INFECTION AND IMMUNITY, May 1988, p. 1194–1202
0019-9567/88/051194-09$02.00/0

Vol. 56, No. 5

A Capsule-Deficient Mutant of *Francisella tularensis* LVS Exhibits Enhanced Sensitivity to Killing by Serum but Diminished Sensitivity to Killing by Polymorphonuclear Leukocytes

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Received 3 August 1987/Accepted 11 February 1988

The live vaccine strain (LVS) of *Francisella tularensis* is killed by human polymorphonuclear leukocytes as a result of strictly oxygen-dependent mechanisms (S. Löfgren, A. Tärnvik, M. Thore, and J. Carlsson, Infect. Immun. 43:730–734, 1984). We now report that a capsule-deficient (Cap^-) mutant of LVS survives in the leukocytes. In contrast to the encapsulated parent strain, the Cap^- mutant was avirulent in mice and was susceptible to the bactericidal effect of nonimmune human serum. The mutant was killed by serum as a result of activation of the classical pathway of complement by naturally occurring immunoglobulin M. This killing by serum was mitigated by the presence of human polymorphonuclear leukocytes. After opsonization in complement component C5-deficient nonimmune serum, the Cap^- mutant was ingested and survived in the leukocytes. Under these conditions, the parent strain was killed. The leukocytes responded to both the parent and the Cap^- strain with a very low chemiluminescent response. Only the response to the parent strain was inhibited by superoxide dismutase. When the Cap^- mutant was opsonized with immunoglobulin G, it induced a higher and superoxide dismutase-inhibitable chemiluminescent response and was killed by the leukocytes. In conclusion, the capsule of *F. tularensis* LVS seemed to protect this organism against the bactericidal effect of serum. When deprived of the capsule, the organism failed to induce an antimicrobial response in polymorphonuclear leukocytes and survived in the leukocytes. Survival in phagocytes is a key characteristic of intracellular parasites. The Cap^- mutant of *F. tularensis* may become a useful tool in experiments to explain the differences between pathways of ingestion of intracellular parasites, evidenced by the death or survival of the parasite.

In phagocytes, microorganisms induce a respiratory burst, which leads to the production of microbicidal oxygen reactants (2). This antimicrobial response may be evaded in more than one way by obligate or facultative intracellular parasites. The parasite may withstand the oxygen reactants (8, 12, 24) or scavenge them (29, 30, 41). The parasite may also slip into the phagocyte without inducing a respiratory burst. This ability has been reported to occur in several intracellular parasites, such as *Salmonella typhi* (18, 26), *Brucella abortus* (19), *Mycobacterium leprae* (14), *Yersinia pestis* (5), and *Toxoplasma gondii* (1, 42).

The present data may support the assumption that a failure to induce an antimicrobial response is a trait native in intracellular parasites. Previous studies in our laboratory have shown that an attenuated live vaccine strain (LVS) of the facultative intracellular parasite *Francisella tularensis* is phagocytosed and killed by human polymorphonuclear leukocytes (PMNs) and that this killing is strictly oxygen dependent (22, 24). We isolated a capsule-deficient (Cap^-) mutant of *F. tularensis* LVS and found that this virtually avirulent mutant could be ingested by PMNs without being killed. The ability of the mutant to survive depended on the method of opsonization.

MATERIALS AND METHODS

Bacteria. A wild strain of *F. tularensis* biovar *palaearctica* (strain SBL R45), was supplied by R. Möllby, National Bacteriological Laboratory, Stockholm, Sweden (22). *F. tularensis* LVS (6) was obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md. Bacteria were cultured on modified Thayer-Martin agar plates (22) at 37°C in 5% CO_2 in air. The Cap^- mutant of *F. tularensis* has been previously used (35), although it has not been described in detail.

Acridine orange treatment. *F. tularensis* organisms (10^{10} CFU) from overnight cultures were suspended in 1 ml of a solution of acridine orange (1,000, 500, 250, 125, 62.5, 31.3, or 15.6 μg/ml; Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (pH 7.4) (12.6 mM KH_2PO_4, 54.0 mM Na_2PO_4, 85.5 mM NaCl) and were incubated at room temperature for 1 h. Thereafter, subcultures were done to screen for rough colony (Cap^-) forms.

Preparation of bacterial membranes. Bacterial colonies were harvested from the surface of agar plates and were suspended at a density of 10^{10} CFU/ml in 10 ml of EDTA in 10 mM Tris hydrochloride (pH 8.0). The bacteria were disrupted by four 15-s bursts at full power of an ultrasonic disintegrator (Branson Sonic Power Co., Danbury, Conn.). Cell debris was removed by centrifugation for 15 min at 2,000 × *g*, and membranes were pelleted by centrifugation for 1 h at 100,000 × *g*.

Analysis of membrane proteins by SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (20). Gradient gels contained 10 to 17.5% acrylamide and 0.20 to 0.46% bisacrylamide. Membranes were suspended in sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 1% SDS, 20% β-mercaptoethanol, 10% glycerol) at a protein concentration of 0.5 mg/ml. Samples (30 μl) were heated at

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95°C for 5 min and were subjected to SDS-PAGE. After overnight electrophoresis, proteins were fixed by immersing the gel for 10 min in a mixture of 45% methanol and 9% acetic acid. The proteins were stained for 30 min in 0.2% Coomassie brilliant blue in 7% methanol–5% acetic acid and were destained in 7% methanol–5% acetic acid. All steps were carried out at 37°C.

Cellular fatty acid composition. Cellular fatty acids were extracted and characterized as described by Miller (25). The percentage value of each fatty acid was calculated on the basis of the individual peak area and expressed as the percentage of total peak area.

Mouse challenge. NRMI mice were injected intraperitoneally with 10^6 bacteria in 0.1 ml of saline. Mice were killed on days 0, 1, 3, and 6. Spleens were homogenized, and viable counts were performed.

