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# Competitive Enzyme Immunoassay for Antibodies to a 43,000-Molecular-Weight *Francisella tularensis* Outer Membrane Protein for the Diagnosis of Tularemia

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**Antibodies against a 43,000-molecular-weight *Francisella tularensis* outer membrane (OM) protein (43K protein) were measured in paired serum specimens from 23 patients with tularemia and matched against antibodies in sera from 25 patients with nontularemic infectious diseases and from 25 blood donors. Antibodies were measured by a competition enzyme-linked immunosorbent assay which tested the ability of human serum to compete with rabbit anti-43K protein antibodies for its binding to the *F. tularensis* OM coat. The sera from nontularemic patients and from blood donors, in dilutions of 1:16 and 1:64, respectively, showed no or very low levels of antibodies. All of the tularemia patients showed positive tests with the first, the second, or both of the serum specimens examined. For instance, with serum diluted 1:64, each of the serum specimens showed a sensitivity of 95.7% and a specificity of 96%. When used for antibody competition in Western blotting (immunoblotting), the rabbit anti-43K selectively blocked the binding of human serum antibodies to the 43,000-molecular-weight protein. This protein was immunoaccessible in Formalin-killed *F. tularensis*. These data indicate an important role of the 43,000-molecular-weight OM protein in the immunobiology of tularemia and emphasize its potential usefulness as an antigen in serodiagnostic tests.**

Cultural confirmation of tularemia usually is unsuccessful. Thus, *Francisella tularensis* was isolated from only 1 out of 57 patients in a recent outbreak (2). Besides clinical signs, the diagnosis of tularemia depends on tests to detect the development of humoral or cellular immunity to *F. tularensis* antigens. For humoral immunity measurements, poorly defined mixtures of *F. tularensis* antigens have been used mostly (13, 21, 24), with the exception of the lipopolysaccharide (5).

Recently, we showed that an outer membrane (OM) preparation of *F. tularensis* was a suitable antigen in enzyme-linked immunosorbent assay (ELISA) for the diagnosis of tularemia (2). We noted that when sera from patients were tested by Western blotting (immunoblotting), considerable case-to-case variation occurred in antibody activity against the various OM antigens (2). However, it appeared that serum antibodies against a 43-kilodalton OM protein (43K protein) was a characteristic feature of the patients.

The present study was undertaken to obtain more exact information of the antibody response against the 43K *F. tularensis* OM protein in patients with tularemia. To test for the antibodies, we used a competition ELISA. Also, experiments are described with results which indicate that the 43K protein is surface exposed in *F. tularensis*.

## MATERIALS AND METHODS

**Patients and serum samples.** A total of 23 patients who acquired tularemia during an outbreak in our area in 1984 to 1985 were studied. The outbreak and the diagnostic criteria have been described previously (2). From all of the 23 patients, the first and the second serum samples received by us for diagnostic serology were tested. The first samples were collected in the acute phase of the disease when tularemia was suspected, and the second samples were collected from 1 to 8 weeks thereafter. Controls included sera from 25 blood donors and from 25 patients with various nontularemic infectious diseases. All of the controls were negative in a bacterial agglutination test (4) for anti-*F. tularensis* antibodies. Sera were stored at −20°C until being tested.

***F. tularensis* strain and OM preparation.** A *F. tularensis* strain which was isolated from a dead hare during the 1984 to 1985 outbreak was used in this study. Glucose-cysteine-blood agar (Difco Laboratories, Detroit, Mich.) was used for primary isolation and subculture (48 h) of the strain as previously described (2). The isolate was harvested in 0.85% NaCl with 0.5% Formalin to prepare whole-cell vaccines or in a $LiCl_2$-containing buffer (15) to prepare the *F. tularensis* OM. Bacteria in the buffer were further processed to prepare the OM as previously described (2). The OM preparation was lyophilized.

