Heat and sodium dodecyl sulfate treatment. Twenty micrograms of the prepared antigen in 200 μl of sodium dodecyl sulfate (SDS) buffer (2% SDS, 25% glycerol in 0.025 M Tris-hydrochloride [pH 6.8]) was immersed at 96°C for 15 min and precipitated with 1 ml of cold acetone. After centrifugation, the precipitate was solubilized in 0.05 M carbonate buffer, pH 9.6. As the control, the antigen in phosphate-buffered saline was precipitated with acetone.

Periodate treatment. Two milligrams of the prepared antigen was treated in 2 ml of 0.01 M sodium periodate at 4°C for 8 days (14). Sodium periodate was removed from the mixture with a G-25 chromatography gel (PD-10; Pharmacia AB, Uppsala, Sweden). As the control, sodium periodate was added to an antigen sample immediately before chromatography on G-25 gel.

Proteinase K treatment. One hundred micrograms of the prepared antigen in 1 ml of SDS buffer was heat treated at

96°C for 15 min, precipitated in 5 ml of cold acetone, and centrifuged. The pellet was solubilized in 1 ml of phosphate-buffered saline containing 0.1 mg of proteinase K (Boehringer Mannheim, Scandinavia AB, Bromma, Sweden) and incubated at room temperature for 30 min. One milliliter of saturated phenol (in the same buffer solution) was added to remove residual proteinase K activity. The mixture was incubated for 60 min at room temperature with occasional shaking. After the centrifugation, the water phase was saved, and the rest of the phenol was removed by extraction with ether until the water phase was clear. As the control, the SDS-heat-treated sample was used.

Phenol-water preparation. The water phase from phenol-water-extracted bacteria was obtained as described by Westphal et al. (32).

Hypertonic treatment. *F. tularensis* LVS organisms were treated with hypertonic sodium chloride as described by Hood (11).

Human sera. Blood was obtained from six persons vaccinated 60 days previously with *F. tularensis* LVS according to the instructions given by the manufacturer. Serum was prepared, pooled, and used as pooled immune serum. A pool of nonimmune serum was prepared from the blood of five persons who denied previous tularemia or tularemia vaccination.

Rabbit sera. Rabbits were immunized with the prepared antigen solubilized in saline (100 μg/ml). On day 1, 0.5 ml of a mixture (1:1) of antigen and Freund complete adjuvant (Difco Laboratories) was injected intracutaneously. On days 3, 5, and 7, 2× 0.5 ml of the mixture was injected subcutaneously. On days 14 and 16 and on day 18, 0.5 and 2× 0.5 ml, respectively, of the antigen solution were injected intramuscularly. Finally, intramuscular boosters of 0.5 ml of antigen solution were given weekly for 6 weeks. The rabbits were bled weekly, and sera were prepared and stored at −70°C.

Hybridoma preparation. BALB/c female mice were immunized as described by Polin et al. (24). A modification (13) of the hybridization method described by Köhler and Milstein (15) was followed.

ELISA. ELISA was performed in microplates essentially by the method of Voller et al. (31). Various preparations of the antigen were solubilized in 0.05 M sodium bicarbonate buffer, pH 9.6, and coated on microplates. Serum was diluted in phosphate-buffered saline containing 0.05% Tween 20 (vol/vol). Swine anti-human immunoglobulin G conjugated with alkaline phosphatase (Orion Diagnostics, Helsinki, Finland) was utilized. The reaction volume was 200 μl, and the absorbance at 405 nm was measured. Means of two to four separate determinations were indicated. To assay monoclonal antibodies of hybridoma supernatant, the supernatant was concentrated 10 times by precipitation with half-saturated ammonium sulfate solution and solubilized in phosphate-buffered saline containing 0.05% Tween 20 (vol/vol). Rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase (Dakopatts A/S, Copenhagen, Denmark) was used, and the enzyme reaction was assessed by visual inspection.