Sera. Blood was obtained from six persons previously vaccinated with *F. tularensis* LVS according to the instructions given by the manufacturer. Serum was prepared, pooled, and used as tularemia-immune serum. The agglutinin titer of the pool was 320. Nonimmune serum referred to a pool of sera from at least five individuals denying tularemia or tularemia vaccination and with agglutinin titers less than 20. Sera were stored in portions at −70°C.

Preparation of immunoglobulin- or complement-deficient serum. Nonimmune human serum was depleted of immunoglobulin G (IgG) by chromatography sequentially on immobilized protein A and anti-IgG. Protein A-Sepharose CL-4B (Pharmacia AB, Uppsala, Sweden) particles (10 g) were swollen, washed in phosphate-buffered saline, and put into a column (300 by 15 mm) at room temperature. Serum (4 ml) was applied and eluted with phosphate-buffered saline at a flow rate of about 12 drops per min. The eluate was concentrated to the original volume by ultrafiltration (Immersible CX-10, 10,000 Da; Millipore Corp., Bedford, Mass.) at 4°C and was applied at room temperature onto a column (300 by 15 mm) containing cyanogen bromide-activated Sepharose 4B (10 g; Pharmacia AB) to which rabbit anti-human IgG (4 ml; Dakopatts A/S, Copenhagen, Denmark) had been coupled. After being eluted with phosphate-buffered saline and concentrated to the original volume, portions were frozen and stored at −70°C. No IgG was detected by a radial immunodiffusion technique with a sensitivity of 0.02 mg/ml.

Aliquots (4 ml) of nonimmune human serum were also chromatographed on columns (300 by 15 mm) of cyanogen bromide-activated Sepharose 4B (10-g) particles to which rabbit anti-human antiserum to IgM, factor B, C4, or C5 (4 ml; Dakopatts A/S) had been coupled. The eluates were handled as described for serum depleted of IgG. IgM-deficient serum contained less than 0.03 mg of IgM per ml according to radial immunodiffusion. C4-deficient serum had normal hemolytic activity via the alternative pathway but had no detectable activity by the classical pathway when tested for hemolysis by gel techniques (9, 21). Factor B-deficient serum had no detectable hemolytic activity by the alternative pathway but activity was preserved by the classical pathway. Serum deficient in both IgG and IgM was obtained by chromatography of IgG-depleted serum on immobilized anti-IgM. For control purposes, serum was run through a column to which bovine serum albumin had been coupled. Sera deficient in immunoglobulins had an intact hemolytic activity by both pathways.

Assay of bactericidal effect of serum. Bacteria (5×10^5 CFU) from 48-h cultures were suspended in 0.5 ml of RPMI 1640 containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (RPMI-HEPES; GIBCO Bio-Cult, Glasgow, Scotland), to which serum had been added at various concentrations. The suspension was incubated at 37°C on a shaking water bath, and viable counts were performed at various intervals.

Deposition of ^{125}I-C3 on bacteria. Purified C3 was a generous gift of Ulf Nilsson, University of Uppsala, Uppsala, Sweden. ^{125}I-labeled C3 was prepared with Enzymobeads (Bio-Rad Laboratories, Richmond, Calif.) as described by the manufacturer, except for the use of Veronal-buffered saline containing $MgCl_2$ and $CaCl_2$ instead of phosphate buffer. For each labeling, 1 mCi of ^{125}I was used for 330 μg of C3, to give a specific activity of 4×10^6 cpm/μg. Bacteria (1.5×10^9 CFU) and ^{125}I-C3 (1.4 μg) were mixed in 1.5 ml of RPMI-HEPES supplemented with 20% C5-deficient nonimmune human serum. The mixture was incubated at 37°C, and 0.1-ml samples were taken at various intervals. The samples were washed by repeated centrifugation for 3 min at 12,000 × *g* in a microcentrifuge. Bound radioactivity was assayed in a gamma counter. The number of C3 molecules bound per CFU was calculated.

Preparation of PMNs. Venous blood was obtained from healthy volunteers with no history of tularemia or tularemia vaccination and no demonstrable serum agglutinins against *F. tularensis*. PMNs were separated by density centrifugation (38) and were suspended in RPMI-HEPES at various densities. Giemsa-stained preparations showed more than 98% PMNs among leukocytes.

Assay of phagocytosis and killing. By using C5-deficient serum for opsonization, phagocytosis and killing of bacteria by PMNs could be studied free from complement-induced lysis. This technique has been recently used also in studies on *Leishmania major* (28). We suspended PMNs (5×10^6) and bacteria (5×10^6 CFU) in 0.5 ml of RPMI-HEPES supplemented with 20% C5-deficient nonimmune human serum. The suspension was incubated at 37°C with rotation end-over-end (60 rpm) in capped plastic tubes (12 by 75 mm; Becton Dickinson Labware, Oxnard, Calif.). For estimates of killing, samples were obtained after 0 and 60 min of incubation, diluted in chilled phosphate-buffered saline, sonicated to lyse the PMNs, and cultured. To estimate phagocytosis, diluted samples were centrifuged at 110 × *g* for 10 min at 4°C to separate PMNs from free bacteria. Viable counts were performed on sonicated supernatants. For estimates of survival of phagocytosed bacteria, sediments obtained after centrifugation of 60-min samples were resuspended and sonicated. Viable counts were performed on the sonicated sediment and compared with viable counts on sonicated 0-min samples.

