

## Comparative Analysis of Antibodies to *Francisella tularensis* Antigens during the Acute Phase of Tularemia and Eight Years Later

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**Approximately 8 years after treatment for tularemia, 14 of 22 (63.6%) individuals tested still had a positive microagglutination test for *Francisella tularensis* antibodies. An enzyme-linked immunosorbent assay for anti-*F. tularensis* outer membrane antibodies was positive for 55% (immunoglobulin A [IgA]), 95% (IgG), and 27% (IgM) of the late-phase sera, but with antibody levels significantly reduced from those in the acute-phase sera. IgG and IgA antibody levels in the late-phase sera showed significant correlation with levels in the acute-phase sera. The IgG/IgM ratio calculation discriminated between acute-phase and persistent antibodies for most sera, but Western blot (immunoblot) patterns did not. Immunoblotting indicated that the *F. tularensis* lipopolysaccharide is a major target for antibodies in both groups of sera. Our results substantiate the need for caution in the interpretation of positive serological test results for tularemia, which could result from disease occurring years earlier.**

Cell-mediated immunity against *Francisella tularensis* antigens plays a major role in protection against tularemia (3), whereas the humoral immunity induced in patients with tularemia is particularly important in the diagnosis of the disease, which depends on serological testing in most cases. However, positive serological test results for *F. tularensis* do not invariably indicate ongoing or recent *F. tularensis* infection, since high levels of anti-*F. tularensis* antibodies may persist for years after the disease (4, 8).

In the present study, we examined the persistence of serum antibodies against antigens of an *F. tularensis* outer membrane (OM) preparation approximately 8 years after the occurrence of tularemia. The individuals had suffered from tularemia during an outbreak in our area in 1984-1985, when a total of 57 cases were reported (1). In collaboration with the primary physicians, we obtained serum specimens from 22 of the individuals in 1992 and tested the late-phase sera along with an acute-phase serum which had been stored at  $-20^{\circ}\text{C}$  since the outbreak. The clinical pictures for the 22 tularemia cases in 1984-1985 were as follows: 3 were glandular type; 6 were ulceroglandular; 9 were oropharyngeal; 3 were febrile illness with myalgia and arthralgia; and no information was available for 1. All of the tularemia patients had received treatment with tetracycline during the acute phase of the disease and were declared cured. None of them had received vaccine. In 1992, they ranged in age from 23 to 71 years. All of them were men.

An *F. tularensis* biovar palaeartica strain which was isolated from the liver of a dead hare was cultured as described before (1). Formalin-killed and washed bacteria were stained with safranin and used as the antigen in a microagglutination (MA) test (1). *F. tularensis* OM was prepared by extraction of the bacteria in buffer containing 0.2 M lithium chloride and 0.1 M sodium acetate (pH 6.0), and partially purified by ultracentrifugation and washing (1).

The MA test was performed as described previously (1) by testing serum dilutions beginning at 1:10 in V-shaped microti-

ter wells. Titers of  $>1:40$  were defined as positive. The *F. tularensis* OM enzyme-linked immunosorbent assay (ELISA) for immunoglobulin A (IgA), IgG, and IgM antibodies was performed as reported previously (1). Briefly, coats were prepared with 25  $\mu\text{l}$  of the OM per ml; alkaline phosphatase-conjugated class-specific antibodies (Sigma Chemical Co., St. Louis, Mo.) were used for antibody detection when human sera were tested at a dilution of 1:1,000, and *p*-nitrophenyl phosphate (Sigma) was used as the substrate. The optical density at 405 nm, corrected for background color (1), was multiplied by the dilution factor (1,000) to calculate ELISA units. The cutoff levels for positive tests (shown in Table 1) were calculated in a previous study (1) and applied in diagnostic serology in our laboratory.

Western blotting (immunoblotting) was performed essentially as described earlier (1, 2), except that electrophoretically separated components were transferred onto polyvinylidene difluoride protein-sequencing membranes (Bio-Rad Laboratories, Richmond, Calif.) instead of nitrocellulose membranes. Sera were tested at dilutions of 1:1,000 for IgG antibodies and 1:250 for IgA and IgM antibodies. Class-specific peroxidase-conjugated rabbit antibodies (Sigma) were used to detect antibody binding.

Statistical analysis was based on Student's *t* test to evaluate differences between means and on the Pearson formulas to calculate correlations.

In the MA test, 21 of 22 (95.4%) of the acute-phase sera and 14 of 22 (63.46%) of the late-phase sera were positive. A significant reduction in titer was recorded, with mean  $\pm$  standard deviation (SD) of titers reduced from  $(\log_{10}) 2.628 \pm 0.442$  to  $(\log_{10}) 1.875 \pm 0.346$  ( $P < 0.001$ ). The *F. tularensis* OM ELISA measured highly elevated levels of antibodies of all three Ig classes in the acute-phase sera (Table 1), in agreement with our earlier study (1). Eight years later, the levels of antibodies for all three Ig classes were significantly reduced ( $P < 0.05$ ). However, considerable levels of antibodies persisted, and 21 of 22 (95.4%) individuals were still positive for IgG antibodies. Also, 55 and 27% of them were still positive for IgA and IgM antibodies, respectively. Our findings substantiate previously described observations of long persistence of anti-

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TABLE 1. Antibodies to the *F. tularensis* OM in sera obtained from 22 patients during the acute phase of tularemia and 8 years later<sup>a</sup>

Sera	IgA		IgG		IgM	
	Mean ELISA units $\pm$ SD (range)	% of sera positive	Mean ELISA units $\pm$ SD (range)	% of sera positive	Mean ELISA units $\pm$ SD (range)	% of sera positive
Acute phase	269 $\pm$ 192 (51-632)	100	988 $\pm$ 306 (140-1,429)	100	381 $\pm$ 233 (12-840)	91
Late phase	107 $\pm$ 111 (0-400)	55	553 $\pm$ 290 (98-1,136)	95	50 $\pm$ 56 (0-192)	27

<sup>a</sup> The cutoff levels for positive tests were defined as  $\geq 47$  ELISA units for IgA,  $\geq 115$  ELISA units for IgG, and  $\geq 56$  ELISA units for IgM antibodies (1).

bodies after tularemia (4, 8) and that this is also the case for IgA and IgM antibodies. If we assume a constant rate of decay of the antibodies and interpolate our data, then we can estimate that it will be another 8 years before the average IgG antibody level has decreased to the cutoff level used to define a positive test.

