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Cell-Mediated and Humoral Immunity Induced by a Live *Francisella tularensis* Vaccine

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Live attenuated *Francisella tularensis* vaccine induced long-lasting humoral and cell-mediated immune responses in all 13 subjects studied. Lymphocyte blast transformation reactivity to *F. tularensis* appeared 2 weeks after vaccination in most subjects and remained unchanged for up to 1.5 years. Similarly, in most recipients, antibodies against *F. tularensis* were detectable by both the enzyme-linked immunosorbent assay (ELISA) and the agglutination method from 2 weeks after vaccination, although diagnostically significant agglutination titers (≥80) were not detectable until 4 weeks after vaccination. Maximal agglutination titers of 80 to 2,560 appeared at 4 to 8 weeks, and in spite of decreasing tendency, titers as high as 320 were still present 1.5 years after vaccination. ELISA showed the simultaneous, but not parallel, appearance of different immunoglobulin classes, immunoglobulin M (IgM) reaching individual maximal values 1.8 months after vaccination on average, at the same time as agglutinating antibodies, 1 week earlier than IgA, and about 1 month earlier than IgG. All of these immunoglobulin classes persisted in significant amounts up to 1.5 years, with IgG generally dominant. Long-lasting IgA and IgM responses after vaccination, as also after infection, suggested that the serodiagnosis of tularemia generally requires two consecutive serum samples with a significant increase in the titer.

Francisella tularensis induces both a humoral and a cell-mediated immunity in various mammals, the latter being the more important in resistance against tularemia (1, 5, 19, 34). In humans, live attenuated vaccine has been shown to be more efficient in preventing *F. tularensis* infection than killed vaccine (3). Recently, it has been shown that lymphocytes from subjects who have had tularemia or been vaccinated with a live vaccine strain of *F. tularensis* respond to *F. tularensis* antigen in vitro by blast transformation and increased DNA synthesis (18, 32, 33). This response is due to T-lymphocytes (31) and, like the purified protein derivative of tuberculin (PPD) response, it is probably an in vitro counterpart of the delayed cutaneous hypersensitivity reaction induced by tularin in the skin test, a diagnostic tool used by clinicians and epidemiologists during the last 50 years (2).

Immunity cannot usually be studied in tularemia patients until 1 to 2 weeks after the onset of symptoms, i.e., 2 to 4 weeks after infection, at which time antibodies and cell-mediated immune response may already be detectable (14, 18, 33). To obtain more exact information on the development and nature of the immune response to *F. tularensis*, we studied lymphocyte blast transformation responses and antibodies as measured by the agglutination method and enzyme-linked immunosorbent assay (ELISA) in healthy subjects immunized with a live attenuated *F. tularensis* vaccine.

MATERIALS AND METHODS

Vaccine. A lyophilized live attenuated tularemia vaccine (BB IND 157, lot 11) was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md.

Subjects. Thirteen healthy volunteers (nine males and four females) without previous tularemia or tularemia vaccination, working with animals or in a bacteriological laboratory, were vaccinated intradermally by the multiple pressure method. They were informed about possible adverse reactions both orally and on a consent form which they then signed.

One subject did not respond at all after the first vaccination, during a follow-up period of 6 months. After revaccination, his responses were similar to those of the subjects vaccinated only once, and these measurements are included in the data.

Blood samples. Venous blood samples were taken before vaccination and at 1, 2, and 4 weeks and 2, 4, 10 to 11, and 17 months after vaccination. The sera for the antibody determinations were immediately separated and stored at −20°C until all samples for the same subject could be studied simultaneously. The blast transformation tests were begun within a few hours of obtaining the heparinized blood samples.

Lymphocyte cultures. Cell-mediated immune response against *F. tularensis* was measured by the lymphocyte blast transformation method, using a whole-blood modification described earlier (18) and

using Formalin-killed whole bacteria of *F. tularensis* (150 μg/ml) as the stimulating agent in 7-day cultures. The results were expressed as median increment counts per minute for triplicate cultures (median counts of stimulated culture minus median counts of unstimulated culture). PPD (State Serum Institute, Copenhagen, Denmark) was used as the control antigen (10 μg/ml). Because of extensive *Mycobacterium bovis* BCG vaccination during the last 40 years, and also because of natural contact with tuberculosis, a considerable proportion of the Finnish population is PPD positive (20). All of our subjects had been vaccinated with BCG in childhood.

