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Humoral Immunity against *Francisella tularensis* after Natural Infection

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Forty-two subjects with acute tularemia were studied for the occurrence of C-reactive protein (CRP), and 73 subjects with acute tularemia or experience of the disease within the last 11 years were studied for immunoglobulin M (IgM), IgA, and IgG class-specific antibodies, agglutinating antibodies, and complement-fixing antibodies to *Francisella tularensis* by using an enzyme-linked immunosorbent assay (ELISA), the tube agglutination test, and a complement-fixing ELISA. The incubation time between infection and the outbreak of symptoms varied from 1 to 10 days, averaging 6.5 days. Elevated CRP concentrations were found in all samples taken in the first 6 days of illness, when the antibodies generally were absent. The highest CRP values, up to 165 mg/liter, occurred in the earliest samples and then decreased rapidly, being undetectable (<1 mg/liter) from 1 month after the onset of symptoms. Simultaneous though individually varying formation of IgM, IgA, and IgG class-specific antibodies to *F. tularensis* was demonstrable by ELISA in all the tularemia patients during the acute stage. In most cases, these antibodies appeared 6 to 10 days after the onset of symptoms, i.e., about 2 weeks after infection, reached their highest values at 4 to 7 weeks, and, despite a decreasing trend in their level, were still present 0.5 to 11 years after onset of tularemia, as demonstrable by the agglutination test and by the complement-fixing ELISA. Of the three methods used, ELISA for IgM, IgA, and IgG proved to be the most efficient for the early serodiagnosis of tularemia.

The humoral immune responses to *Francisella tularensis* are well known as far as agglutinating antibodies are concerned, due to the masterly studies by Francis and Evans (7) and Ransmeier and Ewing (23). These authors demonstrate that agglutinins appear in the second week of illness, reach their maximum in the fourth to the seventh week, and, despite a decreasing trend, are still detectable over 10 years later. Cell-mediated immune responses are also demonstrable years and even decades after tularemia (1, 12).

Immunoglobulin M (IgM) IgA, and IgG class antibodies to *F. tularensis* are all very long lasting, and their presence has been shown by enzyme-linked immunosorbent assay (ELISA) years after tularemia infection and also after vaccination (2, 13). Thus, humoral immunity to *F. tularensis* seems to be exceptional among infectious diseases.

To obtain more exact information on humoral tularemia immunity, we determined the presence of IgG, IgM, and IgA antibodies to *F. tularensis* at various intervals after the onset of tularemia, during a follow-up period extending from the first day of illness to 11 years after disease and studied the ability of the antibodies to agglutinate bacteria and to activate complement. The occurrence of C-reactive protein (CRP) in tularemia was also studied, since this has been found in increased amounts in the serum of patients with a wide variety of diseases associated with active inflammation or tissue destruction, including bacterial infections (see reference 22), and elevated levels of CRP can thus be expected in acute tularemia.

MATERIALS AND METHODS

Subjects and serum samples. A total of 199 sera from 73 subjects with clinically typical tularemia confirmed by antibody determination at the Department of Medical Microbiology, University of Oulu, from 1967 through 1978, were studied. Most subjects had suffered from the ulceroglandular (53, or 73%) or glandular (18, or 25%) form of the disease, but there was one (1%) subject with the oculoglandular form and one (1%) with oropharyngeal tularemia. The subjects consisted of 41 (56%) males and 32 (44%) females, and their ages varied from one to 71 years (average, 38 years). The day and source of infection were known in 14 cases, and the timing of the onset of symptoms was known to the day in 59 cases and to the week in 14.

All the subjects were from rural areas around Oulu, northern Finland, where tularemia epidemics have occurred since 1967. Most cases in the area up to 1978 consisted mainly of ulceroglandular and glandular tularemia, resulting chiefly from bites from blood-sucking insects, such as mosquitos and horseflies, and direct contacts with sick or dead hares.