Lymphocyte stimulation. Lymphocytes were prepared from heparinized blood by centrifugation on Lymphoprep (4), and three to five repeated cultures were established as previously described (29). Each culture contained 3×10^5 lymphocytes and various amounts of antigen preparations in 200 μl of RPMI HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Flow Laboratories, Svenska AB,

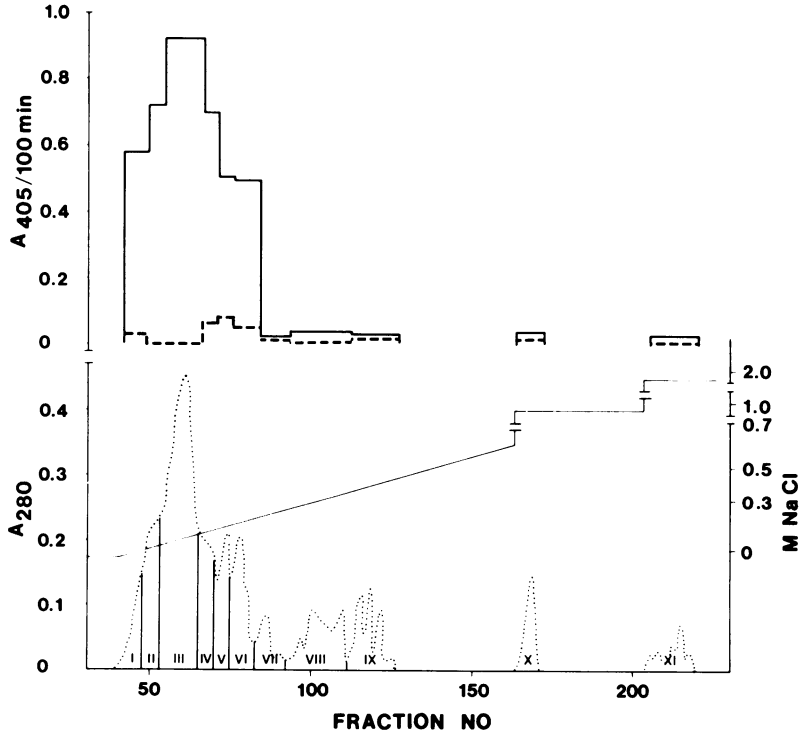


FIG. 1. Diethylaminoethyl-Sephacel chromatography of ammonium sulfate-precipitable material of ether-water extract from *F. tularensis* LVS. The thin solid line (lower part of the figure) shows the concentration of NaCl used for elution, and the dotted line shows the absorbance at 280 nm of the eluted material. Fractions were pooled as indicated (pools I–XI). Each pool was ultrafiltered and dialyzed to give an absorbance at 280 nm of 0.05 in 0.05 M sodium carbonate buffer, pH 9.8, and was coated on microplates. The reactivity of the material of each pool with pooled immune (broad line) and nonimmune (interrupted line) serum in ELISA is shown.