**Isolation of the 43K OM protein.** Preparative sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was performed by using the apparatus manufactured by Shandon Scientific Company Limited, London, England. The apparatus was operated by following the instructions of the producer, with the use of buffers as for slab gel SDS-PAGE (14). *F. tularensis* OM (10 mg) was applied. Fractions (1 ml) of the eluate were collected and analyzed for the 43K protein by Western blotting, using rabbit anti-*F. tularensis* serum (see below). Fractions which contained the protein were pooled, dialyzed against water, and lyophilized. Then, the materials were separated by slab gel SDS-PAGE. The gel section which contained the 43K protein was minced and eluted with the electrode buffer. Fragments were removed by centrifugation, and the supernatant was divided into eight equal portions for immunization. When analyzed by immunoblotting (see below) using the anti-*F. tularensis* serum, only one stained band of 43K was seen (data not shown).

**Immunization.** Antiserum to whole cells of *F. tularensis* was raised in rabbits (2). Antiserum to the 43K OM protein (anti-43K) was raised by intradermal injection of one portion

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of the protein-containing eluate (0.2 ml) with 0.2 ml of complete Freund adjuvant and then intradermally one portion with incomplete Freund adjuvant (0.2 ml) after 6, 12, and 24 weeks. The animals (two rabbits) were bled 7 days after the last injection, and serum from one animal was selected for the experiments.

**Immunoblotting.** SDS-PAGE and immunoblotting were performed as previously described (2). *F. tularensis* OM (10 μg per track) was applied. The binding of human antibodies was detected by using peroxidase-conjugated rabbit antibodies to human immunoglobulin G (IgG), and the binding of rabbit antibodies was detected by using peroxidase-conjugated swine antibodies to rabbit IgG (1:1,000; Dako Patts, Glostrup, Denmark). Western blot inhibition was performed by incubation of paper strips, which contained the *F. tularensis* OM transblots, with the rabbit anti-43K (1:100 in phosphate-buffered saline with 0.05% [wt/vol] Tween 20) at 20°C for 1 h before incubation with human serum (1:300). Control strips were incubated in buffer with normal rabbit serum before incubation with the human serum. Antibody binding was detected by using the anti-human IgG conjugate.

**ELISA and competition ELISA.** Indirect ELISA was performed as described previously (2), by using coats prepared with *F. tularensis* OM (25 μg/ml; 50 μl) and alkaline phosphatase-conjugated antibodies to rabbit IgG (1:1,000; Sigma Chemical Co., St. Louis, Mo.) to detect antibody binding. For the competition ELISA, wells coated with the *F. tularensis* OM were incubated with appropriately diluted human serum (60 μl) at 20°C for 1 h. Then, 60 μl of appropriately diluted rabbit anti-43K was added. The plate was incubated (20°C; 1 h), washed, and processed to measure bound rabbit antibodies as for the indirect ELISA (2). Optical density at 405 nm was recorded and matched against positive controls with no human serum added and against negative controls in which the antigen and the rabbit antiserum were omitted. Inhibition was calculated as percent reduction of the optical density at 405 nm brought about by the human serum. The tests were performed in duplicate. Paired serum specimens were tested on the same ELISA plate with controls included on each plate. Percent inhibition greater than or equal to the mean plus three times the standard deviation of the percent inhibition recorded for the blood donors was defined as a positive competition ELISA.

**Adsorption of serum.** A volume of 0.4 ml of a 1:16 dilution of human serum was mixed with 0.2 ml of pelleted Formalin-killed *F. tularensis* at 20°C for 1 h. The mixture was centrifuged (10,000 × *g*; 15 min), and the supernatant was tested by the inhibition ELISA.

**Immunofluorescence assay.** Serial dilutions of anti-43K in phosphate-buffered saline were tested against Formalin-killed *F. tularensis* by using fluorescein-conjugated antibodies to rabbit IgG (1:40; Sigma). The test was performed and results were recorded as previously described (1).