The majority of the samples (170 sera) had been sent to the laboratory by physicians for serodiagnosis and the monitoring of antibody levels. These samples were all taken at the acute or convalescent stage, between 1 day and 6 months after the onset of symptoms. Blood samples were also taken from 29 subjects 0.5 to 11 years after the onset of tularemia. The sera were stored at −20°C, and all the samples from the same subject were studied simultaneously. Agglutinating antibodies to *F. tularensis* were determined in all 199 samples, class-specific immunoglobulins (IgG, IgM, and IgA) were assayed in 146 samples, and complement-fixing antibodies to *F. tularensis* were assayed in 139 samples. CRP was quantified in 109 sera from 42 subjects with acute tularemia.

ELISA. The antibodies of different immunoglobulin classes were determined by ELISA with bacterial sonicate as the antigen. The procedure was carried out on disposable polystyrene microtiter plates with flat-bottomed wells (Dynatech Laboratories, Inc., Alexandria, Va.).

The antigen was obtained by culturing a strain of live

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attenuated tularemia vaccine (BB IND 157, lot 11) supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md., for 3 days on plates of blood agar enriched with glucose and cysteine. The colonies were suspended in 0.5% Formalin in 0.05 M phosphate-buffered saline, (PBS; pH 7.2) for 20 h. After washing three times with PBS (10 min, 3,000 rpm in MSE Super Minor; MSE Scientic Instruments, Crawley, United Kingdom), the suspension was sonicated for 2 min at an amplitude of 20 μm (model 150-W ultrasonic disintegrator; MSE Scientific Instruments), and the supernatant of the sonic fluid (5 min, 4,000 rpm) was diluted with 0.05 M PBS (pH 7.2) to a suitable antigen concentration determined in preliminary tests against dilutions of pooled sera from 10 tularemia patients. The optimal antigen dilution was found to have a protein content of 2 5 μg/ml. The protein concentration of the sonicate antigen was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Antigen suspension (200 μl) was added to the wells, and the microtiter plates were incubated at 37°C for 6 h and washed three times for 5 min with 0.05 M PBS (pH 7.2) supplemented with 0.05% Tween 20 (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 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, which were found to be optimal in preliminary tests, were used. After incubation for 2 h, the plates were washed as before, and 100 μl of fresh substrate was added to the wells for incubation for 30 min at 37°C. 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-magnesium chloride buffer (pH 10; Orion Diagnostica) was used as the substrate (1 mg/ml). The amount of alkaline phosphatase bound to the wells was determined by photometric estimation of the *p*-nitrophenylate released. A_{405} was measured with a Titertek Multiskan (Eflab, Helsinki, Finland). Measurements were carried out against antigen-sensitized wells treated with 4% PBS-Tween, antisera, substrate, and 2 N NaOH. The levels of antibodies in the sample are given as ELISA titers (5, 11) read at optical density values of 0.3 for IgG and 0.1 for both IgM and IgA limits derived from the mean plus two standard deviations of absorbances recorded at a dilution of 1:100 in 60 control sera without agglutinating antibodies. Titers below 100 were regarded as negative.

CF-ELISA. Complement-fixing antibodies were determined by using CF-ELISA, a complement-fixing modification of ELISA (P. Koskela, Vaccine, in press). The sera, complement, and enzyme conjugate were diluted in 4% PBS-Tween, and the diluted sera were inactivated before the test (30 min at 56°C). Fresh human AB serum without antibodies to *F. tularensis* was used as a source of complement, and alkaline phosphatase-labeled anti-human C3c was used as conjugate. The incubation time for the serum samples, complement, and enzyme-labeled antisera was 1 h. In all other respects, the procedure was similar to that for ELISA. The CF-ELISA titers were read at an absorbance of 0.1, and titers below 100 were regarded as negative.