In one series of experiments, opsonization was designed to express IgG at the surface of the Cap$^-$ mutant. After incubation of bacteria for 30 min at 37°C in RPMI-HEPES containing 75% C5-deficient serum, the bacteria were washed and reincubated in RPMI-HEPES with or without rabbit anti-human C3c antiserum (Dakopatts A/S; 1/16 final dilution). Bacteria were then washed and assayed for phagocytosis and killing. A sandwich enzyme-linked immunosorbent assay (39) was used to confirm that IgG was indeed exposed at the bacterial surface. First, microplates were coated with suspensions of the Cap$^-$ mutant (10^7 or 10^9 CFU/ml). The second layer consisted of 10% C5-deficient nonimmune human serum in RPMI-HEPES, the third layer consisted of rabbit anti-human C3c antiserum, and the fourth layer consisted of alkaline phosphatase-labeled swine anti-rabbit IgG (Orion Diagnostics, Helsinki, Finland). The end-point titer of IgG anti-C3c was 10^{-5} when added to bacteria

opsonized in C5-deficient serum compared with 10^{-2} when added to nonopsonized bacteria.

Assay of respiratory burst. The generation of superoxide anions by PMNs was estimated as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* by a modification (24) of the method of Babior et al. (3). Bacteria were preincubated in 20% C5-deficient nonimmune human serum in RPMI-HEPES and were washed before being added to PMNs.

The chemiluminescent response was measured in a luminometer (Model 1250; LKB-Wallac Oy, Turku, Finland) with plastic vials (LKB-Wallac Oy). Each vial contained PMNs (5×10^5) and luminol (4×10^{-5} M; Sigma) in 0.46 ml of RPMI-HEPES. To activate the PMNs, a 40-μl sample of bacteria (5×10^7 CFU), preincubated for 30 min at 37°C in RPMI-HEPES containing 75% C5-deficient serum, was added and the response was recorded at intervals. In one series of experiments, bacteria were incubated first in the presence of C5-deficient serum and then incubated, after washing, in RPMI-HEPES with or without rabbit anti-human C3c antiserum (1/16 final dilution). Finally, the bacteria were washed and suspended in RPMI-HEPES containing 75% C5-deficient serum.

Electron microscopy. The ultrastructure of different strains of *F. tularensis* and the intracellular localization of the Cap$^-$ mutant were studied. Proper sections were not easily obtained, as the bacteria seemed to be pushed rather than cut by the ultratome, leaving empty holes in the Epon material. To overcome the problem, colony-containing agar plates were overlaid with nutrient broth (Oxoid Ltd., Basingstoke, England), supplemented with 2.5% sodium chloride and 0.6% Bacto-Agar (Difco Laboratories, Detroit, Mich.). Fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.2], containing 0.1 M sucrose) was added to the plate. After being fixed overnight, colonies were excised, washed in cacodylate buffer, dehydrated in a graded series of ethanol, treated for 30 min in propylene oxide, and embedded in Epon 812. Sections were cut with an LKB ultratome and stained with uranyl acetate and lead citrate. A JEOL 100 CX electron microscope was used.

PMNs (5×10^7) and Cap$^-$ mutant bacteria (5×10^9 CFU) were incubated for 60 min at 37°C in 0.5 ml of RPMI-HEPES containing 20% C5-deficient serum. After centrifugation (110 × *g*), the sedimented PMNs were fixed and prepared in the same way as were the excised colonies.

RESULTS

Isolation of a Cap$^-$ mutant of *F. tularensis* LVS. Bacteria of the wild-type strain of *F. tularensis* and of *F. tularensis* LVS were exposed for 1 h to various concentrations of acridine orange and subcultured. Rough colonies were obtained only from the LVS. Using a concentration of 125 μg of acridine orange per ml, the frequency of rough colonies was 10^{-4}. At lower concentrations, only smooth colony forms were found, and at higher concentrations, bacteria did not survive. Electron microscopy showed that cells of the LVS (Fig. 1A) and of the wild-type strain were surrounded by an electron-dense surface layer similar to that previously recognized in other strains of *F. tularensis* and identified as a capsule (15, 33). A mutant, which formed rough colonies, did not have this layer (Fig. 1B). In the following experiments, we used the Cap$^-$ mutant and the parent strain of *F. tularensis* LVS, but not the wild-type strain.

The cellular fatty acid compositions and membrane protein profiles of the two strains were compared. *F. tularensis* has previously been shown to possess a specific fatty acid composition (17, 32). There were only minor differences between the parent strain and the Cap$^-$ mutant (Table 1). In fact, these differences were smaller than those previously found among different geographic variants of *F. tularensis* (17). The parent strain and the Cap$^-$ mutant showed similar membrane protein profiles by SDS-PAGE (Fig. 2). The mutant seemed not to have lost any of the polypeptides present in the parent strain. On the contrary, loss of capsular material by mutation seemed to enable the detection of additional bands (Fig. 2). It should be remarked that the same amount of protein was applied from both strains. When protein concentrations were increased, the profiles of the parent strain became smeary and no further bands were disclosed. In a previous study, T-lymphocyte-stimulating membrane polypeptides of LVS were found to be conserved in the mutant (35).

