

## Enzyme-Linked Immunosorbent Assay for Immunological Diagnosis of Human Tularemia

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The enzyme-linked immunosorbent assay (ELISA) was applied for immunological diagnosis of human tularemia, using lipopolysaccharide from *Francisella tularensis* as antigen. Sera collected from patients, healthy individuals, and vaccinated volunteers were investigated for antibodies against *F. tularensis* by ELISA and tube agglutination. In ELISA all sera were titrated with a polyspecific anti-immunoglobulin enzyme conjugate. A limited number of consecutive sera from individual patients were also investigated for immunoglobulin G (IgG) and IgM antibodies by means of immunoglobulin class-specific conjugates. On an average ELISA was more than 10-fold as sensitive as tube agglutination. Two weeks after onset of disease, sera from patients had significantly higher titers in ELISA than sera from healthy controls. High titers persisted after more than 2 years. Significant amounts of both IgG and IgM antibodies were present within 1 to 2 weeks after infection. The antibody activity increased during the first month, without any significant change of the relation between IgG and IgM titers. After 2.5 years the IgG/IgM titer ratio of sera from patients was significantly increased. Within 6 weeks after vaccination sera from about half of the vaccinees had significantly elevated titers in ELISA. Titers observed after vaccination were generally lower than those found after infection. An elevated ELISA titer can be of diagnostic importance by the end of the first week of illness. A significant increase of titer in consecutive serum samples indicates a diagnosis of tularemia. Determination of IgG and IgM antibodies may be of value in determining whether a positive titer of a single serum sample is of longstanding or recent origin.

In Sweden tularemia is endemic in northern areas of the country, where wild rodents serve as a reservoir for infection. Epidemic outbreaks of the disease occur approximately every third or fourth year. The etiological agent, *Francisella tularensis*, is usually transmitted by mosquitoes, by direct handling of dead rodents and lagomorphs, or by inhalation of infected dust (1, 7).

*F. tularensis* is highly infectious and also difficult to culture. Therefore, laboratory diagnosis is traditionally based on quantitation of specific antibody or demonstration of the pathogen in biopsies or pus by immunofluorescence (16), or on both. The most widely used assay for antibodies against *F. tularensis* is the whole-cell agglutination test (Widal's reaction) (20). This test, as most agglutination tests, is, however, likely to measure predominantly immunoglobulin M (IgM) antibodies.

For immunological diagnosis of other bacterial

infections the enzyme-linked immunosorbent assay (ELISA) has proved superior to tests such as Widal's reaction and passive hemagglutination (4, 18). This is to a large extent the result of the use of more well-defined antigens in ELISA. In this investigation we apply ELISA to serodiagnosis of human tularemia, using phenol-water-extracted lipopolysaccharide (LPS) as antigen. Furthermore, the humoral immune response after vaccination with live attenuated *F. tularensis* is investigated.

### MATERIALS AND METHODS

**Sera.** Sera were obtained from 40 patients with clinically diagnosed tularemia. Diagnosis was confirmed by demonstration of *F. tularensis* in pus or biopsy material, or in both, by immunofluorescence and in a few cases (3 of 16) by positive culture. A total of 79 serum samples were obtained from the patients: 19 were drawn during the first, 13 during the second, and 28 during the third to fifth week of illness. In

addition, sera were collected from 19 patients 2.5 years after their infection. For comparison, 48 serum samples were also collected from the same number of healthy individuals.

From 46 healthy volunteers, inoculated with the "live vaccine strain" (LVS) of *F. tularensis* (Fort Detrick, Frederick, Md.) (8), sera were obtained before and 5 to 6 weeks after vaccination.

Rabbit hyperimmune serum was obtained after intravenous injection of approximately  $10^8$  formalinized *F. tularensis* bacteria (virulent strain SVA; culture collection, National Veterinary Institute, Stockholm, Sweden) twice weekly during 5 weeks. Serum was collected 1 week after the last injection.

All sera were stored at  $-20^\circ\text{C}$  until used.