Tube agglutination. The agglutinating antibodies were determined by using a Widal bacterial agglutination test, as initially described for tularemia by Francis and Evans (7). Serial twofold dilutions of the sera, starting from 1:5, were prepared in PBS (pH 7.2). Tubes containing 0.5 ml of this and an equal amount of a standardized suspension of Formalin-killed whole bacteria (National Bacteriological Laboratory, Stockholm, Sweden) were incubated overnight (20 h) at 37°C. The antibody titers were expressed as the reciprocal of the highest dilution giving visible agglutination with a clear supernatant, and titers below 10 were considered as negative.

CRP determination. CRP was measured by the Mancini single immunodiffusion method (LC-Partigen-CRP, Behringwerke AG, Marburg, Federal Republic of Germany) with a minimum detectable concentration of 1 mg/liter. CRP measurements below this value were regarded as negative or normal.

RESULTS

Incubation time and certain clinical aspects. The incubation time, i.e., the delay between infection and outbreak of symptoms, varied from 1 to 10 days, averaging 6.5 days. The delay between the onset of symptoms and the seeking of treatment ranged from 1 to 45 days, averaging 7 days, whereas that between the onset of symptoms and the first serum sample for tularemia antibodies varied from 1 to 63 days, being 15 days on average. Serological diagnosis of tularemia took place at 4 to 45 days (20 days on average) after the onset of disease.

The fever generally vanished in 2 weeks, and the lymphadenopathy vanished in 5 weeks. Despite adequate antibiotic treatment, two patients with ulceroglandular and glandular tularemia suffered from a persistent fever for 10 weeks and still had enlarged axillary lymph nodes 8 months afterwards. Diseased muskrats in spring (April through May) had served as the source of infection for these patients.

CRP. Elevated levels of CRP, with a range of 14 to 165 mg/liter and a mean of 49.3 mg/liter, were found in all patient sera during the first 6 days of tularemia (Fig. 1), at a time when the antibodies to *F. tularensis* were undetectable (Table 1; Fig. 2), but then CRP decreased rapidly. About half of the patients had a normal CRP value at 11 to 20 days, and from 1 month, all the measurements were negative for CRP (Fig. 1). In all cases, the highest CRP values were observed in the first serum sample, a decrease setting in from the second samples onwards.

Antibodies. Individual differences were observed in the immunization time and the level and persistence of the antibodies appearing 6 to 10 days after the onset of symptoms in most patients, i.e., about 2 weeks after infection.

The earliest IgG class antibodies were found on the first day of tularemia, and the latest ones appeared 12 days after the onset of symptoms. The corresponding ranges for IgM, IgA, and agglutinating antibodies were 2 to 14 days, 2 to 18 days, and 4 to 11 days, respectively, and 4 to 11 days for complement-fixing antibodies.

IgM antibodies occurred in 18.2% of the samples in the first 5 days of tularemia, IgA antibodies occurred in 9.1%, and IgG antibodies occurred in 36.4%. The corresponding frequencies for days 6 to 10 were 75.0, 58.3, and 91.7%, respectively (Table 1), the differences in these frequencies being without statistical significance in the chi-square test.

In 23 subjects, the number of serum samples and the duration of the follow-up period were sufficient to show the timing of the maximal antibody levels. The highest individual IgG titers (ranging from 640 to 110,000) were found at 16 to 90 days (average, 41 days) after the onset of tularemia,