Tube agglutination. The agglutination procedure was performed according to Widal (11, 36), as initially described for tularemia by Francis and Evans (13). Serial twofold dilutions of the sera, starting from 1:10, were prepared in phosphate-buffered saline, pH 7.2 (PBS). Tubes containing 0.5 ml of this and an equal amount of a standardized suspension of Formalin-killed whole bacteria of *F. tularensis* (National Bacteriological Laboratory, Stockholm, Sweden) were incubated for 18 h at 37°C. Antibody titers were expressed as the reciprocal of the highest dilution giving visible agglutination with a clear supernatant.

ELISA. The antibodies of different immunoglobulin classes were quantified by using ELISA carried out on disposable polystyrene microtiter plates with flat-bottomed wells (Dynatech Laboratories, Inc., Alexandria, Va.).

A Formalin-killed vaccine strain of *F. tularensis* was sonicated for 2 min at an amplitude of 20 μm (MSE 150-W ultrasonic disintegrator; MSE Scientific Instruments, Crawley, England), and the supernatant of the sonic fluid (4,000 rpm/5 min) diluted in 0.05 M PBS (pH 7.2) and coupled to the wells was used as the antigen. Two hundred microliters of antigen suspension with a protein concentration of 2.5 μg/ml, found to be optimal against serial dilutions of serum from tularemia patients (P. Koskela and A. Salminen, manuscript in preparation), was added to each well. The microtiter plates were incubated at 37°C for 6 h and washed three times for 5 min with PBS (pH 7.2) supplemented with 0.05% Tween (PBS-Tween).

Serial twofold dilutions of the sera, starting from 1:100, were prepared in 4% PBS-Tween. One hundred microliters of the serum dilutions was added to the sensitized wells and incubated for 2 h at 37°C. After washing as before, 100 μl of alkaline phosphatase-labeled swine anti-human immunoglobulin G (IgG), IgM, and IgA (Orion Diagnostica, Espoo, Finland) was added to the wells. These anti-heavy-chain antisera were diluted in 4% PBS-Tween. Dilutions of 1:450 for IgG and 1:300 for IgM and IgA were used. After incubation for 2 h, the plates were washed as before. One hundred microliters of fresh substrate was added to the wells, which were incubated for 30 min at 37°C before the enzyme reaction was stopped by the addition of 50 μl of 2 N NaOH. *p*-Nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo.) in diethanolamine-$MgCl_2$ buffer, pH 10 (Orion Diagnostica), was used as the substrate. The amount of alkaline phosphatase bound to the wells was determined by photometric estimation of the *p*-nitrophenylate released. The optical absorbance was measured at a wavelength of 405 nm in a multichannel photometer adapted for microtiter plates (Titertek Multiskan, Eflab, Helsinki, Finland). Measurements were carried out against antigen-sensitized wells treated with antisera, substrate, and 2 N NaOH. All tests were performed in duplicate. The concentrations of antibodies in the sample are given as ELISA titers (6, 17) read at optical density values of 0.3 for IgG and 0.1 for both IgM and IgA (Koskela and Salminen, manuscript in preparation).

Detection and elimination of rheumatoid factor. Since rheumatoid factor can modulate the levels of immunoglobulins found by ELISA (21, 28), its amount was determined in all sera by the ELISA procedure described above for IgM, but using highly purified human IgG as the antigen (28). Human IgG was obtained from the Finnish Red Cross Transfusion Service (Helsinki, Finland).

Only in the samples from one vaccinee could rheumatoid factor activity (i.e., IgM–anti-IgG antibodies) be detected by ELISA, and this was then removed by using latex-rheumatoid factor reagent (Behringwerk AG, Marburg, West Germany) before classification of the anti-*Francisella* antibodies.