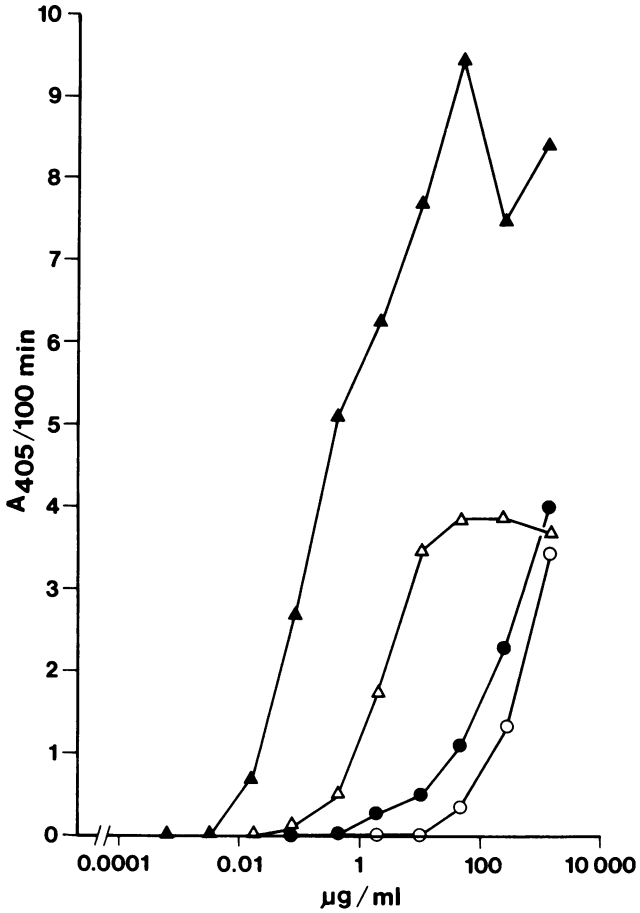


FIG. 2. Reactivity in ELISA of ether-water and phenol-water extracts of *F. tularensis* LVS with human immune and nonimmune serum. Symbols indicate the reaction of various concentrations of ammonium sulfate-precipitable material of the ether-water extract with pooled immune (▲) and nonimmune (△) serum and of the phenol-water extract with pooled immune (●) and nonimmune (○) serum diluted to 10^{-2}.

Stockholm, Sweden) supplemented with 10% pooled nonimmune serum. Antigen preparations were dialyzed against RPMI HEPES before use. After incubation at 37°C for 6 days, the cultures were pulsed with [^{14}C]thymidine and harvested (10).

Antigen preparation. Cells of *F. tularensis* LVS were treated with ether-water (2.5:1), and the water phase was precipitated with saturated ammonium sulfate (2.26 M) at pH 7.2 (21, 22).

Ammonium sulfate-precipitable material was dialyzed against 0.015 M Tris-hydrochloride buffer, pH 8.0, and diluted to an absorbance of 7.5 at 280 nm. Two milliliters were applied to a column (25 by 280 mm) of diethylaminoethyl-Sephacel (Pharmacia AB) equilibrated with this buffer solution. The column was eluted with a linear gradient of NaCl from 0 to 0.6 M in 500 ml and with 1 M and 2 M NaCl. Fractions of 4 ml were collected, and the absorbance at 280 nm was monitored. Fractions were pooled (pools I–XI, Fig. 1), and the pools, adjusted to equal concentrations on a protein content basis, were tested by ELISA for antigen activity against pooled immune serum. Antigen activity was found in pools I–VI, with a maximum in pool III (Fig. 1).

Material from pools II–IV obtained by diethylaminoethyl-Sephacel chromatography was dialyzed against 4 M NaCl in 0.01 M sodium phosphate buffer, pH 7.0, and concentrated by ultrafiltration (PE 809; Millipore Corp., Bedford, Mass.) to an absorbance of 1.5 at 280 nm. Six milliliters were applied to a column (16 by 150 mm) of Phenyl-Sepharose CL-4B (Pharmacia AB) equilibrated with this buffer solution for hydrophobic interaction chromatography. Adsorbed material was eluted by stepwise decreasing NaCl concentration (4, 3, 2, 1 and 0 M), and the absorbance at 280 nm was monitored. High antigen activity was found in the breakthrough volume, whereas low or no activity was found in fractions eluted with lower salt concentrations.

Material not retained by hydrophobic interaction in the Phenyl-Sepharose column was dialyzed against water and lyophilized. Twenty-five milligrams of lyophilized material were solubilized in 1 ml of 0.05 M carbonate buffer, pH 9.6, and applied to a column (16 by 750 mm) of Ultrogel A2 (Pharmacia AB) equilibrated with this buffer solution. The absorbance at 280 nm was monitored. Most of the antigenically active material was found in the void volume. This material of the void volume was referred to as the prepared antigen. The yield of antigenic material was ca. 1 μg per 10^{11} *F. tularensis* LVS cells.

RESULTS

Reactivity in ELISA and the lymphocyte stimulation test of an ether-water extract of *F. tularensis* LVS. Ammonium