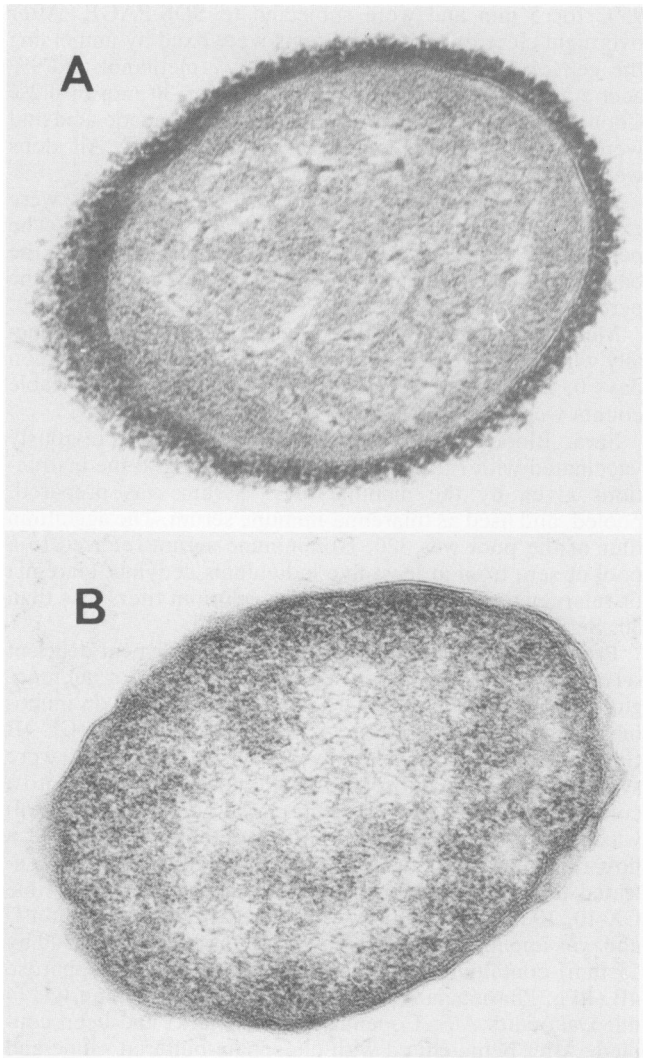

FIG. 1. Morphology of parent strain (A) and Cap$^-$ mutant (B) of *F. tularensis* LVS. Magnification, ×60,000.

Lack of virulence to mice of Cap$^-$ mutant. The parent strain or the Cap$^-$ mutant (10^6 CFU) was injected intraperitoneally in mice, and viable counts from spleen homogenates were performed at various intervals during 6 days. The

TABLE 1. Fatty acid composition of parent strain and Cap⁻ mutant of *F. tularensis* LVS

Fatty acid[a]	% Total peak area[b]	
	Parent strain	Cap⁻ mutant
$C_{10:0}$	11.54 ± 1.21	13.04 ± 0.69
2-OH-$C_{10:0}$	1.21 ± 0.40	0.69 ± 0.11
$C_{14:0}$	6.99 ± 0.25	8.82 ± 0.38
$C_{16:0}$	9.43 ± 0.18	10.53 ± 0.29
3-OH-$C_{16:0}$	2.81 ± 0.14	2.83 ± 0.09
$C_{18:1\omega9}$[c]	9.19 ± 0.39	9.20 ± 0.14
$C_{18:0}$	2.15 ± 0.10	2.32 ± 0.11
3-OH-$C_{18:0}$	14.44 ± 0.34	13.37 ± 0.53
$C_{20:1\omega11}$	1.99 ± 0.14	1.90 ± 0.06
$C_{20:0}$	2.12 ± 0.16	1.95 ± 0.13
$C_{22:1\omega13}$	5.38 ± 0.26	5.10 ± 0.29
$C_{22:0}$	6.35 ± 0.43	5.64 ± 0.31
$C_{24:1\omega15}$	19.05 ± 0.83	17.60 ± 0.74
$C_{24:0}$	4.93 ± 0.61	4.90 ± 0.10
$C_{26:1\omega17}$	1.79 ± 0.14	1.49 ± 0.09
$C_{26:0}$	0.62 ± 0.11	0.60 ± 0.03

[a] Number of carbon atoms and number of double bonds.
[b] Means ± standard deviations of four experiments.
[c] Position of double bond. All double bonds were determined to be *cis* configuration.

numbers of bacteria of the parent strain increased 10^3 times, whereas those of the Cap⁻ mutant decreased during the period (Fig. 3). Only rough colony forms appeared in cultures from the spleens of animals injected with the mutant.

Serum sensitivity of Cap⁻ mutant. The parent strain or the Cap⁻ mutant was incubated in the presence of 0 to 40% nonimmune human serum in RPMI-HEPES for 60 min at 37°C. The parent strain survived at all serum concentrations, whereas the Cap⁻ mutant was efficiently killed at concentrations higher than 1% (Fig. 4A). Similar results were obtained with any of 14 individual sera. In 2.5% nonimmune serum, more than 99% of the Cap⁻ bacteria were killed within 30 min of incubation (Fig. 4B).

The bactericidal effect of nonimmune human serum against the Cap⁻ mutant was abrogated by incubation of serum for 30 min at 56°C (data not shown). Depletion of IgM or of complement component C4 or C5 also abrogated the bactericidal effect of serum, whereas depletion of factor B or of IgG had little effect (Table 2). This suggests that killing of the Cap⁻ mutant by nonimmune human serum resulted from IgM activation of the classical pathway of complement.

Protection by PMNs from the bactericidal effect of serum. Cap⁻ bacteria were incubated for 60 min at 37°C in the presence of 2.5% nonimmune human serum, either in the absence or in the presence of PMNs. PMNs were found to protect the bacteria from the bactericidal effect of serum (Table 3).

Survival of Cap⁻ mutant in human PMNs. When the Cap⁻ mutant was incubated with PMNs for 60 min at 37°C in the absence of serum or in the presence of heat-inactivated serum, no phagocytosis occurred (data not shown). C5-deficient serum was used to obtain opsonization and still circumvent the bactericidal activity of serum. Then, bacteria of the Cap⁻ mutant strain were readily phagocytosed without being killed (Table 4). When the parent strain was similarly incubated, phagocytosis and killing occurred (Table 4).