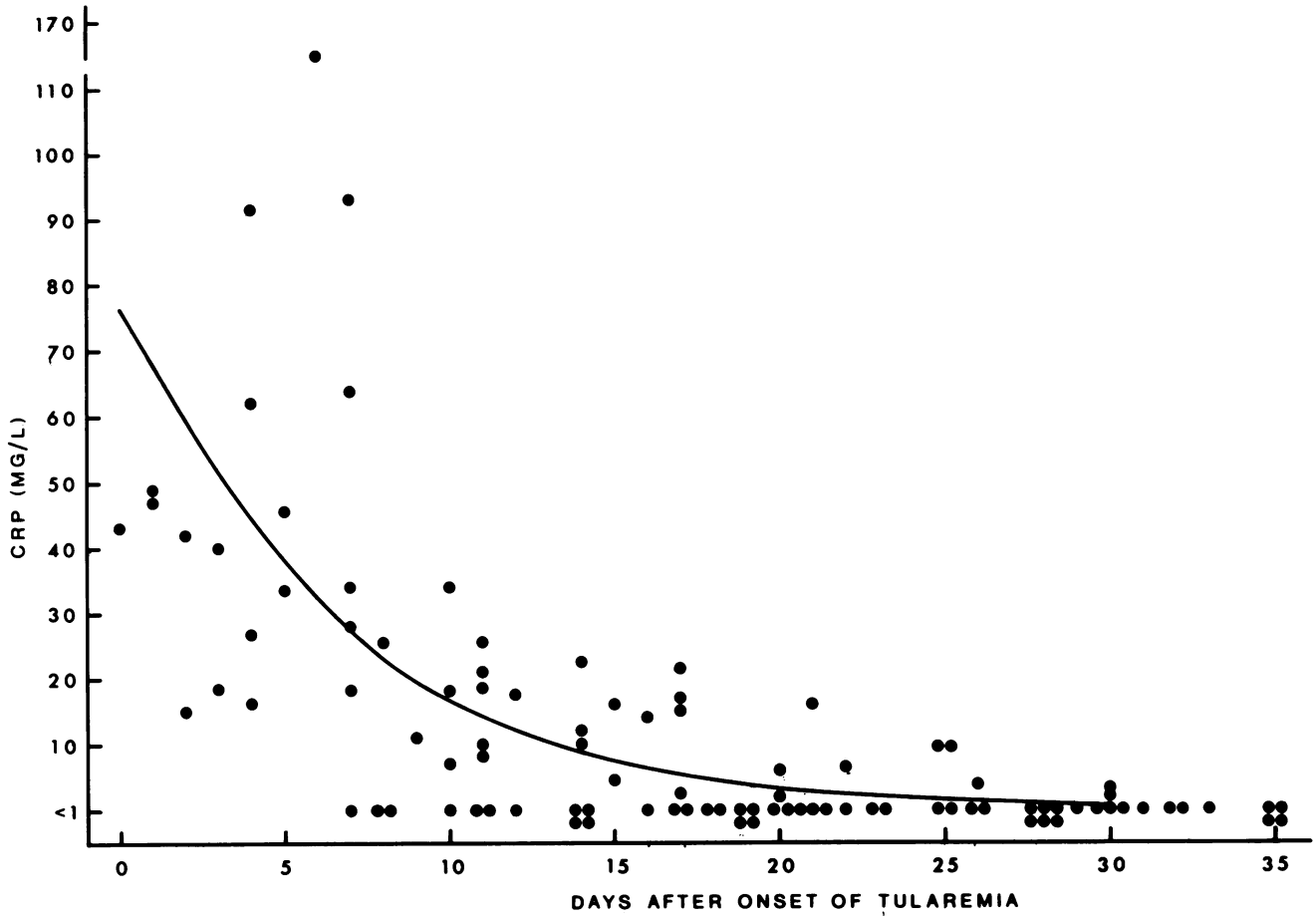

FIG. 1. CRP concentrations in the serum at various intervals after the onset of tularemia.

whereas the corresponding time for IgM (430 to 16,100) was 16 to 71 days (average, 38 days) and that for IgA (410 to 20,500) was 16 to 71 days (average, 33 days). The maximum individual agglutinating titers (ranging from 80 to 20,480) were found at 13 to 71 days (average, 38 days), whereas the corresponding time for complement-fixing antibodies (200 to 30,000) was 11 to 71 days (average, 35 days). An analysis of variance did not reveal any differences in the timing of the highest levels of the different immunoglobulin classes, agglutinating antibodies, or complement-fixing antibodies. The behavior of IgG nevertheless differed from those of IgM and IgA later by decreasing more slowly: IgM and IgA showed a clear declining trend at 3 to 6 months, whereas IgG remained at high levels (Table 1).