**Statistical analysis.** Student's *t* test was used to compare differences between means. Indices of diagnostic reliability of the test were calculated as outlined by Galen (8).

## RESULTS

**Anti-43K.** Western blots of the *F. tularensis* OM developed with antiserum to whole cells of the bacteria showed numerous antigenic OM components (Fig. 1, lanes A and B). Blots developed with anti-43K produced only the 43,000-molecular-weight band (Fig. 1, lane C). The 43K protein required heating in SDS for migration into the polyacrylamide gel (Fig. 1, lanes A and B). Figure 2 shows the

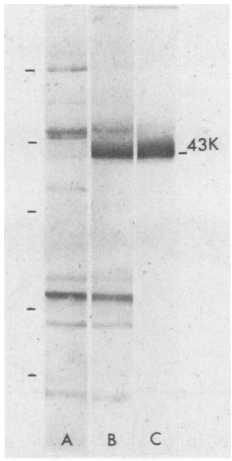


FIG. 1. Western blots of *F. tularensis* OM developed with rabbit anti-*F. tularensis* serum (1:1,000; lanes A and B) and anti-43K (1:1,000; lane C). SDS-PAGE was performed with unheated OM (lane A) or heat treated (100°C) OM in the sample buffer (lanes B and C). Positions of standard 66.2K, 45K, 31K, 21.5K, and 14.4K proteins (top to bottom) are shown to the left.

titration curves for anti-43K and the preimmunization serum as recorded in ELISA with the *F. tularensis* OM coat. On the basis of these results, we chose a final dilution of 1:150 for anti-43K in the competition ELISA. Repeated testing of anti-43K at this dilution showed optical densities at 405 nm of 0.726 ± 0.132 (standard deviation).

**Competition ELISA.** Serial dilutions of pooled serum samples from blood donors ($n$ = 30) and second serum specimens from two randomly selected tularemia patients were tested by the competition ELISA (Fig. 3). Pooled serum showed no anti-43K binding inhibition. The tularemia sera showed complete binding inhibition at dilutions of ≤1:32 and thereafter showed inverse linear dependence of the inhibition with the logarithm of the serum dilution factor. These results led us to select the dilutions of 1:16 and 1:64, respectively, for the ELISA inhibition testing of sera from patients and controls.

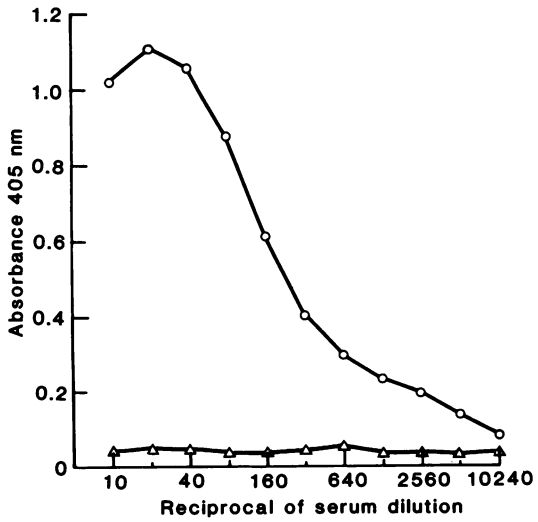


FIG. 2. Serial dilutions of rabbit anti-43K (○) and of preimmunization serum (△), tested by indirect ELISA for IgG antibodies against the *F. tularensis* OM.