There was strong evidence that PMNs protected the Cap⁻ bacteria by ingesting them. First, bacteria of the Cap⁻ mutant were seen intracellularly by electron microscopy

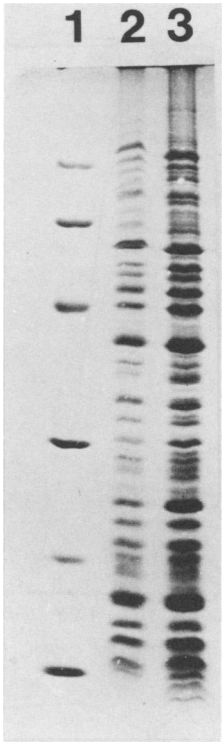

FIG. 2. SDS-PAGE profile of membranes from parent strain and Cap⁻ mutant of *F. tularensis* LVS. Lanes: 1, molecular weight markers, namely, phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400); 2, parent strain; 3, Cap⁻ mutant.

(Fig. 5). Second, the bacteria survived after they had been incubated with PMNs and the PMNs had been isolated, washed, and exposed to 20% fresh serum, which would be expected immediately to kill extracellular bacteria (Table 5).

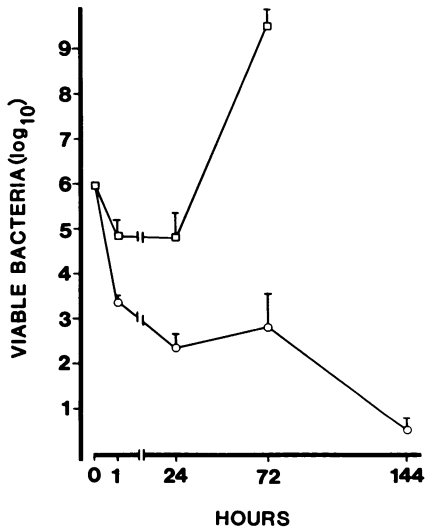

FIG. 3. Infectious course in mice inoculated with 10^6 organisms of parent strain (□) or Cap⁻ mutant (○) of *F. tularensis* LVS. Cultures were performed from the homogenized spleen of each animal. Means ± standard deviations of viable counts from four animals are indicated.

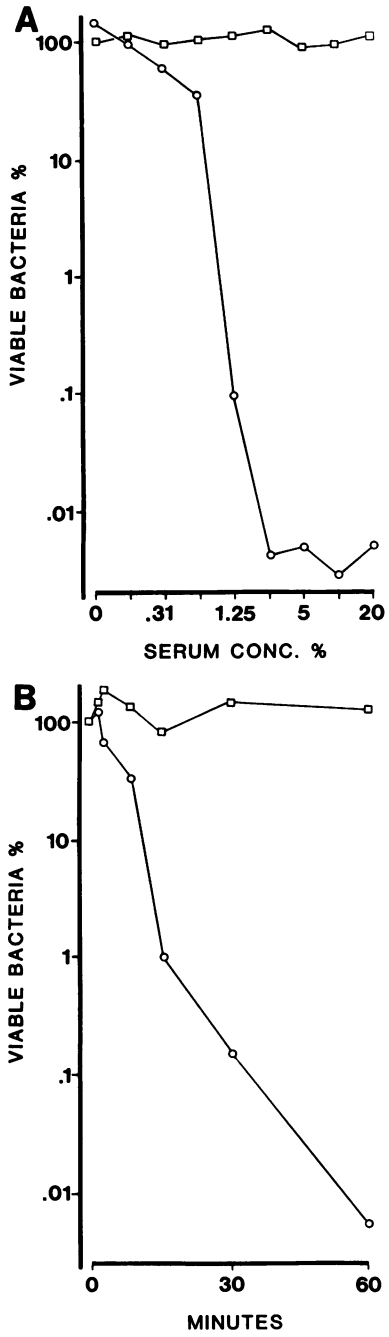

FIG. 4. Killing of bacteria of parent strain (□) or Cap⁻ mutant (○) of *F. tularensis* LVS by nonimmune human serum. Bacteria were incubated at various serum concentrations for 60 min (A) or for various time periods in 2.5% serum (B). The percentage of inoculum viable after incubation was recorded. Means of values from two experiments are indicated.

Finally, sonicated PMNs did not protect the bacteria from being killed by serum (Table 5).

Differing ability of Cap⁻ mutant and parent strain to induce a respiratory burst in PMNs. We examined the possibility that the difference between the Cap⁻ mutant and the parent strain in intracellular survival was accompanied by a difference in ability to induce a respiratory burst in the leukocytes. First, we found that no evident superoxide dismutase-inhibitable ferricytochrome *c* reduction was induced after opsonization in C5-deficient nonimmune serum, either by the Cap⁻ mutant or by the parent strain. We then tried the more sensitive luminol-enhanced chemiluminescent technique. For control purposes, the parent strain in tularemia-immune serum was shown to induce a high chemiluminescent response in PMNs (Fig. 6A). This was in agreement with previously reported data (23). When compared with this response, very low responses were induced in the presence of C5-deficient nonimmune serum by the parent strain, as well as by the Cap⁻ mutant (Fig. 6A). The response to the parent strain was inhibited by superoxide dismutase. The response to the Cap⁻ mutant had different kinetics and was unaffected by superoxide dismutase (Fig. 6B).

TABLE 2. Importance of complement pathways, IgG, and IgM to killing of Cap⁻ mutant of *F. tularensis* LVS by nonimmune human serum

Serum deficiency[a]	% Viable bacteria[b]	No. of expts
None (control)	0.2 ± 0.2	6
C5	97.5 ± 3.5	4
C4	75.0 ± 32.0	4
Factor B	0.1 ± 0.1	4
IgG and IgM	66.6 ± 16.4	10
IgG	3.9 ± 5.2	14
IgM	67.0 ± 31.0	8

[a] Bacteria of the Cap⁻ mutant (5×10^5 CFU) were incubated for 60 min at 37°C in 0.5 ml of RPMI-HEPES supplemented with 2.5% serum, from which various serum proteins had been removed by affinity chromatography. A control serum was chromatographed on immobilized bovine serum albumin.
[b] Mean ± standard deviation of 4 to 14 experiments.