The data were analyzed to test whether a correlation existed between the ELISA units recorded for the acute-phase sera and those for the late-phase sera. For IgG antibodies, a highly significant correlation was found ( $r = 0.809$ ;  $P < 0.001$ ). Correlation was also evident for the IgA antibodies ( $r = 0.691$ ;  $P < 0.001$ ) but was less convincing for the IgM antibodies ( $r = 0.473$ ;  $0.02 < P < 0.05$ ). Thus, the antibody level reached in the acute phase of the disease is one of the factors which determine the levels of antibodies which persist at a given point in time.

The long persistence of anti-*F. tularensis* antibodies indicates that physicians should be cautious in interpreting positive serological tests for tularemia as an indication of current infection. Estimation of the IgG/IgM ratio has been proposed as a means to discriminate between acute-phase and persistent antibodies (4). We found a mean IgG/IgM ratio  $\pm$  SD of  $4.9 \pm 7.4$  (range, 0.9 to 36.2) for the acute-phase sera, and 18 of 22 (81.8%) sera had a ratio of  $\leq 5$ . For the late-phase sera, a ratio of  $46.6 \pm 113.9$  (range, 3.9 to 547) was found, and 2 of 22 (9.1%) sera had a ratio of  $\leq 5$ . Our findings indicate that ratio calculation will be of use in diagnosing an infection in progress, if it is based on well-standardized ELISA results.

Next, we analyzed whether the banding patterns obtained by immunoblotting could discriminate between acute-phase and persisting antibodies. Figure 1 shows the results obtained for

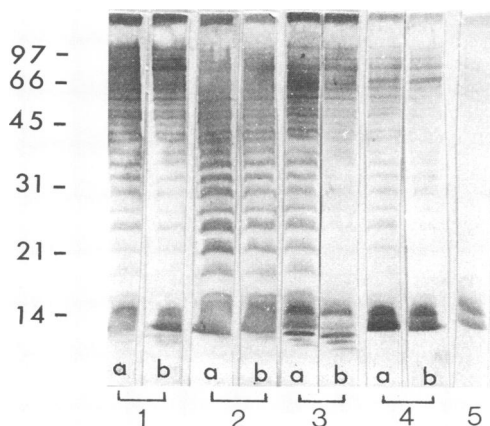


FIG. 1. Western blots of *F. tularensis* OM developed with IgG antibodies in early-phase (lanes a) and late-phase (lanes b) sera from four patients (lane pairs 1 to 4) treated for tularemia 8 years earlier and of pooled serum from 50 blood donors (lane 5). The positions of size standard proteins are shown to the left (in kilodaltons).

early- and late-phase sera when four different individuals were tested for IgG antibodies. All sera except the pool of blood donor sera (right lane) were positive for IgG in the ELISA but had different levels of antibodies. All sera from the patients reacted against a high-molecular-weight antigenic complex at the top of the gel, and they produced a ladder-like banding pattern within the range from 12.5 to 97.5 kDa. The distance between neighboring bands in the ladder corresponded to differences of approximately 3 kDa. The patterns obtained with the late-phase sera were essentially similar to those of the acute-phase sera except for differences related to reductions in antibody levels, mirrored in the number and staining intensity of bands. The patterns obtained with sera from other individuals in the experimental group were similar to those shown in Fig. 1, and probing for IgA or IgM antibodies in selected sera resulted in patterns similar to those obtained for IgG antibodies.

The periodicity of the bands strongly suggested that the *F. tularensis* lipopolysaccharide is involved (5, 9). Partially purified lipopolysaccharide showed a similar ladder formation, and its formation was not affected by digestion of the OM with proteinase K (not shown). The banding patterns were obscured if distinct bands resulting from protein antigens were present. This was shown to be the case in a previous study with acute-phase sera (1, 2), particularly for a 43-kDa OM protein band which was prominent in some Western blot experiments, in which the ladder-like banding was less distinct than in our present study. Our findings argue against the possibility that immunoblotting can be used to discriminate between acute-phase and persisting antibodies.

The data presented here support the assumption that the *F. tularensis* lipopolysaccharide is a major immunogen in the acute phase of tularemia and is also an important target for persisting IgA, IgG, and IgM antibodies. It is thus possible that the *F. tularensis* lipopolysaccharide continues its immunostimulation by persistence in the body after cure of the disease, as enterobacterial lipopolysaccharides persist in compartments of the body (6, 7).

In summary, our study confirms that antibodies, including IgA and IgM antibodies, against *F. tularensis* OM antigens, notably the lipopolysaccharide, persist for a long time; that the duration of high levels of IgG antibodies is strongly influenced by the acute-phase levels; and that calculation of the IgG/IgM antibody ratio is of utility for discrimination between acute-phase and persisting antibodies. Banding patterns in Western blotting had no discriminatory power.

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