**Antigen.** *F. tularensis* LVS was cultured under aerobic conditions in semisynthetic medium at  $37^\circ\text{C}$  for 18 h under constant shaking, as described by Scharer et al. (17). The antigenically unrelated organisms *Brucella abortus* strain 544 and *Yersinia enterocolitica* O-type 3 (O3), strain 482 (culture collection, Department of Bacteriology, National Bacteriological Laboratory, Stockholm, Sweden), were cultured in Roux flasks with meat extract agar as described earlier (13). The bacteria were killed by treatment with 1% Formalin for 24 h, washed in 0.05 M phosphate-buffered saline (pH 7.2), centrifuged, and extracted with phenol-water (19). For *F. tularensis* and *Y. enterocolitica* the aqueous-phase LPS was collected and for *B. abortus* phenol-phase LPS was used (14, 15). If necessary nucleic acid was removed from the LPS-containing phase by treatment with ribonuclease (5 $\times$  crystalline, Nutritional Biochemicals Corp., Cleveland, Ohio). The purified LPS was lyophilized.

**Tube agglutination.** Whole-cell agglutination was performed according to Widal (20) as modified by Felix (11). To serial twofold dilutions of serum in phosphate-buffered saline, equal amounts (0.5 ml) of a standardized suspension of Formalin-killed *F. tularensis*, heat-killed *Y. enterocolitica* O3, or *B. abortus* bacteria were added. Agglutination was recorded after incubation for 18 h at  $37^\circ\text{C}$ . Titers were expressed as reciprocals of the last serum dilution giving visible agglutination.

**ELISA.** ELISA was performed in disposable polystyrene tubes (11 by 55 mm; Heger Plastics AB, Stal-larholmen, Sweden) according to Engvall and Perlman (10) as modified by Carlsson et al. (5), using 1 M diethanolamine-HCl (pH 9.8) as substrate buffer. Phenol-water-extracted LPS from *F. tularensis*, *Y. enterocolitica* O3, and *B. abortus* were used as antigens. For *Y. enterocolitica* and *B. abortus* 5  $\mu\text{g}$  of LPS per ml was used for coating (3). Sera were tested diluted in phosphate-buffered saline containing 0.05% Tween 20 in duplicates (1-ml aliquots). Sheep anti-rabbit or anti-human immunoglobulin, purified by affinity chromatography on insolubilized rabbit or human immunoglobulin reacting with both heavy and light chains and conjugated with alkaline phosphatase (calf intestinal mucosa, type VII; Sigma Chemical Co., St. Louis, Mo.) as described by Engvall and Perlmann (9), was utilized. Heavy-chain-specific anti-human IgG and anti-human IgM from swine serum, purified and conjugated with alkaline phosphatase as above, was obtained from Orion Diagnostica OY, Helsinki, Fin-

land. Enzyme activity was determined by photometric estimation of released *p*-nitrophenylate at 400 nm. All absorbance values were extrapolated to 100 min.

## RESULTS

**Optimal antigen dose for ELISA.** The optimal amount of *F. tularensis* LPS for coating in ELISA was established by incubating the plastic tubes overnight at  $25^\circ\text{C}$  with different concentrations of LPS in 0.05 M carbonate buffer (pH 9.6), using hyperimmune rabbit anti-*F. tularensis* antiserum diluted  $10^{-4}$  or a patient serum diluted  $10^{-3}$  (Fig. 1). Bound specific antibody was determined as enzyme activity (absorbance at 400 nm per 100 min) of bound anti-rabbit or anti-human immunoglobulin conjugated with alkaline phosphatase. With both antisera, 0.1 to 1.0  $\mu\text{g}$  of LPS per ml gave optimal antibody binding, and 0.5  $\mu\text{g}$  of *F. tularensis* LPS per ml was used for coating throughout this investigation.