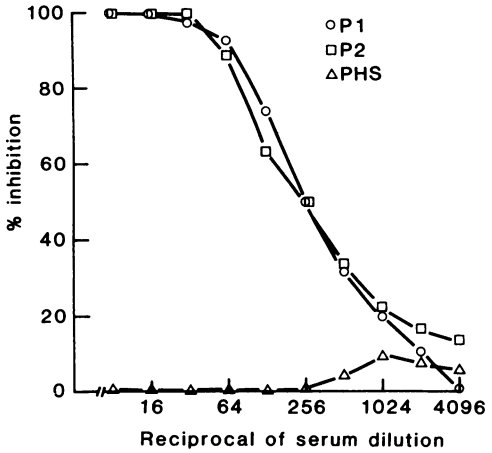


FIG. 3. Serial dilutions of second serum specimens from two patients (P1 and P2) with tularemia and of pooled serum from blood donors (PHS), tested for binding inhibition of anti-43K IgG to *F. tularensis* OM coat.

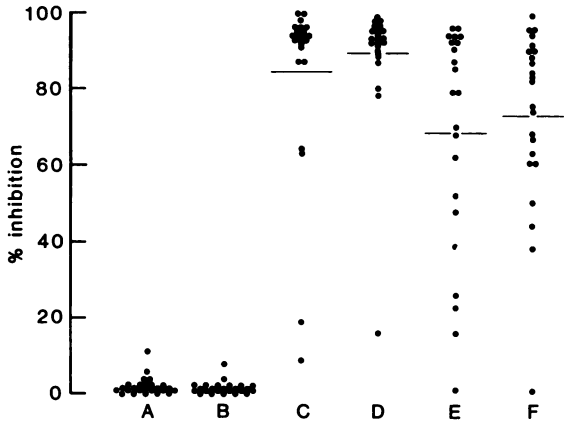


FIG. 4. Competition ELISA results with sera from blood donors (A; $n = 25$), sera from patients with nontularemic infections (B; $n = 25$), and first (C and E) and second (D and F) serum specimens from patients with tularemia ($n = 23$). Sera were tested in a dilution of 1:16 (C and D), or 1:64 (A, B, E, and F). Horizontal lines indicate means of percentage inhibition.

Table 1 and Fig. 4 present the results obtained when single serum specimens from blood donors ($n = 25$) and from nontularemic patients with various infectious diseases ($n = 25$) and paired serum specimens from tularemia cases ($n = 23$) were tested. The blood donors either showed very weak anti-43K blocking activity or no activity. None of the blood donors showed a positive competition ELISA. Although the mean of the levels of anti-43K antibodies in sera from the patients with nontularemic infectious diseases did not differ significantly from the mean of those from the blood donors ($P > 0.1$), three (12%) of the samples from nontularemic patients had a false-positive competition ELISA with serum diluted 1:16, and one (4%) nontularemic sample had a false-positive competition ELISA with serum diluted 1:64. However, none of the false-positive sera showed more than 23% inhibition. On the average, both of the serum specimens from the tularemia patients at either of the serum dilutions of 1:16 and 1:64 showed strong elevation of the anti-43K antibody levels, when matched against those of the blood donors ($P < 0.001$) or the nontularemic patients with various infectious diseases ($P < 0.001$). The antibody levels in the second serum samples were only slightly elevated over those of the first serum samples. With serum diluted 1:16, one of the tularemia patients (4.3%) gave a false-negative competition ELISA with serum sample 1, whereas none of the tularemia patients had negative results with serum sample 2. When serum diluted 1:64 was used, the only tularemia patient (4.3%) with a false-negative first serum specimen had a positive second serum specimen, and the only patient (4.3%) with a false-negative second serum sample had a positive first sample. Thus, all of the patients showed positive inhibition ELISA with one or both of the serum samples. With both samples 1 and 2 and with serum diluted 1:64, the competition ELISA for the diagnosis of tularemia showed a sensitivity of 95.7%, a specificity of 96%, and predictive values of positive and negative test results of 95.7 and 96%, respectively. The test tended to be less reliable when serum was examined in a dilution of 1:16, since its specificity was 88% compared with 96% with serum diluted 1:64.