IgG, IgM, and IgA class antibodies to *F. tularensis* were still present 2 to 11 years after the onset of the disease and retained the ability to agglutinate bacteria and fix complement (Table 1; Fig. 2). The only exceptions consisted of a lack of IgM at 5 months and at 3 years after the onset of tularemia in two subjects and an absence of IgA at 11 years in one subject (Table 1).

Comparison of ELISA, CF-ELISA, and the agglutination test. ELISA results for IgG, IgM, and IgA correlated highly significantly with results obtained by CF-ELISA and also with those obtained by the agglutination test, when assessed by linear regression analysis with logarithmic transformation of titers (Table 2). Highly significant correlation ($r = 0.82$) was found also between CF-ELISA and the agglutination test. Results of both the CF-ELISA and the agglutination test correlated slightly more strongly with IgM, but the differences between the correlation coefficients for different immunoglobulin classes remained without statistical significance.

The frequencies for the three single immunoglobulin classes detected by ELISA in samples from days 0 through 5, 6 through 10, and 11 through 20 of tularemia, and the corresponding frequencies for CF-ELISA and the agglutination test were statistically similar when assessed by the chi-square test (Tables 1 and 2).

Antibodies of IgM, IgA, or IgG were found by ELISA in 36.4% of the samples from the first 5 days of tularemia, in 91.7% of those from days 6 through 10, and 96.7% of those from days 11 through 20, the corresponding frequencies for CF-ELISA being 18.2, 70.0, and 88.0%. The agglutination test similarly found antibodies with titers of $\geq$10 in 7.1, 65.0, and 97.8% of cases, respectively; i.e., it was just as efficient as ELISA and CF-ELISA, as evaluated by the chi-square test, whereas the frequencies of 0.0, 15.0, and 71.7%, respectively, for titers of $\geq$80 were significantly lower than those observed in ELISA ($P < 0.05$, $P < 0.001$, and $P < 0.01$) and in CF-ELISA (not significant, $P < 0.01$, and not significant).

The ELISA finding was positive in five cases without agglutinins, four of them consisting of IgG and one consisting of IgA antibodies, whereas the agglutination test was positive in one case with no detectable antibodies in ELISA.

TABLE 1. Percentage distribution of ELISA titers (IgM, IgA, and IgG) and CF-ELISA titers at various intervals after the onset of tularemia

Time after onset of tularemia	% Distribution of ELISA titers										
	<100	100–249	250–499	500–999	1,000–1,999	2,000–3,999	4,000–7,999	8,000–15,999	16,000–31,999	>32,000	*n*
IgM											
0–5 days	81.8		18.2								11
6–10 days	25.0	41.7	16.7	16.7							12
11–20 days	10.0	13.3	26.7	13.3	6.7	10.0	13.3	3.3	3.3		30
21–40 days		5.3	13.2	7.9	21.1	29.0	13.2	10.5			38
41–60 days			10.5	21.1	10.5	21.1	15.8	21.1			19
3–6 mo	7.7		15.4	7.7	23.0	30.8	7.7	7.7			13
7–12 mo			57.1	28.6		14.3					7
2–4 yr	14.3	14.3	71.4								7
11 yr			100.0								9
IgA											
0–5 days	90.9	9.1									11
6–10 days	41.7	25.0	25.0	8.3							12
11–20 days	10.0	16.7	16.7	20.0	10.0	10.0	16.7				30
21–40 days		5.3	7.9	23.7	18.4	23.7	10.5	10.5			38
41–60 days		5.3	10.5	21.1	26.3	21.1	5.3	5.3	5.3		19
3–6 mo				61.5	30.8	7.7					13
7–12 mo			28.6	57.1	14.3						7
2–4 yr		28.6	28.6	28.6		14.3					7
11 yr	11.1	11.1	77.8								9
IgG											
0–5 days	63.6	9.1	18.2	9.1							11
6–10 days	8.3	16.7	41.7	8.3	16.7	8.3					12
11–20 days	10.0	3.3	23.3	10.0	13.3	6.7	16.7	13.3	3.3		30
21–40 days			7.9	21.1	13.2	10.5	10.5	26.3	10.5		38
41–60 days			5.3		31.6		15.8	26.3	15.8	5.3	19
3–6 mo					23.1	7.7		38.5	30.8		13
7–12 mo			14.3	14.3	14.3		14.3	42.9			7
2–4 yr			14.3	28.6	28.6	28.6					7
11 yr		11.1	11.1	33.3	44.4						9
Complement-fixing antibody											
0–5 days	81.8		18.2								11
6–10 days	30.0	40.0	20.0		10.0						10
11–20 days	12.0	48.0	16.0			16.0	4.0		4.0		25
21–40 days		21.1	29.0	10.5	15.8	2.6	7.9	10.5	2.6		38
41–60 days		5.3	10.5	21.1	31.6	10.5	21.2				19
3–6 mo		7.7	15.4	30.8	23.1		15.4	7.7			13
7–12 mo		14.3	42.8	28.6	14.3						7
2–4 yr		57.1	28.6	14.3							7
11 yr		88.9	11.1								9