RESULTS

General. All 13 subjects developed both humoral and cell-mediated immune responses to *F. tularensis*, although one did so only after revaccination. Individual differences were found in the time required for immunization, in the level of agglutinins and various immunoglobulin classes, and also in the strength of the cell-mediated response. In most subjects, the appearance of antibodies coincided with that of cell-mediated reactivity. In one recipient, antibodies were demonstrable 1 week later, and in one, 4 weeks earlier, than the lymphocyte blast transformation response.

Cell-mediated response. The lymphocytes from the subjects did not respond to *F. tularensis* antigen in vitro before vaccination, whereas they responded strongly to PPD (Fig. 1). In most subjects, cell-mediated reactivity was detectable 2 weeks after vaccination, the interval varying from 1 to 8 weeks. The strength of the response remained essentially unchanged up to 17 months (Fig. 1).

Humoral response. All prevaccination samples were negative for agglutinins (Fig. 2), as they were for IgM, IgA, and IgG antibodies to *F. tularensis* (Table 1). Agglutinating antibodies began to appear 2 weeks after vaccination, the titers being low: 20 to 40 in seven subjects, 80 in one, and totally negative in three. The individual highest titers, varying from 80 to 2,560, were reached at 4 to 8 weeks (1.8 months on average) (Table 2). Although the agglutinating antibodies tended to decrease later, titers as high as 160 to 320 were found 17 months after vaccination (Fig. 2 and 3).

Vaccination induced the simultaneous, but not parallel, appearance of IgM, IgA, and IgG, as identified by ELISA, from 2 to 4 weeks after inoculation (Table 1), at the same time that