**Antibody binding inhibition in Western blotting.** The results described above are consistent with the generation of antibodies against the 43,000-molecular-weight protein in all of the tularemia patients. We performed a Western blot antibody inhibition assay to further address the specificity of the human serum antibodies which caused binding inhibition of the rabbit anti-43K antibodies in the competition ELISA. As shown in Fig. 5, lane A, blots of the OM developed with a second serum sample from a tularemia patient showed at least four distinct lines with molecular weights of ≥43,000 and several less distinct bands of lower molecular weights. Incubation of the nitrocellulose paper strip which contained

TABLE 1. Antibodies to a 43,000-molecular-weight *F. tularensis* OM protein in sera from patients and controls, measured by a competition ELISA

| Subject group | % Inhibition with serum diluted: | | | |
|---|---|---|---|---|
| | 1:16 | | 1:64 | |
| | Mean ± SD (range) | No. positive/ no. tested (% positive)[a] | Mean ± SD (range) | No. positive/ no. tested (% positive) |
| Tularemia patients | | | | |
| Serum sample 1 | 84.4 ± 24.3 (8–100) | 22/23 (95.7) | 68.4 ± 29.6 (0–96) | 22/23 (95.7) |
| Serum sample 2 | 89.1 ± 16.8 (16–99) | 23/23 (100) | 72.9 ± 23.5 (0–99) | 22/23 (95.7) |
| Nontularemia patients | 3.3 ± 7.2 (0–23) | 3/25 (12) | 0.6 ± 1.8 (0–8) | 1/25 (4) |
| Blood donors | 2.1 ± 3.4 (0–11) | 0/25 (0) | 0.9 ± 2.2 (0–7) | 0/25 (0) |

[a] % Inhibition greater than or equal to the mean plus three times the standard deviation of the results from blood donors.

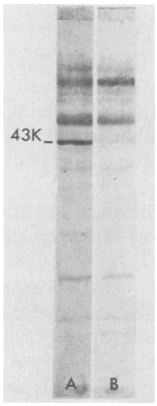


FIG. 5. Western blots of *F. tularensis* OM developed with the second serum specimen (1:300) from a patient with tularemia. The nitrocellulose membrane strips were incubated in phosphate-buffered saline with 0.05% (wt/vol) Tween 20 and normal rabbit serum 1:100 (lane A) or with anti-43K (1:100) in the buffer (lane B) before incubation with the human serum.

the OM transblots with anti-43K before incubation with serum samples from the patient, resulted in abrogation of the 43,000-molecular-weight band but not of other bands. These results are consistent with the notion that human serum antibodies capable of blocking the binding of anti-43K were directed against determinants of the 43,000-molecular-weight protein.

**Interaction of anti-43K protein antibodies with *F. tularensis*.** The possibility that antigenic determinants of the 43,000-molecular-weight OM protein are surface exposed in *F. tularensis* bacteria was explored. Second serum samples (1:16) from 4 tularemia patients were adsorbed with Formalin-killed bacteria. The adsorbed samples showed 5.5 ± 4.2% inhibition (range, 0 to 10%) in the competition ELISA, versus 68.5 ± 17.9% inhibition (range, 57 to 95%) recorded when the sera were tested unadsorbed ($P < 0.001$). In the immunofluorescence assay the bacteria showed strong binding of rabbit antibodies in the anti-43K serum, in dilutions of ≤1:64. Preimmunization serum showed a negative immunofluorescence assay. These results are consistent with immunoaccessibility of antigenic determinants of the 43,000-molecular-weight protein in intact *F. tularensis*.

## DISCUSSION

*F. tularensis* OM contained a variety of immunogenic components as shown in this and in a previous study (2). In the present study, we isolated a 43K *F. tularensis* OM protein which, on immunization, resulted in the generation of rabbit anti-43K protein serum (anti-43K). Western blots developed with anti-43K showed strong antibody binding only to the 43K protein (Fig. 1), and in blots developed with serum from a patient with tularemia, the antiserum selectively blocked the binding of human serum antibodies to the 43K protein (Fig. 5). These results accord with specificity of anti-43K for the 43,000-molecular-weight OM protein. In light of these results, we anticipated that blocking effects by human sera of the binding of rabbit anti-43K IgG to *F. tularensis* OM coats were due to human serum antibodies to this protein. On this assumption, the competition ELISA as performed by us, should specifically measure anti-43K antibodies in human sera.