With these exceptions, the ELISA and agglutination test findings were parallel. In no case was CF-ELISA positive earlier than ELISA or the agglutination test.

DISCUSSION

CRP is synthesized by hepatocytes (10) and is a normal trace constituent in the serum, occurring in concentrations of 0.070 to 0.580 mg/liter in healthy individuals (4). Increased CRP levels have been found in various diseases with active inflammation or tissue destruction, including bacterial infections (see reference 22). The maximum CRP values in the tularemia patients (14 to 165 mg/liter) were observed in the first serum samples, taken during the first few days of the disease, at a time when antibodies to *F. tularensis* were absent. By far the highest CRP levels, however, occurred earlier on, since CRP decreased in the later samples.

CRP binds specifically to a variety of substances originating from microbes or damaged autologous cells (8, 21, 28, 29), and complexed CRP will potentially activate complement (classical pathway), mediating complement-dependent adherence (20, 25). Thus, CRP may generate a protective mechanism which acts before specific immunity has developed. According to Pepys (22) the main role of CRP, however, may be to recognize toxic autogenous materials in the plasma when these have been released from damaged tissues, to bind and detoxify them, and to facilitate their clearance.

The CRP concentration in serum is higher in severe forms of tularemia than in milder forms, and it tends to be higher in pulmonary tularemia than in the ulceroglandular type (H. Syrjälä, unpublished data). In these respects, it differs from specific antibodies, which show no difference in level between different clinical forms or severities of tularemia (H. Syrjälä, P. Koskela, T. Ripatti, A. Salminen, and E. Herva, J. Infect. Dis., in press).

The IgM, IgA, and IgG antibodies induced by *F. tularensis* infection generally occurred simultaneously, as also observed in the various clinical types and severities of the disease (Syrjälä et al., in press) and after vaccination (13). The tendency for IgG to appear earlier than other antibodies