Deposition of C3 on Cap⁻ mutant. When the Cap⁻ mutant was incubated in the presence of 20% C5-deficient nonimmune serum, 8×10^4 molecules of C3 were bound per CFU within 60 min of incubation (Fig. 7). The parent strain, on the other hand, bound virtually no C3 at all. The mechanism, whereby C5-deficient nonimmune serum promoted ingestion of the parent strain, was not clarified. Using an indirect immunofluorescence technique, we were not able to demonstrate an evident occurrence of IgG on its surface (data not shown).

Response of PMNs to Cap⁻ mutant opsonized with IgG. From the experiments on killing of the Cap⁻ mutant by sera deficient in specific proteins (Table 2), it seemed probable that the deposition of C3 in the presence of C5-deficient serum was mediated by naturally occurring IgM antibodies. To obtain opsonization with IgG, we incubated the Cap⁻ mutant first in C5-deficient nonimmune serum and then in anti-C3c antiserum. It was confirmed that IgG was expressed at the bacterial surface (Materials and Methods). In this situation, Cap⁻ bacteria induced a higher and superoxide

TABLE 3. Protection by PMNs from serum-induced killing of Cap⁻ mutant of *F. tularensis* LVS

Incubation[a] of bacteria in:	% Viable bacteria[b]
RPMI-HEPES	96.6 ± 13.1
RPMI-HEPES + serum	0.03 ± 0.02
RPMI-HEPES + serum + PMN	8.8 ± 1.9

[a] Bacteria of the Cap⁻ mutant (5×10^6 CFU) were incubated for 60 min at 37°C in 0.5 ml of RPMI-HEPES, RPMI-HEPES with 2.5% nonimmune human serum, or RPMI-HEPES with 2.5% serum and 5×10^6 PMNs.
[b] Cultures were performed on sonicated samples to calculate survival as percentage of inoculate. Mean ± standard deviation of four experiments.

TABLE 4. Phagocytosis and killing by PMNs of parent strain and Cap⁻ mutant of *F. tularensis* LVS

Bacterial strain[a]	% Bacteria[b]		
	Phagocytosed	Killed	Phagocytosed but still viable
Mutant	97.2 ± 1.2	12.3 ± 14.5	82.0 ± 12.1
Parent	95.1 ± 3.8	75.5 ± 6.2	15.3 ± 4.3

[a] Bacteria (5×10^6 CFU) were incubated for 60 min at 37°C with PMNs (5×10^6) in 0.5 ml of RPMI-HEPES supplemented with 20% C5-deficient serum.
[b] Mean ± standard deviation of four experiments.

TABLE 5. Unaccessibility of PMN-associated Cap⁻ mutant of *F. tularensis* LVS to the bactericidal effect of serum

Bacterial prepn[a]	% Viable bacteria[b]
PMN-associated bacteria	89.3 ± 14.0
Bacteria + disintegrated PMNs	0.002 ± 0.001
Bacteria	0.004 ± 0.001

[a] Bacteria of the Cap⁻ mutant were prepared in various ways and exposed to nonimmune human serum (20% in RPMI-HEPES) for 60 min at 37°C, before samples were obtained for culture.
[b] Mean ± standard deviation of four experiments.

dismutase-inhibitable chemiluminescent response (Fig. 8) and were killed by the PMNs (Table 6).

Thus, the antimicrobial response of PMNs to the Cap⁻ mutant depended on the method of opsonization of the mutant. The results contradicted the possibility that the Cap⁻ mutant resisted PMNs by displaying some leukotoxic activity and that the mutant was resistant to antimicrobial agents produced by properly stimulated leukocytes.

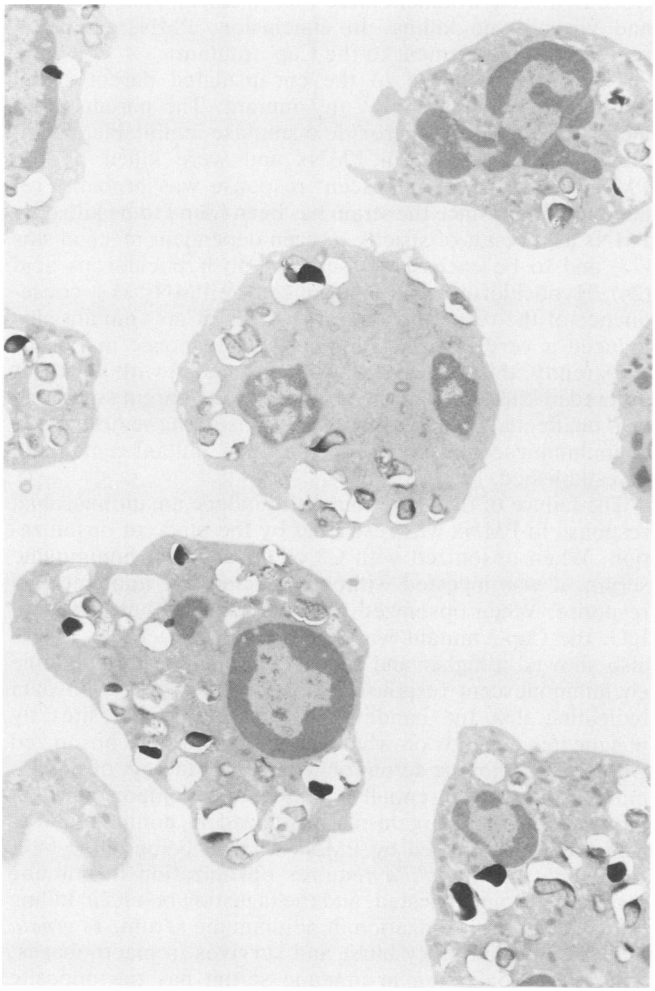

FIG. 5. Intracellular localization of Cap⁻ mutant of *F. tularensis* LVS in PMNs. After incubation of bacteria with PMNs in the presence of C5-deficient serum, PMNs were sedimented and prepared for electron microscopy. Magnification, ×5,200.