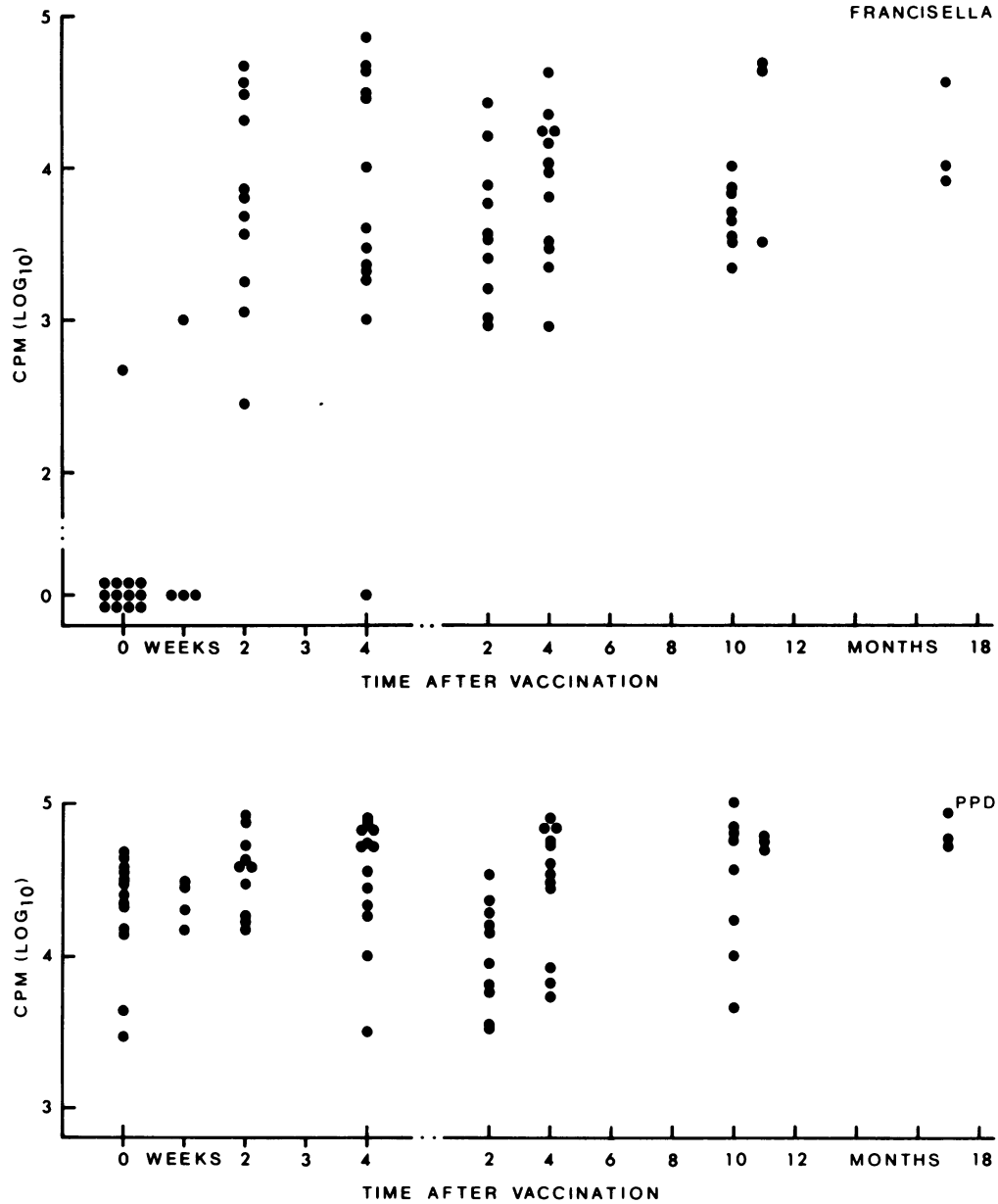

FIG. 1. Lymphocyte blast transformation responses to *F. tularensis* antigen and PPD (control antigen) at various intervals after vaccination with a live attenuated strain of *F. tularensis*. Responses less than cpm ($\log_{10}$) 2.7 (500 cpm) are regarded as negative.

agglutinating antibodies were also detectable (Fig. 2 and 3). The timing of the maximal responses was different in these immunoglobulin classes (Table 2). The individual maximal IgM titers (ranging from 170 to 4,160) were reached 1.8 months after vaccination on average, whereas the corresponding time for IgA (150 to 3,010) was 2.0 months and that for IgG (<100 to 3,080) was 3.0 months; the difference between IgM and IgG was significant ($t = 3.38$, $P < 0.01$), as was that between IgA and IgG ($t = 3.03$, $P < 0.01$). All of these immunoglobulin classes persisted in significant amounts up to 17 months, with IgG generally dominant, although in four subjects IgM was most pronounced.

In the subject with the lowest agglutination values, having a maximal titer of 80, ELISA did not detect any IgG antibodies, and IgM was