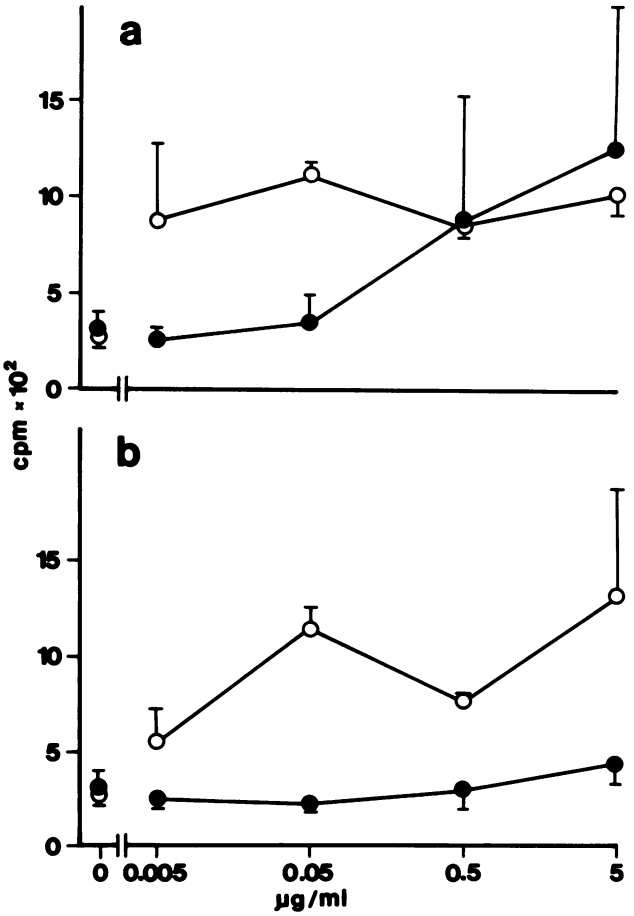
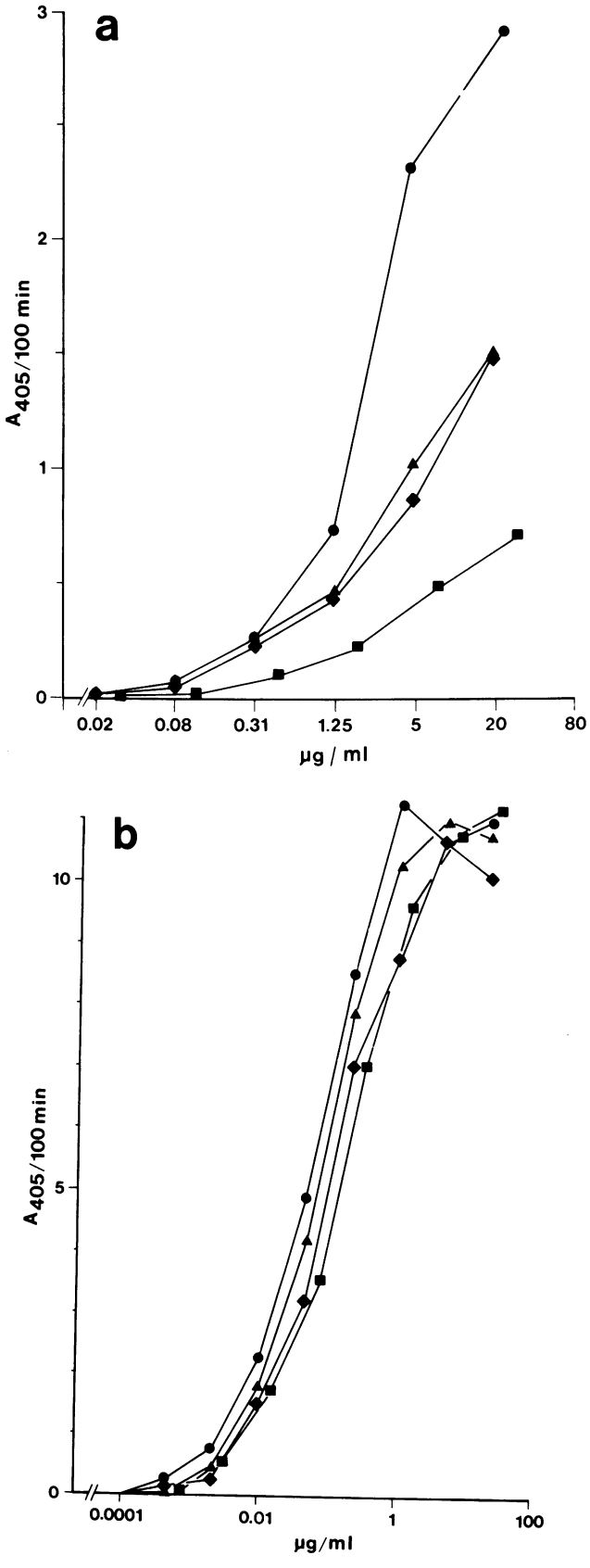


FIG. 3. The response of lymphocytes from tularemia-vaccinated (○) and nonimmunized (●) individuals to ammonium sulfate-precipitable material of the ether-water extract (a) and to prepared antigen (b). Mean counts per minute ± standard deviation represent tests on five nonimmunized and three tularemia-vaccinated individuals.



sulfate-precipitable material of the water phase of the ether-water extract of *F. tularensis* LVS reacted in ELISA with homologous immune serum much more efficiently than did a phenol-water extract (Fig. 2). However, it reacted to a fairly high extent also with nonimmune serum (Fig. 2).