Human serum antibodies against various microbial antigens have been measured by competitive inhibition of monoclonal antibody binding (3, 11, 12, 19). It may be that the polyclonal anti-43K contained antibodies against several antigenic domains of the 43K protein. Strong anti-43K antibody inhibition by serum samples from humans with tularemia presumably would require that the patients with tularemia elicited antibodies against the same 43K protein regions as did the rabbit immunized with the SDS-denatured protein. Our data indicate that this was the case (e.g., Fig. 3), in agreement with the observation that animals of diverse species recognized the same antigenic structures of a protein (23). Although we anticipate that the magnitude of the inhibiting effects was a measure of the amount of the human serum antibodies, additional factors may affect the results, such as individual variations in the affinity of the blocking human antibodies compared with that of the detecting rabbit antibodies.

Blood donors showed very weak anti-43K antibody activity, which accords with the fact that tularemia occurs very rarely in our area. Also, this observation is concordant with the lack of cross-reactivity of the 43K *F. tularensis* OM protein with antigens of commensal bacteria which may induce normal human serum antibodies. However, the possibility of cross-reactivity of the *F. tularensis* OM protein with certain other human pathogens can not be excluded, since 3 of 25 of the patients with nontularemic infections had weakly positive competition ELISAs. These sera were collected during a period in which no cases of tularemia were recorded. All of the tularemia patients showed positive inhibition ELISAs with serum in one or both of the dilutions of 1:16 and 1:64, with both of the serum samples tested, or with only the first or the second sample. Thus, the sensitivity of the competition ELISA for anti-43K protein antibodies for the diagnosis of tularemia compared favorably with that of tests using antigens which contained multiple *F. tularensis* components, such as the bacterial agglutination test (4, 5, 24) or indirect ELISA for antibodies against OM (2) or against sonic extracts (21, 24) or the bacteria. For diagnostic serology, testing in the competition ELISA of serum diluted 1:64 provided higher diagnostic reliability than testing of serum diluted 1:16, without much loss of sensitivity. Unfortunately, the advantage gained by the testing of class-specific antibody response in tularemia (13, 21) will not be provided by the competition ELISA. Our finding of fully developed anti-43K protein response at the time when the first serum specimens were collected accords with the results of testing for anti-OM antibodies and agglutinins to *F. tularensis* (2).

Previously, we noted considerable case-to-case variation in the antibody response in humans against the various *F. tularensis* OM antigens (2). However, the data in this study underscore the fact that the 43K protein is particularly apt to induce immune response in patients with tularemia. Thus, the 43K protein may be the OM antigen of choice in serological tests for tularemia, although its cross-reactivity with other *F. tularensis* strains and other bacteria must be tested before final assessment of the applicability of the protein. Our finding that the 43K protein required pretreatment in hot SDS for migration into the polyacrylamide gel is consistent with the assumption that this protein is the porin of *F. tularensis* (7, 17). However, conclusive evidence for this supposition is lacking. Antibodies against porin proteins of gram-negative bacteria may or may not be protective (9, 10, 16, 20). In the present study, we showed that antigenic sites of the 43K protein were immunoaccessible on the bacterial cell surface. When seen in association with the strong immunogenicity of the 43K protein in humans, this makes the protein an attractive *F. tularensis* antigen for

studies, for instance, to elucidate its potentiality as a component vaccine against tularemia. In this context, assessment of the propensity of the 43K protein to induce cell-mediated immunity is particularly important (6, 18, 22).

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