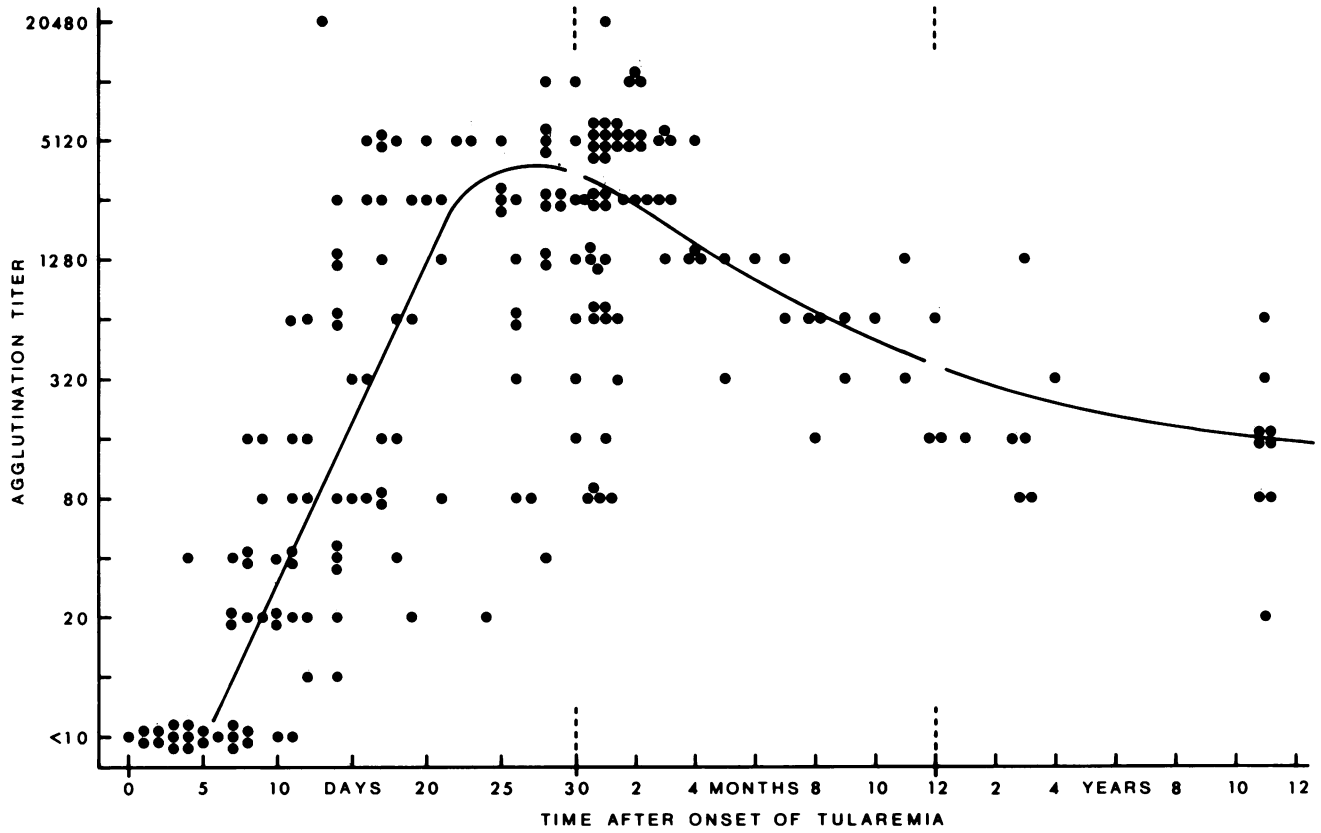

FIG. 2. Agglutination antibody titers at various intervals after the onset of tularemia.

agrees with the results of Viljanen et al. (30). The occurrence of IgG and IgA in samples without any positive reaction in the agglutination test indicates that they are weak agglutinogens, as reported for antibodies against *Brucella* spp. (31).

Carlsson et al. (2) have shown that IgM and IgG antibodies to *F. tularensis* are demonstrable 2.5 years after tularemia infection. In this study, we found that IgM, IgA, and IgG may all persist for at least 11 years and retain their ability to agglutinate bacteria and fix complement (see below). The present agglutination results were identical with those of Francis and Evans (7) and Ransmeier and Ewing (23) as far as timing, level, and persistence were concerned.

F. tularensis seems to be exceptional among infectious diseases in that it induces a pattern of humoral immunity which includes continued synthesis of IgM, IgA, and IgG for years after both infection and vaccination, as IgM and IgA antibodies generally concern primary immune responses and vanish within some months after contact between the immune mechanism and the causative agent, as is mostly the case in acquired toxoplasmosis (9). IgM antibodies occasionally may be present for years after the acute stage of toxoplasmosis (9), however, as has also occurred after rubella infection (18, 26). Long-lasting IgM have also been found after Japanese encephalitis (6) and yellow fever vaccination (19).