**Titration of sera from patients and controls.** Ten sera were randomly selected from each of the groups of patients (weeks 3 to 5) and healthy controls. These sera were tested in 10-fold dilutions ( $10^{-2}$  to  $10^{-5}$ ) by ELISA. The  $10^{-3}$  dilution gave the best discrimination between patients and control subjects (Fig. 2). At higher

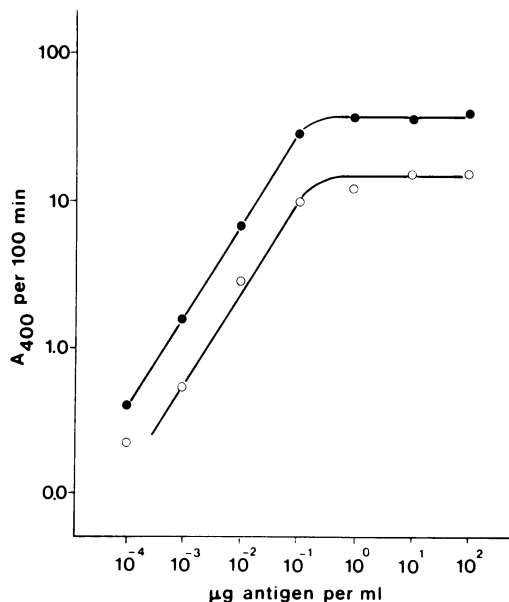


FIG. 1. Determination of optimal concentration of *F. tularensis* LPS for coating of plastic tubes in ELISA. The tubes were incubated with LPS dissolved in 0.05 M carbonate buffer (pH 9.6) overnight at  $25^\circ\text{C}$ . The amount of antigen adsorbed to the tube was estimated as bound antibody from hyperimmune rabbit antiserum diluted  $10^{-4}$  (●) and patient serum diluted  $10^{-3}$  (○).

dilutions most sera gave low or background absorbance, and at a  $10^{-2}$  dilution controls showed relatively high activity. Accordingly, the  $10^{-3}$  dilution was used for titrations in this work. The titers were expressed as absorbance at 400 nm per 100 min multiplied by the dilution factor ( $10^3$ ) and are referred to as relative ELISA titers.

Sera obtained during weeks 3 to 5 of illness from 28 patients as well as sera from 48 healthy controls were investigated for antibodies against *F. tularensis*, *B. abortus*, and *Y. enterocolitica* O3 by ELISA and Widal's reaction (Fig. 3, Table 1). In ELISA, sera from 23 patients had relative titers against *F. tularensis*  $\geq 2,000$  and only one serum sample had a titer  $< 500$ . Of the sera from healthy individuals 47 had relative titers  $< 500$  and one had a titer of 1,400. In tube agglutination, sera from 22 patients had reciprocal titers  $\geq 320$  against *F. tularensis*, four sera had a titer of 160, and two had titers  $< 40$ . Among control subjects sera from 47 had reciprocal agglutination titers  $\leq 40$ , and serum from 1 had a titer of 160 (ELISA titer, 1,400). In both assays the difference between patients and controls was highly significant statistically ( $P < 0.001$ , median test).

Against the *Y. enterocolitica* O3 antigen, sera from 25 patients and 45 healthy individuals had titers  $< 500$  in ELISA. Against the *B. abortus*

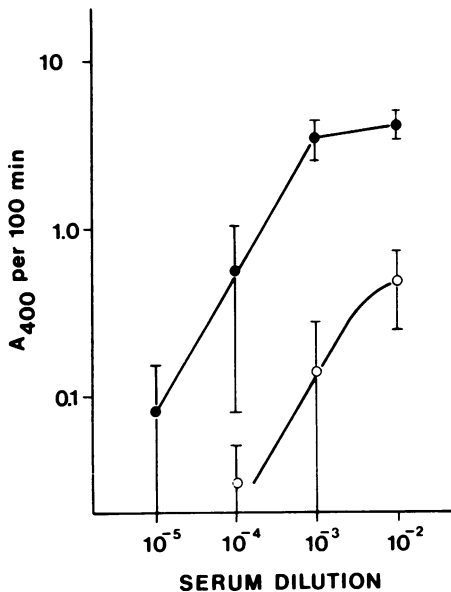


FIG. 2. Determination of the serum dilution giving maximal difference in antibody activity against *F. tularensis* LPS between patients' (●) and healthy controls' (○) sera by ELISA. The curves represent mean values of 10 sera from each group, and the horizontal bars indicate the standard deviation.  $A_{400}$ , Absorbancy at 400 nm.

