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Phagocytosis and Killing of *Francisella tularensis* by Human Polymorphonuclear Leukocytes

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Bacteria of a wild strain of *Francisella tularensis* were less efficiently killed by human polymorphonuclear leukocytes than were bacteria of an attenuated strain. This finding was explained to some extent by a less efficient phagocytosis, but bacteria of the wild strain also seemed to be more resistant to killing after ingestion.

Cell-mediated immunity is considered to be crucial for the defense of mammals against *Francisella tularensis*, whereas humoral immunity does not seem to be sufficient for protection (5, 10, 14, 22). However, the possible contribution of serum components and polymorphonuclear leukocytes (PMNs) to the defense mechanisms has been but little studied. There is some evidence that humoral immunity may interfere with strains of low virulence. Thus, the transfer of immune serum to nonimmune animals has afforded some protection against strains of *F. tularensis* of low virulence but not against strains of high virulence (16). A variance between strains in sensitivity to the bactericidal effect of serum seems improbable since no such effects have been observed, even in bacteria of attenuated strains (19). More likely, strains differ in their resistance to ingestion or intracellular killing by PMNs. An attenuated strain of *F. tularensis* when opsonized with immune serum induces a respiratory burst in simian (3) and human (12) PMNs. Simian leukocytes have been shown to ingest and kill the bacteria (19).

No data are available on the interaction between bacteria of virulent strains and PMNs. The purpose of the present study was to compare an attenuated and a wild strain of *F. tularensis* with respect to their interaction with human PMNs.

MATERIALS AND METHODS

Bacteria and growth conditions. An attentuated strain of *F. tularensis* was supplied by the U.S. Army Research Institute of Infectious Diseases, Fort Detrick, Md. (7). A wild strain of *F. tularensis* (strain SBL R45) was supplied by R. Möllby, National Bacteriological Laboratory, Stockholm, Sweden. It was isolated during an outbreak that led to more than 400 cases of the ulceroglandular type of tularemia in northern Sweden in 1981. The strain was identified through culture characteristics, fermentation pattern, and surface antigens. It did not utilize glycerol or produce citrulline ureidase and so was identified as *F. tularensis* subsp. *palaearctica* (15). The bacteria were kept frozen at −70°C. At intervals of 1 month, a sample was thawed, and the bacteria were cultivated on modified Thayer-Martin agar containing Gc-Medium Base (36 g/liter; Difco Laboratories, Detroit, Mich.), hemoglobin (10 g/liter, Difco) and IsoVitaleX (10 ml/liter; BBL Microbiology Systems, Cockeysville, Md.) at 37°C in 5% CO_2 in air. For each experiment, bacteria grown for 2 days were harvested from the surface of the agar and suspended in RPMI 1640 with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (RPMI-HEPES; Gibco Bio-Cult, Glasgow, Scotland) at a density of 5×10^7 bacteria per ml.

Serum. Blood was obtained from six persons vaccinated 60 days previously with the attenuated strain of *F. tularensis* according to the instructions given by the manufacturer. Serum was prepared and pooled (vaccinee serum). Patient serum was prepared from the blood of a man who had suffered from tularemia 1 month previously. A serum pool was also prepared from five persons who denied either tularemia or tularemia vaccination (nonimmune serum). The agglutinin titers of vaccinee, patient, and nonimmune serum against *F. tularensis* were 320, 1,280, and <20, respectively. Serum was stored at −70°C.

Separation of IgM and IgG. Immunoglobulin M (IgM) and IgG were separated in the following manner. Fractionation by gel chromatography was performed on Sephadex S-300 (Pharmacia AB, Uppsala, Sweden). A 3-ml sample of vaccinee serum was applied to a column (900 by 26 mm) and was eluted at 4°C with phosphate-buffered saline (12.6 mM KH_2PO_4, 54.0 mM Na_2HPO_4, 85.5 mM NaCl; pH 7.4) at a flow rate of 9 ml per h. Portions of 4.5 ml were collected and tested for the presence of IgM and IgG by radial immunodiffusion in Partigen plates (Behringwerke AG, Marburg, West Germany). Portions containing detectable IgM or IgG, but not both, were pooled. The pooled portions were concentrated 20 to 40 times by ultrafiltration (Immersible CX-10; Millipore Corp., Bedford, Mass.) at 4°C and were then stored at −70°C.