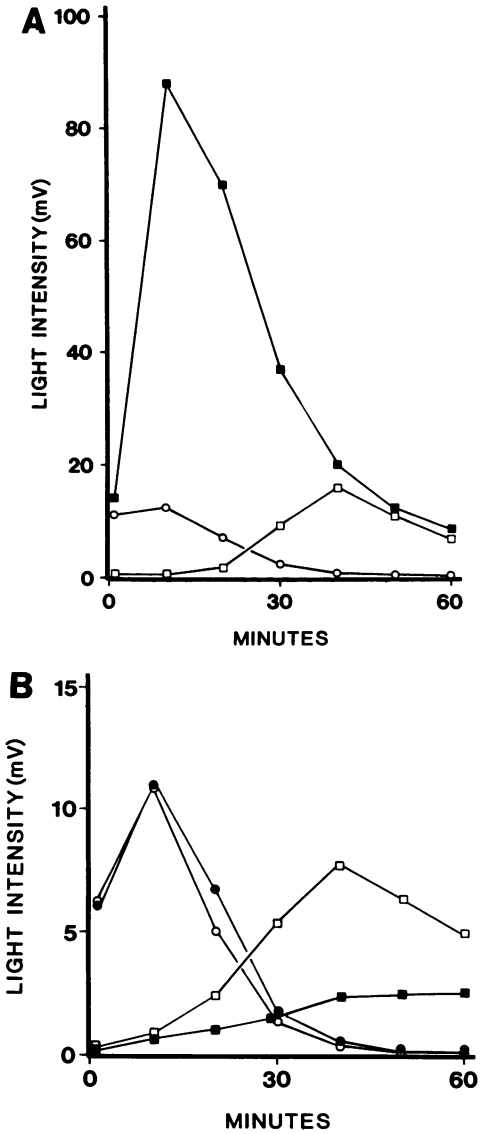

FIG. 6. Chemiluminescent response of PMNs to the two strains of *F. tularensis* LVS in the presence of C5-deficient nonimmune serum. (A) Response to parent strain (□) and Cap⁻ mutant (○). For controls, the response to the parent strain in the presence of tularemia immune serum (■) is also shown. (B) Response to parent strain in the absence (□) and in the presence (■) of superoxide dismutase and to Cap⁻ mutant in the absence (○) and in the presence (●) of superoxide dismutase. Three repeated experiments gave similar results.

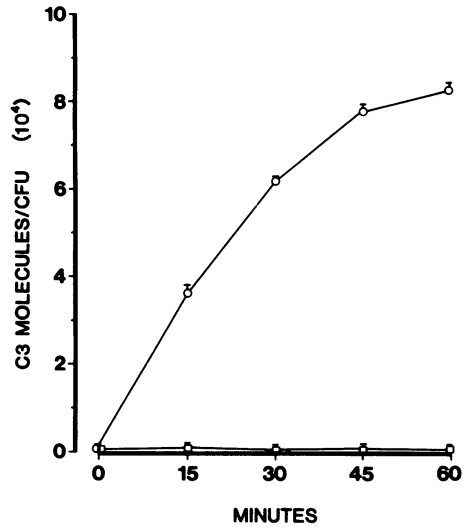

FIG. 7. Deposition of C3 on parent strain (□) and Cap⁻ mutant (○) of *F. tularensis* LVS after incubation for various time periods in the presence of C5-deficient nonimmune serum. Means ± standard deviations of four experiments are indicated.

DISCUSSION

In contrast to the parent strain of *F. tularensis* LVS, the Cap⁻ mutant was highly susceptible to the bactericidal effect of nonimmune human serum. The mutant was protected from the killing effect of serum by being ingested by PMNs. In an environment containing serum and PMNs, mutant bacteria obviously had to rely on a narrow time schedule to survive. After opsonization with complement components, the bacteria must be engulfed before the membrane attack complex of complement became active. According to studies on *Escherichia coli*, there may indeed be a time interval of a few minutes between insertion of C3b and formation of membrane attack complex in the outer membrane (37). By using C5-deficient serum, phagocytosis of the Cap⁻ mutant in PMNs could be studied free from complement-induced lysis. In this environment there was an efficient phagocytosis and virtually no killing. In conclusion, PMNs offered a protective compartment to the Cap⁻ mutant.

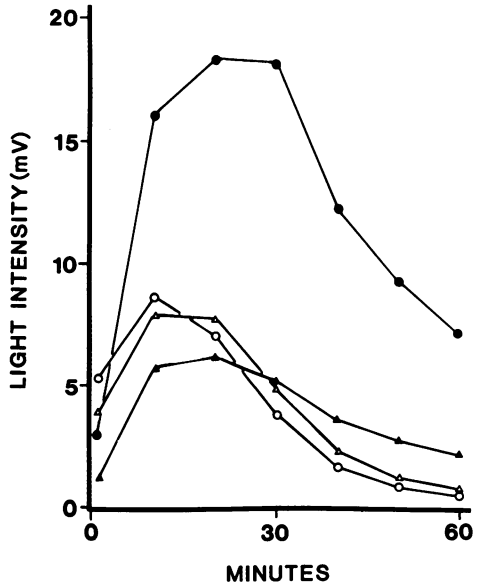

FIG. 8. Chemiluminescent response of PMNs to bacteria of Cap⁻ mutant of *F. tularensis* LVS opsonized in C5-deficient serum (open symbols) or subsequently opsonized in anti-human C3c antiserum also (closed symbols). Incubation was performed in the absence (circles) or in the presence (triangles) of superoxide dismutase. Four repeated experiments gave similar results.