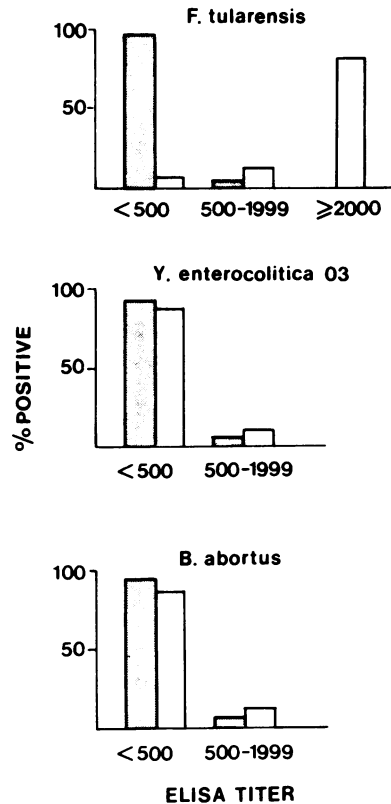


FIG. 3. Distribution of antibody titers in ELISA against LPS from *F. tularensis*, *Y. enterocolitica* O3, and *B. abortus* in sera from 28 patients with tularemia (open bar) and 48 healthy controls (closed bar). Sera were tested at a  $10^{-3}$  dilution.

TABLE 1. Incidence of positive titers in tube agglutination (Widal's reaction) ( $\geq 160$ ) and ELISA ( $\geq 500$ ) against *F. tularensis*, *B. abortus*, and *Y. enterocolitica* O3 antigens in sera obtained from 28 patients with tularemia during days 15 to 35 after onset of disease

Assay	Test antigen	No. of positive titers	Median titer
ELISA	<i>F. tularensis</i>	27	4,400
	<i>B. abortus</i>	4	$< 10$
	<i>Y. enterocolitica</i>	3	45
Widal	<i>F. tularensis</i>	26	320
	<i>B. abortus</i>	0	40
	<i>Y. enterocolitica</i>	0	$< 40$

antigen corresponding values were 24 and 45, respectively. All sera tested had titers  $< 2,000$  against both of these antigens. In tube agglutination, all sera tested had titers  $< 160$  against *Y. enterocolitica* and *B. abortus*.

If a relative titer  $\geq 500$  was considered positive in ELISA, 27 of 28 patients scored as positive

against *F. tularensis* LPS, with a median titer of 4,400 (Table 1, Fig. 3). Against *Y. enterocolitica* and *B. abortus* LPS, 3 and 4 of 28 patients, respectively, were positive, all with titers < 2,000. Median titers against these antigens were 45 and < 10, respectively. Among healthy controls one scored as positive against *F. tularensis* LPS and three were positive against *Y. enterocolitica* and *B. abortus* LPS (Fig. 3). Median titers were 40, 30, and 20, respectively.

The distribution of ELISA titers against *F. tularensis* LPS of patients' sera at different times after onset of disease is described in Fig. 4. During week 1 of illness, 3 of 19 patients had ELISA titers  $\geq 500$ ; during week 2, 7 of 13 had positive titers; and during weeks 3 to 5, 27 of 28 were positive. More than 2.5 years after their infection 18 of 19 reinvestigated patients still had titers  $\geq 500$ . The incidence of relative titers  $\geq 500$  was significantly higher in patients during week 2 of illness and later than in healthy control subjects (1 of 48) ( $P < 0.001$ , chi square). All anti-*Yersinia* and anti-*Brucella* titers in patients' sera were unchanged in ELISA during the whole course of disease (data not shown).

#### IgG and IgM responses during and after

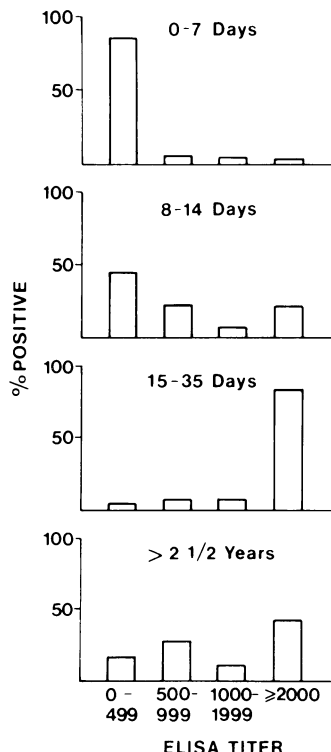


FIG. 4. Distribution of antibody titers in ELISA against *F. tularensis* LPS in sera from patients with tularemia at different times after onset of disease. Sera were tested at a  $10^{-3}$  dilution.