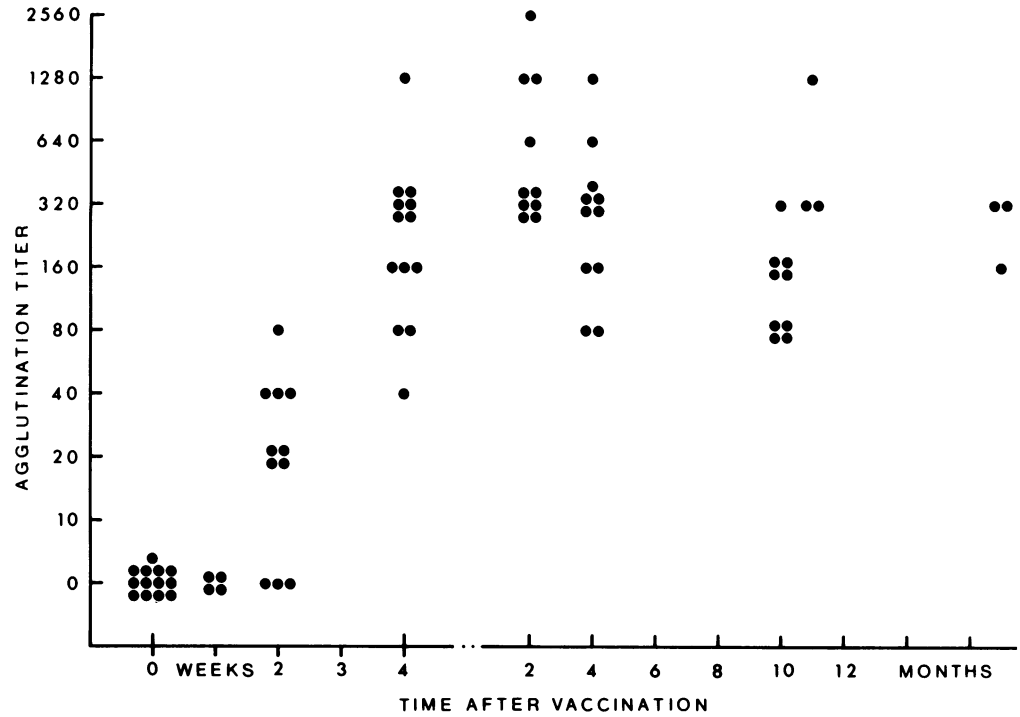

FIG. 2. Agglutination antibody titers at various intervals after vaccination with a live attenuated strain of *F. tularensis*. (Titer of ≥80 is usually regarded as diagnostically significant).

TABLE 1. Percentage distribution of ELISA titers (IgM, IgA, and IgG) at various intervals after vaccination with a live attenuated strain of *F. tularensis*[a]

Time after vaccination	% Distribution of ELISA titers							
	<100	100–199	200–399	400–799	800–1599	1,600–3,199	>3,200	*n*
IgM								
0	100.0							13
1 wk	100.0							4
2 wk	45.5	55.5						11
4 wk		23.1	46.1	23.1			7.7	13
2 mo		10.0	20.0	50.0	10.0		10.0	10
4 mo	16.7	8.3	41.7	33.3				12
10–11 mo	25.0	16.7	33.3	25.0				12
17 mo			66.7	33.3				3
IgA								
0	100.0							13
1 wk	100.0							4
2 wk	72.7	27.3						11
4 wk		46.1	30.8	15.4	7.7			13
2 mo			40.0	50.0		10.0		10
4 mo		25.0	41.7	33.3				12
10–11 mo		41.7	41.7	16.6				12
17 mo		33.3	33.3	33.3				3
IgG								
0	100.0							13
1 wk	100.0							4
2 wk	55.5	27.3	9.1	9.1				11
4 wk	7.7	23.0	7.7	38.5	23.0			13
2 mo			10.0	20.0	20.0	40.0	10.0	10
4 mo	8.3		8.3	25.0	25.0	33.3		12
10–11 mo	8.3	8.3		33.3	33.3	16.7		12
17 mo			33.3		67.7			3

[a] Titers of <100 considered negative.

TABLE 2. Timing of maximal antibody values in 10 subjects after vaccination with a live attenuated strain of *F. tularensis*

Titer	Mo after vaccination	
	Mean ± SE	Range
Agglutination	1.8 ± 0.13[a]	1–2
ELISA		
IgM	1.8 ± 0.13	1–2
IgA	2.0 ± 0.00	
IgG	3.0 ± 0.33	2–4

[a] Agglutination titer.

positive at 4 weeks only, whereas IgA was detectable from 4 weeks to the end of the 10-month follow-up period. This vaccinee also had a relatively weak lymphocyte blast transformation response.

In one serum sample without demonstrable agglutinins at 2 weeks after vaccination, ELISA detected IgG antibodies. With this exception, ELISA and agglutination test findings were parallel.

DISCUSSION

Vaccination with a live attenuated strain of *F. tularensis* induced long-lasting humoral and cell-mediated immune responses in all 13 subjects. In most recipients, antibodies as well as lymphocyte reactivity to *F. tularensis* were demonstrable at 2 weeks after vaccination. Simultaneous appearance of humoral and cell-mediated response after vaccination has earlier been described by Tärnvik et al. (33), who found lymphocyte reactivity to *F. tularensis* membranes and agglutinins 2 to 4 weeks after inoculation.