Lymphocytes from tularemia-vaccinated individuals responded to the ammonium sulfate-precipitable material at all doses tested, from 0.005 to 5 μg/ml (Fig. 3a). Lymphocytes from nonimmunized individuals also responded to the material, although only at the highest doses (Fig. 3a).

Increase of immunological specificity by preparation of the ether-water extract of *F. tularensis* LVS. The reactivity of the ammonium sulfate-precipitable material of the ether-water extract with nonimmune serum could be successively reduced by different preparatory steps (Fig. 4a), although the reactivity with immune serum was retained (Fig. 4b). Thus, nonspecifically reacting material was removed.

The nonspecific lymphocyte-stimulating effect of the ammonium sulfate-precipitable material of the ether-water extract was also reduced. Irrespective of antigen dose, the prepared antigen stimulated lymphocytes from immunized individuals only (Fig. 3b).

Nature of antigen prepared. The antigen prepared had a high content of carbohydrate and protein and a low content of lipid (1:1:0.01). It was not retained on Ultrogel A2 chromatography, indicating a molecular weight larger than 23×10^6.

When antiserum raised in rabbits against the prepared antigen was tested at a dilution of 10^{-2} by ELISA towards the prepared antigen (2 μg/ml) and phenol-water-extractable material (2 μg/ml) of whole *F. tularensis* cells, the values obtained were 11.2 ± 0.9 and 0.4 ± 0.1, respectively (mean of four experiments ± standard deviation). The results contradicted the possibility that the antigen prepared was a lipopolysaccharide.

A surface localization of the antigen prepared was indicated by the finding that the reactivity of pooled immune serum with the antigen in ELISA could be removed by adsorption of the serum with *F. tularensis* LVS organisms. This was also supported by the finding that monoclonal antibodies of five different hybridomas raised against the antigen reacted with material released by hypertonic treatment of the LVS organisms (data not shown).

Discrimination of the lymphocyte-stimulating activity of the prepared antigen from its reactivity with humoral antibody. SDS-heat treatment had little effect on any of the two activities of the antigen (data not shown). Sodium periodate treatment reduced the reactivity of the prepared antigen in ELISA with individual or pooled immune serum but did not reduce its lymphocyte-stimulating capacity (Table 1). Proteinase K treatment of the SDS-heat-treated antigen, however, abolished the lymphocyte-stimulating capacity without reducing the reactivity in ELISA (Table 1). Control experiments were performed to exclude the possibility that the lack of lymphocyte response to proteinase K-treated antigen was due to a cytotoxic effect of residual proteinase K. This

FIG. 4. Removal of nonspecifically reacting material by preparation of ether-water extract of *F. tularensis* LVS. Ammonium sulfate-precipitable material of the ether-water extract (●), material of pools II–IV obtained by chromatography on diethylaminoethyl-Sephacel (▲), material eluted at 4 M NaCl by chromatography on Phenyl-Sepharose CL-4B (◆), and material eluted in the void volume by chromatography on Ultrogel A2 (■) were tested in various concentrations by ELISA towards pooled nonimmune (a) and immune (b) serum diluted to 10^{-2}.