**tularemia.** The IgG and IgM antibody content against *F. tularensis* antigen was determined using immunoglobulin class-specific enzyme conjugates. Initially the relative IgG titers were approximately as high as the IgM titers, and the relation between IgG and IgM antibody content did not change significantly during the first month of disease. In sera collected during weeks 1 and 2 of illness the median IgG/IgM ratio was 0.8, during weeks 3 to 5 it was 1.1, and after 2.5 years it was 2.6 (Table 2). Compared with serum samples obtained during the first month after infection, the IgG/IgM antibody ratio was increased in samples obtained 2.5 years after infection in 18 of 19 investigated patients. In 13 cases the ratio was at least doubled ( $P < 0.01$ , sign test). Furthermore, an IgG/IgM ratio of 2 or more was significantly more frequent in sera obtained after 2.5 years (13 of 19) than in sera obtained during the acute phase of disease (5 of 19) ( $P < 0.05$ , chi square).

**ELISA and tube agglutination.** To evaluate the relationship between ELISA and tube agglutination, the ELISA titers against *F. tularensis* antigen of all patient sera (79 tested) were plotted versus the corresponding agglutination titers (Fig. 5). Despite the apparently wide scatter of values, a statistically highly significant correlation between the two methods was assessed by a nonparametric correlation test ( $n = 79$ ;  $r_s = 0.61$ ;  $P < 0.001$ , Spearman rank).

**Humoral antibody response after *F. tularensis* vaccination.** The humoral immune response after vaccination was investigated in 46 healthy volunteers inoculated with *F. tularensis* LVS (Tables 3 and 4). All individuals were bled immediately before vaccination and 5 to 6 weeks after. Sera were tested against LPS from *F. tularensis*, *Y. enterocolitica* O3, and *B. abortus* by ELISA and against *F. tularensis* by tube agglutination. Before vaccination all sera had a relative titer < 500 in ELISA. After vaccination a more than twofold increase against *F. tularensis* was observed in 44 individuals ( $P < 0.01$ , sign test). The remaining two subjects had unchanged titers. Of the 44 samples for which the relative titer increased more than twofold, 22 had relative titers  $\geq 500$ , but all had titers < 2,000. Against *B. abortus* and *Y. enterocolitica* LPS, 30 and 33 individuals, respectively, had unchanged titers. The number of significantly increased and decreased titers was approximately equal (Table 3), all with a difference less than 50% from the prevaccination value. In tube agglutination all sera were negative before vaccination. After vaccination 40 individuals developed fourfold titer increases against *F. tularensis* ( $P < 0.01$ , sign test); of these, 19 had titers of 160 to 320.

TABLE 2. Median relative IgG and IgM ELISA titers against *F. tularensis* LPS and ratio of relative IgG/IgM titers of sera obtained from healthy controls and from patients with tularemia in the acute phase of disease and 2.5 years after infection

Subjects	No. of sera tested	Titer		IgG/IgM ratio
		IgG	IgM	
Healthy controls	13	160 (60-390) <sup>a</sup>	170 (80-340)	0.7 (0.4-2.5)
Patients				
1-2 wk	12	400 (70-2,800)	230 (100-2,660)	0.8 (0.3-4.1)
3-8 wk	19	2,290 (200-6,630)	2,950 (130-6,750)	1.1 (0.5-3.9)
2.5 yr	19	4,610 (360-8,320)	1,150 (170-3,570)	2.6 (0.7-5.8)

<sup>a</sup> Numbers in parentheses are ranges.