Diagnostic significant agglutination values, i.e., titers of ≥80 (7), were found in most subjects at 4 weeks, i.e., 2 weeks later than lymphocyte reactivity. This response time is identical to that reported by Buchanan et al. (2), who found positive skin tests in most subjects at 2 weeks and positive agglutination tests only at 4 weeks

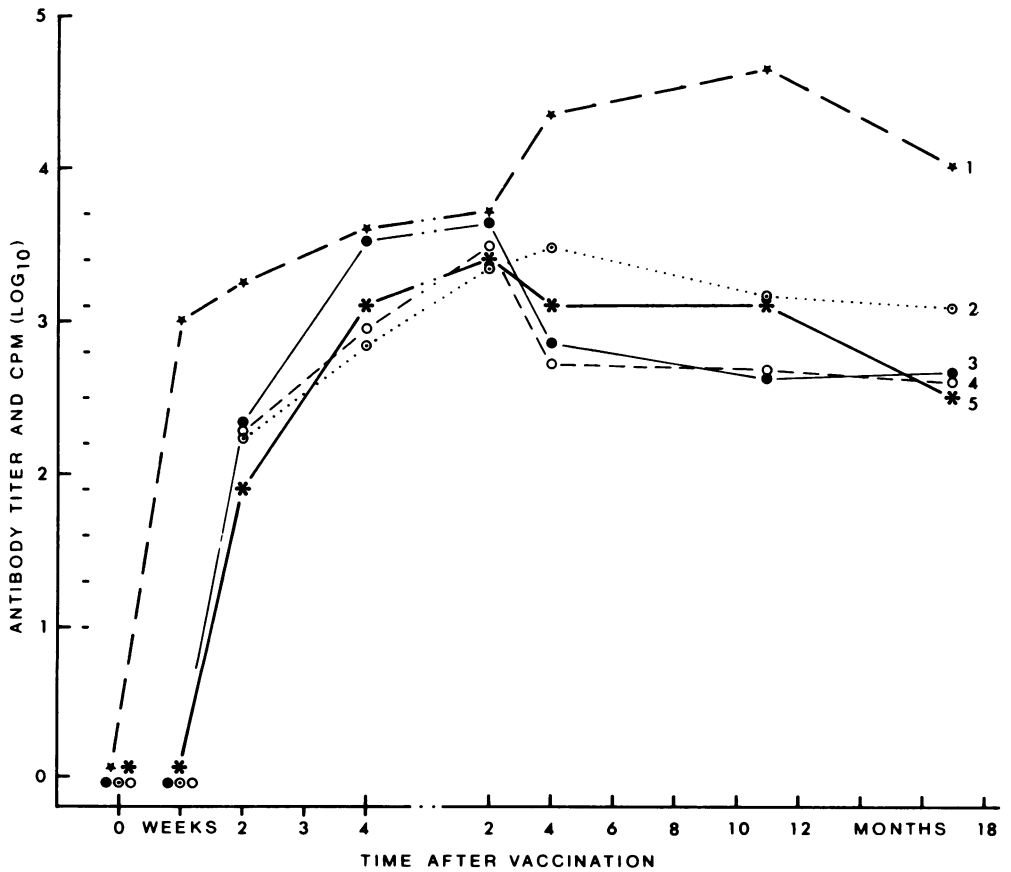

FIG. 3. Cell-mediated (1) and humoral (2–5) immune responses in a 28-year-old man after vaccination with a live attenuated strain of *F. tularensis*. 1, counts per minute; 2, IgG titer; 3, IgM titer; 4, IgA titer; 5, agglutination titer.

TABLE 3. Timing of the humoral response (agglutination titer ≥80) and cell-mediated response after inoculation with live tularemia vaccine (A) in the present study and (B) in that of Buchanan et al. (2)[a]

Wk after vaccination	Humoral response		Cell-mediated response	
	A	B	A	B
1	0/4	0/21	1/4	6/21
2	1/11	1/19	10/11	19/20
3		5/17		13/15
4	13/13	16/19	12/13	
8	10/10	17/20	10/10	

[a] The figures indicate positive findings/subjects studied. Buchanan et al. measured cell-mediated response by using the skin test, whereas we used the blast transformation test.

after inoculation with a live attenuated *F. tularensis* vaccine (Table 3). In tularemia infections, a skin test similarly becomes positive before the formation of agglutinating antibodies with a significant titer (8, 9, 12, 14).