TABLE 1. Effect of proteinase K and periodate treatment of the prepared antigen on its reactivity with serum and lymphocytes from tularemia-vaccinated individuals

Expt no.[a]	Assay	Antigen dose (μg/ml)	Proteinase K treatment		Periodate treatment	
			Treated antigen	Control antigen	Treated antigen	Control antigen
1	Lymphocyte stimulation	2.0	0.2[b] ± 0.03	1.8 ± 0.85	0.6 ± 0.16	1.0 ± 0.19
		0.2	0.0 ± 0.00	1.0 ± 0.51	0.6 ± 0.33	0.7 ± 0.34
	ELISA	2.0	3.9[c]	3.2	0.7	6.1
		0.2	1.2	0.5	0.0	1.7
2	Lymphocyte stimulation	2.0	0.1 ± 0.10	0.4 ± 0.16	0.6 ± 0.32	0.2 ± 0.09
		0.2	0.1 ± 0.27	0.9 ± 0.51	0.1 ± 0.21	0.5 ± 0.14
	ELISA	2.0	3.2	2.8	0.1	1.2
		0.2	2.1	0.8	0.0	0.4
3	ELISA	2.0	4.5	4.1	1.1	6.2
		0.2	1.6	1.6	0.2	2.1

[a] Experiment no. 1 and 2 were done with cells and serum from two different individuals, and experiment no. 3 was done on the pooled immune serum. Sera were diluted to 10^{-3}.

[b] Count × 10^{-3} per min (mean ± standard deviation) of five repeated cultures. Background values from cultures without antigen have been subtracted.

[c] Absorbance at 405 nm per 100 min. Mean of two determinations. Background values obtained in the absence of antigen have been subtracted.

seemed to be excluded, since the addition of proteinase K-treated antigen did not inhibit the lymphocyte response to nontreated antigen (data not shown). The results suggest that the determinants responsible for lymphocyte stimulation resided in protein, whereas the major ELISA reactivity was due to carbohydrate determinants.

DISCUSSION

Antigen for immunoprecipitation was more efficiently extracted from *F. tularensis* cells by ether-water treatment than by phenol-water extraction. This is in accordance with immunoprecipitation studies on various extracts of *F. tularensis* (20). To take full advantage of the high sensitivity of ELISA, the specificity of the antigen prepared is important (18). The antigen reacted strongly with homologous immune serum, even when used in a dose as low as 1 μg/ml of coating buffer solution, but reacted poorly with a pooled human nonimmune serum. The diagnostic usability of the antigen should be evaluated.

In the lymphocyte stimulation test, the development of highly specific microbial antigens is warranted. Certainly, whole killed *F. tularensis* LVS cells can be used as the antigen in the lymphocyte test (29). However, this involves the risk of exposure of bacterial components, which stimulate lymphocytes nonspecifically. Such components may be common antigens for various bacteria or polyclonal lymphocyte activators as has been shown for many other bacteria (2, 27). The present results also show that *F. tularensis* LVS contains components which stimulate lymphocytes nonspecifically. These components were apparently removed by the preparatory procedure used. Lymphocytes responding specifically to *F. tularensis* have been reported to be the T lymphocytes mainly (28), and the antigen prepared should improve the test as a parameter of cell-mediated immunity. This is important, since the presence of cell-mediated but not humoral immunity correlates with protection against tularemia (17).

From experiments in which the prepared antigen was treated with either proteinase K or periodate, it was concluded that ELISA activity of the antigen was mainly due to carbohydrate determinants and the lymphocyte-stimulating activity to protein determinants. This is compatible with results indicating that polysaccharides from *F. tularensis* act efficiently as the antigen in the passive hemagglutination test (1). The results are also compatible with the view that various microbial protein antigens are able to induce specific lymphocyte stimulation (23). The results may help to explain the poor correlation found between magnitudes of antibody and lymphocyte responses towards *F. tularensis* in immunized individuals.

The usefulness of an antigen for assay of cell-mediated immunity does not necessarily mean that it would be effective as a vaccine. Only viable bacteria have been found so far to induce cell-mediated immunity and protection in vivo (5, 8). The reason for this requirement is unknown. One possible explanation might be that relevant antigen reaches the critical targets in effective doses only when present on bacteria which multiply in macrophages. Purification of such antigen may be a way to render it protective. The present preparatory procedure seems to be a suitable basis, and further purification should, according to the results, be focused on the protein part of the antigen.

ACKNOWLEDGMENTS

We thank Lena Öhlund for skillful technical assistance. We are also grateful to Thorild Eriksson for help with the carbohydrate analysis.

Grants (no. B84-16X-06562-02) for this research were received from the Medical Research Council of Sweden and the Medical Faculty, University of Umeå.

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