TABLE 6. Phagocytosis and killing of Cap⁻ mutant of *F. tularensis* LVS opsonized in different ways

Opsonin[a]	% Bacteria[b]		
	Phagocytosed	Killed	Phagocytosed but still viable
C5-deficient nonimmune serum	98.2 ± 0.5	10.6 ± 9.7	85.0 ± 15.8
C5-deficient nonimmune serum + anti-C3c antiserum	96.8 ± 1.8	71.7 ± 10.3	28.0 ± 7.1

[a] Bacteria (5×10^6 CFU) were incubated for 30 min at 37°C in RPMI-HEPES supplemented with 75% C5-deficient nonimmune human serum, washed, and reincubated in RPMI-HEPES with or without anti-human C3c antibodies. After being washed, the bacteria were incubated for 60 min at 37°C with PMNs (5×10^6) in RPMI-HEPES supplemented with 20% C5-deficient serum at a total volume of 0.5 ml.

[b] Mean ± standard deviation of four experiments.

The PMN response to the encapsulated parent strain differed from that to the Cap⁻ mutant. The parent strain induced a very low superoxide dismutase-inhibitable chemiluminescent response in PMNs and were killed by the PMNs. This chemiluminescent response was probably related to killing, since the strain has been found to be killed by PMNs as a result of strictly oxygen-dependent mechanisms (22) and to be excessively sensitive to hypochlorous acid (24). Hypochlorous acid is produced by PMNs as a consequence of the respiratory burst (10). The Cap⁻ mutant also induced a very low chemiluminescent response in PMNs. Apparently, this response was not associated with killing. It differed in kinetics from the response to the parent strain and was unaffected by superoxide dismutase. The nature of the chemiluminescent response to the Cap⁻ mutant remains to be established.

The failure of the Cap⁻ mutant to induce an antimicrobial response in PMNs was restricted by the mode of opsonization. When opsonized with C3 of C5-deficient nonimmune serum, it was ingested without inducing any antimicrobial response. When opsonized not only with C3 but also with IgG, the Cap⁻ mutant was killed by PMNs. Then, PMNs also showed a higher and superoxide dismutase-inhibitable chemiluminescent response. These results agree with data indicating that the handling of intracellular parasites by phagocytes depends on whether the parasites are opsonized with IgG of immune serum or with serum factors of nonimmune serum, preferentially complement components. *Legionella micdadei* organisms opsonized in nonimmune serum are phagocytosed by PMNs without being killed (40). *Legionella pneumophila* requires opsonization in immune serum to become ingested, and the ingestion results in killing (16, 40). After opsonization in nonimmune serum, *T. gondii* induces no respiratory burst and survives in macrophages, whereas opsonization in immune serum has the opposite effects (1). Similarly, *Histoplasma capsulatum* requires immune serum to induce a respiratory burst in macrophages (43). These results all support the view that the phagocytosis of IgG-opsonized particles is firmly associated with induc-

tion of a respiratory burst, whereas complement-opsonized particles may, under some conditions, be ingested without inducing this burst (4, 13, 31, 45, 46). An important task will be to define the ligand-receptor interaction which leads to ingestion of capsule-deficient *F. tularensis* in PMNs without induction of an antimicrobial response in the PMNs. Complement component C3 is then of primary interest. Studies on the intracellular parasite *Leishmania major* have indicated that C3-opsonized organisms survive better after phagocytosis in macrophages than do nonopsonized organisms, and this finding was attributed to an ability of C3-opsonized *L. major* to be ingested without inducing a respiratory burst (28). If so, it is still unknown in which form C3 should be presented to the phagocyte to promote ingestion without inducing a respiratory burst. *L. major* (27) and *Leishmania donovani* (44) have been reported to be ingested in macrophages preferentially by opsonization with iC3b. However, an ability of iC3b-coated particles to promote ingestion without inducing a respiratory burst (45) is not a general finding (11, 34, 36). Possibly, intracellular parasites have a yet undefined ability to bring iC3b or some other complement-derived fragment to an interaction with the phagocyte membrane, resulting in phagocytosis but not in respiratory burst.

When first observed, the failure to induce the respiratory burst was interpreted to be a virulence trait among intracellular parasites, since it was detected in a virulent but not in an avirulent strain of *S. typhi* (26). Although failure to induce an antimicrobial response may obviously be regarded as a virulence trait, it seems important to state that this trait is not confined to virulent organisms. It has been previously demonstrated in an avirulent strain of *H. capsulatum* (7) and now in an avirulent mutant of *F. tularensis*. The failure to induce an antimicrobial response in phagocytes may be a fundamental trait in intracellular parasites but not equally dominant in all organisms. Special surface structures, such as the capsule of *F. tularensis* LVS, are required to protect parasites against lytic agents, such as serum complement, and these structures may bring the parasite to induce an antimicrobial response.

The Cap^- mutant of *F. tularensis* may offer a useful model in experiments to explain why ingestion of intracellular parasites leads in one situation to induction of an antimicrobial response and in another to intracellular survival. This expectation is based on (i) its rapid growth in vitro, (ii) the ease by which it is separated from phagocytes, thus enabling simple, reproducible, and objective techniques to estimate phagocytosis and killing, and (iii) the efficient phagocytosis with virtually complete intracellular survival. The last finding implicates that at least one of the ways by which the Cap^- mutant and PMNs interact can be studied free from contribution of the other.

ACKNOWLEDGMENTS

We thank Wigert Sjöberg, Lena Öhlund, and Leonore Johansson for skillful technical assistance, Anders Edlund for performing fatty acid analyses, and Jan Carlsson for valuable criticism of the manuscript.

This work was supported by the Medical Faculty, University of Umeå.

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