Antibodies have a tendency to decrease with time, as found in this study, and agglutinins may disappear within 2 years after tularemia vaccination (33), although they are demonstrable in most vaccinees up to 6 years after inoculation (27). In contrast to the antibodies, cell-mediated immunity, which represents the most important mechanism in resistance against *F. tularensis* (1, 5, 19, 34), seems to remain unchanged for years after vaccination as well as after natural tularemia (2, 18). In the present study, lymphocyte reactivity remained unchanged for up to 1.5 years, and in long-term follow-up studies, positive skin tests have been found in most subjects 6 (27) and even 8 (29) years after vaccination. Tärnvik et al. (33) have reported strong lymphocyte blast transformation responses in subjects without demonstrable agglutinins 2 years after tularemia vaccination. These results verify that the blast transformation test (18, 33), which demonstrates cell-mediated immunity, is a suitable method for evaluating immunity to *F. tularensis* and also for diagnosing early cases of tularemia before the development of significant agglutination titers.

Although the synthesis of IgM, IgA, and IgG, as determined by ELISA, began simultaneously, and all of these immunoglobulin classes were still detectable 1.5 years after vaccination, their response curves were different, with IgM the earliest to attain its peak and IgG the latest. The agglutination test preferentially measures IgM antibodies, as reflected in the similar curves for agglutinins and IgM antibodies (Fig. 3, Table 2), whereas the effect of the other antibodies is more clearly noticeable in the complement fixation test. The highest values for complement-binding antibodies are found 2.4 months after tularemia vaccination (P. Koskela and E. Herva, unpublished data), 2 weeks later than those for IgM and 2 weeks earlier than those for IgG.

Carlsson et al. (4) have shown that in tularemia patients IgM and IgG are demonstrable 2.5 years after infection, and we have found that IgM, IgA, and IgG all may persist for up to 11 years (Koskela and Salminen, manuscript in preparation). Therefore, the presence of IgM in a single serum sample, contrary to the case of yersinosis (15), does not always indicate acute disease. For this reason, in addition to the similarity in the timing of the ELISA and agglutination test findings shown here, ELISA does not appear to be superior to the classic tube agglutination method for the serodiagnosis of tularemia, generally requiring two consecutive serum samples with a significant increase in the titer.

Long-lasting IgA and IgM anti-*Francisella* antibodies induced by both vaccination and natural infection are exceptional among infectious diseases. Generally these antibodies concern primary immune responses and disappear within some months after contact between the immune mechanism and the causative agent, as in the majority of acquired toxoplasmosis cases (16). Sometimes, however, IgM antibodies may persist for years after the acute stage of toxoplasmosis (16), as also after rubella infection (22, 30). Prolonged virus-specific IgM responses have also been described after Japanese encephalitis (10) and yellow fever vaccination (23). IgA persisting for years has been reported in patients with yersinia arthritis, whereas IgM in these cases and others without arthritis vanishes within some months of the onset of symptoms (15).

The mechanism inducing continuous synthesis of IgA and IgM has no explanation at present, although it may be assumed that intracellular *F. tularensis* bacteria or their structures remain in the host for a long time, boosting antibody formation and cell-mediated immunity.

Although the duration of immunity induced by live *F. tularensis* vaccine seems to extend to many years, this does not provide absolute host resistance against *F. tularensis*. According to Burke (3), laboratory-acquired cases of typhoidal tularemia decreased very significantly, and the clinical signs and symptoms of ulceroglandular tularemia, the incidence of which remained unchanged, were milder after the live vaccine had replaced the phenol-killed Foshay vaccine in the United States. Mass inoculations with viable *F. tularensis* vaccine have been performed in the Soviet Union since 1946 (35), and the interval for revaccination for people living in endemic areas is officially set at 5 years (24). According to Olsuf'ev (25, 26), a sharp, stable

reduction in the incidence of human tularemia in the USSR has been achieved by this program of extensive vaccination.

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