IgA was removed from the pooled IgG-containing portions by affinity chromatography. Particles of cy-

anogen bromide-activated Sepharose 4B (Pharmacia AB) (10 g) were swollen and washed in 1 mM HCl and were equilibrated in 0.1 M sodium bicarbonate buffer, pH 9.0, containing 0.5 M NaCl. The excess bicarbonate buffer solution was removed, and 4 ml of rabbit anti-human IgA antiglobulin (DACO-Immunoglobulin A/S, Copenhagen, Denmark) in 36 ml of bicarbonate buffer solution was added. The material was then treated according to the directions of the manufacturer, except that 0.67 M instead of 1 M ethanolamine (BDH, Poole, England) was used to block the remaining reactive groups. The material was then introduced into a column (300 by 15 mm). A 3-ml sample of the pooled IgG-containing portions was applied to the column at room temperature and was eluted with phosphate-buffered saline at a flow rate of about 12 drops per min. The eluate was concentrated by ultrafiltration at 4°C to restore the original serum IgG concentration. The concentrated eluate is referred to as the IgG fraction and was stored at −70°C.

Traces of IgG were similarly removed from the pooled IgM-containing portions by means of affinity chromatography with rabbit anti-human IgG antiglobulin (DACO-Immunoglobulins A/S). The resulting IgM fraction was stored at −70°C.

The concentrated immunoglobulin fractions were assayed for IgG, IgM, and IgA by radial immunodiffusion with Partigen plates. No IgM or IgA was detected in the IgG fraction, and no IgA or IgG was detected in the IgM fraction.

Removal of complement component C3 or C5 from serum. Affinity chromatography was used to remove C3 or C5 from serum as previously described (12, 13). Cyanogen bromide-activated Sepharose 4B particles (30 g) were prepared and divided into three equal parts. To these parts were added 4 ml of goat anti-human C3 antiserum, goat anti-human C5 antiserum, or normal goat serum (Meloy Laboratories, Springfield, Va.), respectively in 36 ml of bicarbonate buffer solution. After coupling, the materials were transferred to three columns (300 by 15 mm).

Vaccinee serum (3 ml) was applied to each column at room temperature and was then eluted, pooled, concentrated, and stored as previously described (12). The concentrated eluates were assayed for IgG, IgM, IgA, C3, and C4 by radial immunodiffusion with Partigen plates; assays for C5 used human anti-C5 plates (Meloy Laboratories). There was no detectable C3 in the serum that had been chromatographed on the column containing C3 antiserum and no C5 in the serum that had been chromatographed on the column containing C5 antiserum. There were otherwise no quantitative differences between the three chromatographed sera.

Preparation of human PMNs. Venous blood was obtained from healthy volunteers with no history of tularemia or tularemia vaccination and lacking demonstrable serum agglutinins against *F. tularensis*. The PMNs were prepared as previously described (12). Dextran sedimentation of the erythrocytes (20) was performed, and the remaining erythrocytes were lysed with 0.87% ammonium chloride (6). The PMNs were suspended in RPMI-HEPES to a density of 5×10^7 per ml. Giemsa-stained preparations of the suspension contained 75 ± 5% PMNs, 20 ± 5% lymphocytes, and 5 ± 3% monocytes.

Preparation of human mononuclear leukocytes. Control experiments were performed to exclude the possible contribution to phagocytosis and killing of the small amounts of monocytes in the PMN preparation. Venous blood was obtained from the same donor used for preparation of PMNs. Mononuclear leukocytes were prepared from heparinized venous blood by centrifugation in sodium metrizoate Ficoll (Lymphoprep, Nyegaard & Co. As, Oslo, Norway) as described by Bøyum (2). Monocytes were identified by staining for nonspecific esterase (18). The cells were suspended at appropriate density in RPMI-HEPES.

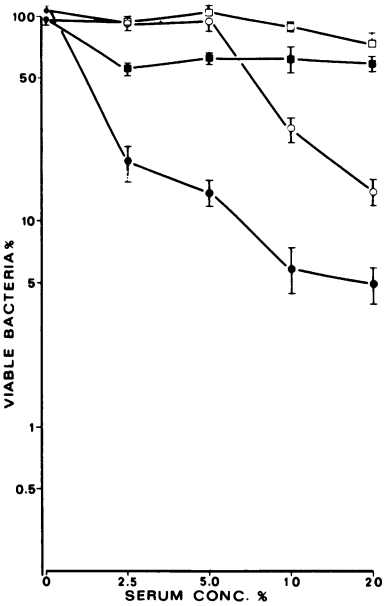

FIG. 1. Effects of various concentrations of vaccinee and nonimmune serum on phagocytosis and killing of bacteria of a wild strain of *F. tularensis* by human PMNs. Bacteria and PMNs were incubated for 60 min in the absence of serum or in the presence of various concentrations of vaccinee or nonimmune serum. To estimate phagocytosis and killing, viable counts were made on the cell-free supernatant of the incubation mixture and on the lysed mixture, respectively. The percentages of surviving bacteria of the initial inoculum were calculated. Means ± standard errors of the mean of six experiments are shown. Symbols: ●, Phagocytosis, vaccinee serum; ○, phagocytosis, nonimmune serum; ■, killing, vaccinee serum; □, killing, nonimmune serum.