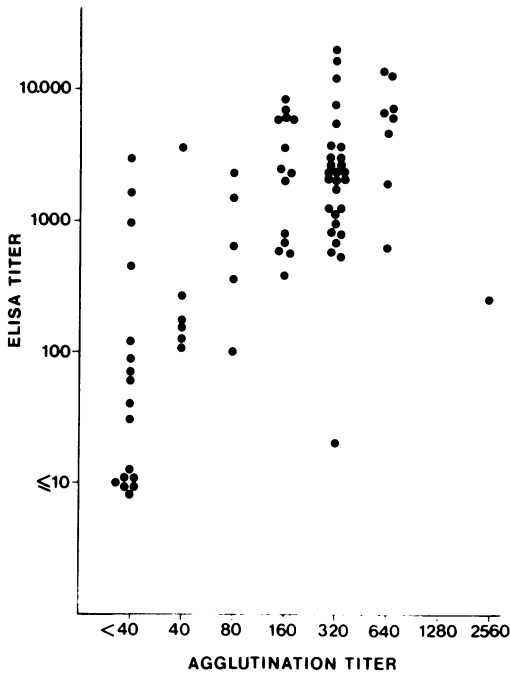


FIG. 5. Correlation between antibody titers in ELISA and tube agglutination against *F. tularensis* antigen of all sera obtained from patients with tularemia (79). The statistical correlation was calculated by the nonparametric Spearman rank correlation test.

TABLE 3. Change of humoral antibody response as measured by ELISA in sera from 46 volunteers after vaccination with *F. tularensis* LVS

Antigen (LPS)	No. of sera with:		<i>P</i> <sup>b</sup>
	In-creased <sup>a</sup> titer	In-creased or de-creased <sup>a</sup> titer	
<i>F. tularensis</i>	44	44	<0.01
<i>B. abortus</i>	6	16	>0.05
<i>Y. enterocolitica</i> O3	5	13	>0.05

<sup>a</sup> A change in titer was considered significant if greater than 20%.

<sup>b</sup> Sign test.

TABLE 4. Distribution of ELISA titers against *F. tularensis* LPS in sera from 46 volunteers before and after vaccination with *F. tularensis* LVS

ELISA titer before vaccination	No. of volunteers with ELISA titer after vaccination of:				
	0-99	100-199	200-499	500-599	>1,000
0-99	6 <sup>a</sup>	8	7	11	4
100-199		1	1	3	5
>200			1 <sup>a</sup>		

<sup>a</sup> One individual without significant increase of ELISA titer after vaccination.

DISCUSSION

Our results establish the usefulness and specificity of ELISA, using phenol-water-extracted antigen from *F. tularensis*, to detect the humoral antibody response after tularemia in the acute phase and also several years after the infection.

During week 1 of illness there was no difference in anti-*F. tularensis* titers in ELISA between sera sampled from either patients or controls. However, already during week 2 the incidence of elevated ELISA titers against *F. tularensis* LPS was significantly higher in patients' sera. Also, in sera obtained  $\geq 2.5$  years after the infection the incidence of ELISA titers  $\geq 500$  against *F. tularensis* was significantly higher than in control sera. Antibody titers against *B. abortus* and *Y. enterocolitica* were low, and there was no difference in distribution of titers between the groups. An ELISA titer  $\geq 500$  may, therefore, be of diagnostic value already 1 week after onset of disease, provided that vaccination against tularemia or a previous history of tularemia can be excluded.

Estimation of the IgG and IgM antibody content proved to be useful in evaluating the significance of a positive anti-*F. tularensis* titer in a serum obtained late after infection. Since the efficiency of the anti-IgG and anti-IgM enzyme conjugates may differ and since the titer levels of individual patients varied, it was considered better to compare the ratio between IgG and IgM titers rather than the titers themselves. In

the early phase of disease (i.e., the first month after infection) the relative IgG and IgM titers were approximately equal. However, the relative amount of IgM antibodies was significantly higher at that time than 2.5 years after infection, when IgG antibodies predominated. Accordingly, under the experimental conditions applied in this work, an IgG/IgM ratio  $\geq 2$  statistically suggests a titer of longstanding origin. These results are in accordance with earlier investigations (K. Carlsson, H. E. Carlsson, R. Neringer, and A. A. Lindberg, *Scand. J. Infect. Dis.*, in press; B. Svenungsson, H. Jörbeck, and A. A. Lindberg, *J. Infect. Dis.*, in press).