The mechanism stimulating the continuous formation of IgM, IgA, and IgG is unknown at present, but it may be that intracellular *F. tularensis* bacteria or their structures remain in the host for a long time, boosting humoral and cell-mediated immunity persisting for years after natural tularemia (12) or vaccination (13).

Antibody classes differ greatly in their ability to fix complement (15): IgM, IgG3, and IgG1 antibodies are the most efficient, and IgG2 is weaker, while IgG4 and IgA fail entirely to activate the first component of complement, i.e., the classical pathway. IgM and IgG antibodies to *F. tularensis* have been shown to promote phagocytosis and the killing of *F. tularensis* bacteria by human polymorphonuclear leucocytes in the presence of complement, these events being supported by IgG even in the absence of complement, though to a lesser extent if C3 is removed (17). Opsonization and complement adherence reactions are mediated largely by the component C3b and Fc fragments of IgG, for which receptors exist on the membranes of various phagocytic cells (16). These data give us reason to assume that antibodies with complement-fixing activity in the humoral immune system provide a better protection against tularemia than those without complement-fixing activity.

TABLE 2. Correlation coefficients between ELISA and CF-ELISA and between ELISA and the agglutination test for antibodies to *F. tularensis*[a]

ELISA	Correlation with	
	CF-ELISA	Agglutination test
IgM	0.77	0.84
IgA	0.67	0.76
IgG	0.74	0.74
IgM plus IgA plus IgG	0.78	0.78

[a] Values were assessed by linear regression analysis with logarithmic transformation of titers. $n = 139$.

Moreover, the role of cell-mediated immunity is most important in resistance to *F. tularensis* (3, 14, 27).

As a rule, immunity induced by tularemia infection provides absolute host resistance against *F. tularensis*, and no case of clinical tularemia reinfection has yet been found in Finland.

The agglutination test is easy and reliable, and it is the most widely used assay for serodiagnosis of tularemia. It preferentially measures IgM antibodies but is affected also by IgA and IgG, which are poor agglutinogens. Because of possible cross-reactions with low titers in the agglutination test for tularemia (7), a titer of ≥80 and a fourfold change of titer in consecutive samples are generally considered as diagnostically significant. In the present study, serological confirmation for every patient was performed by the agglutination test, and generally it is the only assay required for the diagnosis of tularemia. Only a few exceptional tularemia cases with totally negative results or with insignificant low titers in the agglutination test are found. In these cases, seroconversion has been detected by ELISA (30; Syrjälä et al., in press).

Sandström et al. (24) report that ELISA measures mostly antibodies to carbohydrate determinants of *F. tularensis*. The comparative study by Viljanen et al. (30) nevertheless suggests that sonicate antigen is at least as good an antigen in ELISA for *F. tularensis* as is lipopolysaccharide. Their inhibition tests with various bacteria such as *Brucella* and *Yersinia* spp. also show that ELISA with a sonicate of *F. tularensis* as the antigen is highly specific.

The main advantages of ELISA to the serodiagnosis of many infections are its high sensitivity and its ability to determine IgM and IgA antibodies indicating acute disease. The presence of long-lasting IgM and IgA after tularemia infection or vaccination (13) nevertheless suggests that serodiagnosis of tularemia, especially in epidemic areas, also by ELISA generally requires two consecutive serum samples with a significant change in the titer. In early tularemia, ELISA found antibodies more frequently than did the agglutination test (titers of 10 to 40 included) but due to scanty material the differences did not attain a statistical significance; the differences, however, were significant when compared with agglutination titers of >80. The ability of ELISA to detect tularemia cases overlooked in the agglutination test and the rapidity of ELISA in diagnosis of tularemia are advantages with benefits in hastening the beginning of adequate antibiotic treatment.

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