Phagocytosis and killing. PMNs (5×10^6) were mixed in capped plastic tubes (12 by 75 mm; Falcon Plastics, Oxnard, Calif.) with 5×10^6 bacteria and serum or serum fraction, and RPMI-HEPES was added to a final volume of 500 μl. Unless otherwise stated, the concentration of serum in the reaction mixture was 2.5%, and serum fractions were used in equivalent concentrations with respect to serum immunoglobulin contents. The tubes were incubated at 37°C under rotation (60 rpm).

To estimate killing, 50-μl samples were drawn from the tubes immediately after mixing and at various intervals of incubation. Each sample was added to 4.95

TABLE 1. Phagocytosis and killing of wild and attenuated strains of *F. tularensis* by human PMNs in the presence of serum

Bacterial strain	Interaction with PMNs	% Bacteria affected in presence of serum[a]: Patient	Vaccinee
Wild	Phagocytosis	93 ± 2	97 ± 1.5
	Killing	37 ± 7	31 ± 5
Attenuated	Phagocytosis	99.5 ± 0.5	99.7 ± 0.1
	Killing	97.6 ± 0.7	98.1 ± 0.6

[a] Serum (20%) was taken from a tularemia patient or from a pool drawn from vaccinated volunteers. Agglutinating titers of serum were 1,280 (patient) and 320 (vaccinee). Numbers show percentages of inoculum phagocytosed or killed after incubation for 60 min; means ± standard errors of the mean for four experiments are shown.

ml of ice-cold phosphate-buffered saline in siliconized (Sigmacote SL-2; Sigma Chemical Co., St. Louis, Mo.) glass tubes and was sonicated in a 150-W MSE sonicator with a 145 by 35 mm tip at an amplitude of 18 μm for 8 s. This treatment was sufficient to lyse the PMNs without notably affecting the number of viable bacteria. In the experiments where the wild strain was included, hypotonic lysis of the leukocytes in distilled water was performed instead of sonication because of hazardous aerosol formation with the sonicator. Viable counts were made after dilution of the samples, and the number of surviving bacteria were calculated. In control experiments on the attenuated strain, results were similar whether hypotonic lysis or sonication was used.

To estimate phagocytosis, 50 μl was drawn from each tube immediately after mixing and at various intervals of incubation. These samples were added to 4.95 ml of ice-cold phosphate-buffered saline in siliconized glass tubes. To separate phagocytosed from nonphagocytosed bacteria, the cells were sedimented by centrifugation at 110 × *g* for 10 min at 4°C (24), and the number of nonphagocytosed viable bacteria remaining in the supernatant fluid was calculated. In control experiments, cells and bacteria were suspended separately and centrifuged at 110 × *g* for 10 min at 4°C. This centrifugation was found to sediment more than 99% of the cells and less than 1% of the bacteria.

For control purposes, the number of bacteria associated to PMNs but still viable was estimated. The sediment was suspended in 5.0 ml of ice-cold phosphate-buffered saline, and the cells were lysed. Viable counts were made, and the number of viable bacteria was expressed as a percentage of the initial inoculum. The sum of the number of viable bacteria in the sediment and in the supernatant equalled roughly the number of bacteria of the sonicated reaction mixture.

To exclude the possible contribution to phagocytosis by the monocytes in the PMN preparation, a mononuclear leukocyte preparation containing 5×10^5 monocytes was mixed with 5×10^6 bacteria of the attenuated strain and with 2.5% vaccinee serum in 500 μl of RPMI-HEPES in siliconized glass tubes. No phagocytosis could be detected during 60 min of incubation. Experiments were performed in a P3 laboratory.

Electron microscopy. The cell sediment was fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The cells were then washed in cacodylate buffer containing 0.1 M sucrose. They were postfixed in 1% osmium tetroxide for 1.5 h, washed in cacodylate buffer, and dehydrated in a graded series of ethanol. After treatment for 30 min in propylene oxide, they were embedded in Epon 812. Sections were cut on an LKB ultratome and were stained with uranyl acetate and lead citrate. Specimens were examined with a Jeol 100 CX electron microscope.

Statistical analysis. Student's *t* test for paired differences was used.