Previously, a relative ELISA titer  $\geq 500$  could discriminate between sera collected from patients with salmonellosis and from normal controls (Carlsson et al., in press; Svenungsson et al., in press). In the present study the same limit could be used to differentiate between patients with tularemia and healthy individuals. However, serum collected from one patient 3 weeks after infection had a titer  $< 500$ , and serum from one of the controls had a titer  $> 500$ . The patient with tularemia was a 75-year-old female with a positive agglutination titer, who had developed a significant increase in ELISA titer after 5 weeks. The healthy control with an ELISA titer  $> 500$  lived in an endemic area and a previous history of tularemia could not be excluded. The IgG/IgM antibody ratio in his serum was unchanged in all three samples drawn over a 2.5-year period, which further supports a longstanding titer.

The correlation between ELISA and tube agglutination titers against *F. tularensis* antigen was statistically highly significant. The relatively wide scatter of values is not unexpected since tube agglutination measures only agglutinating antibodies (prevalently IgM). In ELISA, all antibody classes can be detected with about equal efficiency by a polyspecific anti-immunoglobulin-enzyme conjugate (5). Furthermore, only antibodies with specificity for antigens in purified soluble LPS will be measured.

Other workers have shown that vaccination with *F. tularensis* LVS can elicit humoral antibodies which may cause problems for the immunological diagnosis of tularemia (2). Sera from healthy volunteers were therefore investigated for antibodies against *F. tularensis*, *Y. enterocolitica* O3, and *B. abortus* before and after vaccination with LVS. A specific and significant increase of antibody titer against *F. tularensis* after vaccination was established in 44 of 46 individuals, confirming that LVS gives rise to humoral antibody production (2). Moreover, no increase of antibodies against *B. abortus* or *Y. enterocolitica* antigen was observed, fur-

ther supporting the hypothesis that the anti-*F. tularensis* antibodies reflect a specific immune response against the vaccine. Although serum samples from most vaccinees (44 of 46 = 96%) had significantly increased titers, only 22 (48%) had relative ELISA titers  $\geq 500$ , on an average considerably lower than patients ( $< 2,000$ ).

The reason for using relative titers derived from one serum dilution is largely a practical one. Antibody titers can also be determined as the serum dilution giving a predetermined absorbance value (endpoint titer) (6, 10). This procedure, however, requires production of complete titration curves, which is laborious and consumes large amounts of reagents. Recently, we could show that the endpoint titer in ELISA correlated well with the absorbance value obtained at a given serum dilution (Carlsson et al., in press). For titration of *Salmonella* O antibodies the best discrimination between sera from patients and healthy controls was obtained at a  $10^{-3}$  dilution (Carlsson et al., in press; Svenungsson et al., in press). The same dilution was also optimal in this study.

It is important to choose the right concentration of antigen for coating the solid phase. An optimal or slightly suboptimal concentration of antigen is preferable since an excess of antigen may give rise to prozone-like phenomena (5). At the same time the amount of antigen must be high enough to allow discrimination between positive and negative sera. The optimal concentration of antigen during coating may vary over several orders of magnitude (4). Therefore, the concentration to be used must be determined for each individual antigen. For *F. tularensis* LPS the optimal concentration was 0.1 to 1.0  $\mu\text{g}/\text{ml}$ .

From the results presented in this work we conclude that ELISA is well suited for immunological diagnosis of tularemia. Even a moderately elevated ELISA titer against *F. tularensis* LPS may be of diagnostic value in nonendemic areas. However, since repeated infections of tularemia have been described (12), the immunological diagnosis may not be as simple in endemic areas. An elevated anti-*F. tularensis* titer may reflect a previous infection as well as a recent one and can also be elicited by vaccination. This investigation has shown that elevated titers may persist for more than 2.5 years after infection. Although, the relative amount of IgG and IgM antibodies can give an indication as to the time elapsed from antigen exposure, a significant increase of *F. tularensis* titer must be established before a clinical diagnosis can be considered serologically confirmed.

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