RESULTS

Survival of *F. tularensis* in the presence of serum. The effect of vaccinee, patient, and nonimmune serum on the survival of a wild and an attenuated strain of *F. tularensis* was tested in the absence of PMNs. Bacteria of both the wild

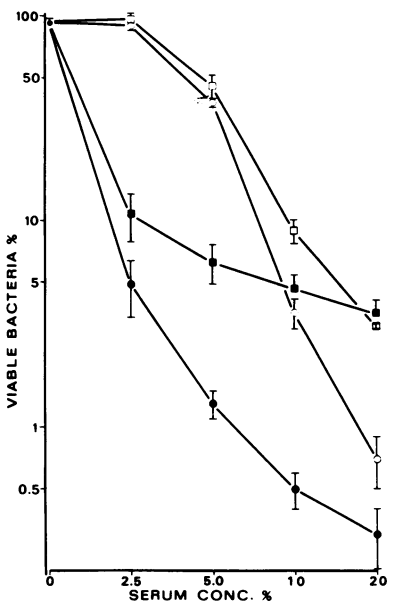

FIG. 2. Effects of various concentrations of vaccinee and nonimmune serum on phagocytosis and killing of bacteria of an attenuated strain of *F. tularensis* by human PMNs. Bacteria and PMNs were incubated for 60 min in the absence of serum or in the presence of various concentrations of vaccinee or nonimmune serum. To estimate phagocytosis and killing, viable counts were made on the cell-free supernatant of the incubation mixture and on the sonicated mixture, respectively. The percentages of surviving bacteria of the initial inoculum were calculated. Means ± standard errors of the mean of three experiments are shown. Symbols: ●, Phagocytosis, vaccinee serum; ○, phagocytosis, nonimmune serum; ■, killing, vaccinee serum; □, killing, nonimmune serum.

and the attenuated strain survived 1 h of incubation in nonimmune serum, but the number of viable bacteria of both strains was reduced by about 20% when incubated in 2.5 to 20% of vaccinee or patient serum.

Effect of serum on phagocytosis and killing of bacteria of the wild strain by PMNs. In the absence of serum, no bacteria of the wild strain were phagocytosed during 60 min of incubation. In the presence of 2.5, 5, 10, or 20% vaccinee serum, 80 to 95% of the bacteria were phagocytosed (Fig. 1). In the presence of 2.5 or 5% nonimmune serum, no phagocytosis occurred, whereas in the presence of 10 and 20% nonimmune serum, 70 and 80% of the bacteria were phagocytosed, respectively (Fig. 1). Killing was poor irrespective of serum source and serum concentration (Fig. 1). Similar results were obtained when either vaccinee or patient serum was used (Table 1).

Effect of serum on phagocytosis and killing of bacteria of the attenuated strain by PMNs. In the absence of serum, no bacteria of the attenuated strain were phagocytosed during 60 min of incubation. In the presence of 20% vaccinee or nonimmune serum, more than 99% of the bacteria were phagocytosed, and 95% were killed (Fig. 2). At lower serum concentrations, vaccinee serum promoted phagocytosis and killing more efficiently that did nonimmune serum. Thus, in the presence of 2.5% vaccinee serum, 93% of the bacteria were phagocytosed, and 85% were killed. In the presence of 2.5% nonimmune serum, less than 20% of the bacteria were phagocytosed and killed (Fig. 2). Compared with the wild strain, bacteria of the attenuated strain were more efficiently phagocytosed ($P < 0.05$) and killed ($P < 0.001$).

The magnitude of phagocytosis and killing was decreased ($P < 0.05$) by the depletion of C3 from vaccinee serum (Fig. 3A). Depletion of C5 from vaccinee serum did not affect phagocytosis or killing (data not shown). The IgM and IgG fractions of vaccinee serum were heat treated (56°C, 30 min) and supplemented with an equal volume of heat-treated or fresh nonimmune serum to study the phagocytosis and killing induced by these fractions in the absence and in the presence of intact complement. The IgM fraction induced phagocytosis and killing only if intact complement was present (Fig. 3B). The IgG fraction induced phagocytosis and killing both in the absence ($P < 0.001$) and in the presence ($P < 0.001$) of intact complement (Fig. 3C), although the rate was higher in the presence of intact complement ($P < 0.05$). In all situations phagocytosis was followed by killing.

Verification of the ingestion of *F. tularensis* in PMNs. PMNs and bacteria of the wild or attenuated strain were incubated for 60 min in the presence of 2.5% vaccinee serum. Cell sediments were prepared and investigated by electron microscopy. Bacteria of the wild as well as of the attenuated strain were found to be localized intracellularly, and no adhered bacteria were seen extracellularly.

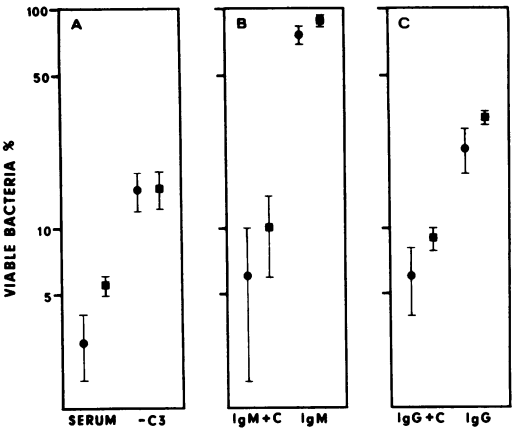

FIG. 3. Effects of complement and of IgM and IgG of vaccinee immune serum on the phagocytosis (●) and killing (■) of an attenuated strain of *F. tularensis* by PMNs. Bacteria and leukocytes were incubated for 60 min in the presence of (A) vaccinee serum (serum) or C3-depleted serum (−C3); (B) the IgM fraction of vaccinee serum and a fresh nonimmune serum supplement (IgM + C) or the IgM fraction and a heat-treated (56°C, 30 min) supplement (IgM); and (C) the IgG fraction of vaccinee serum and a fresh nonimmune serum supplement (IgG + C) or the IgG fraction and a heat-treated supplement (IgG). Means ± standard errors of the mean of three to seven experiments are shown.

DISCUSSION

Bacteria of an attenuated and a wild strain of *F. tularensis* were phagocytosed by human PMNs. Bacteria of the attenuated strain were readily killed, whereas killing of the wild strain was poor. These results agree with those obtained in studies on *Brucella abortus* in that a smooth strain of *B. abortus* was more resistant to killing by human PMNs than was its rough mutant (11). Results with the wild strain of *F. tularensis* also agree with those obtained with *Legionella pneumophila*, which was efficiently phagocytosed but not at all killed by human PMNs (8). Thus, intracellular bacteria of three different species have been found to resist killing by human PMNs, and low-virulence strains of two of the species have been found to be more susceptible.

Bacteria of the wild strain were ingested at a slower rate than were those of the attenuated strain ($P < 0.05$). However, this seemed only

partly to explain the difference in killing between the strains. Thus, killing of the attenuated strain was more efficient ($P < 0.001$) than that of the wild strain in situations where phagocytosis was equivalent. For example, with 2.5% vaccinee serum, 95% of the bacteria of the attenuated strain were phagocytosed, and 90% were killed. A similar degree of phagocytosis of bacteria of the wild strain occurred with 20% vaccinee serum; less than 40% of the bacteria were killed. The reason for the difference between the strains in their interaction with PMNs is unknown. The wild strain has a greater amount of lipid-rich surface structure, which might be of importance (4). Production of cytotoxins by the wild strain seems less probable since *F. tularensis* has not been found to produce any potent toxins (21).

Phagocytosis of *F. tularensis* occurred only if serum was present. The effect of various opsonins in serum on phagocytosis and killing of the attenuated strain was studied. In high concentrations, nonimmune serum supported phagocytosis and killing as efficiently as vaccinee serum did. In suboptimal concentrations, vaccinee serum supported phagocytosis, as did IgG and IgM fractions of vaccinee serum in the presence of complement and the IgG fraction in the absence of complement. Irrespective of manner of opsonization, phagocytosis was followed by killing. *Toxoplasma gondii*, another intracellular parasite, is ingested by mononuclear phagocytes in the presence of nonimmune serum but is killed only if immune serum is present (1).

The augmenting effect of vaccinee serum on phagocytosis and killing was diminished by removing complement component C3 from the incubation mixture but not by removing C5. This indicates that complement components participating later in the sequence than C3 do not affect the opsonization of the attenuated bacteria insofar as phagocytosis and killing are concerned. Previous results have indicated that such components are required for the attenuated bacteria to induce an early chemiluminescent response in the leukocytes (13). The intracellular killing of other bacteria has been found to be enhanced by chemotactic agents such as C5a (9, 17, 23).

The poor killing of the wild strain of *F. tularensis* is in accordance with data indicating that humoral immunity alone does not protect against tularemia (5, 10, 14, 22). Moreover, the more efficient killing of the attenuated strain and the enhancement of phagocytosis and killing of this strain by vaccinee serum are in accordance with data suggesting that specific antibodies may protect various animals against *F. tularensis* strains of low but not of high virulence (16). The resistance of the wild strain against PMNs may explain, at least in part, why it is more virulent than the attenuated strain in humans.

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