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A Wild and an Attenuated Strain of *Francisella tularensis* Differ in Susceptibility to Hypochlorous Acid: A Possible Explanation of Their Different Handling by Polymorphonuclear Leukocytes

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We have previously reported that a wild strain of *Francisella tularensis* is much less efficiently killed by human polymorphonuclear leukocytes than is an attenuated strain. In the present study, the killing of the attenuated strain was found to be strictly oxygen dependent. The wild and the attenuated strains both induced a respiratory burst in the leukocytes. The difference between the strains in susceptibility to agents produced at the burst could be explained by a difference in susceptibility to hypochlorous acid.

A live attenuated strain of *Francisella tularensis* (*F. tularensis* LVS) is used as vaccine for prevention of tularemia (10). The low virulence of this strain may be at least partly explained by the finding that it is more readily killed by human polymorphonuclear leukocytes (PMNs) than is a wild strain (26). The reason for the different handling of the two strains by PMNs is unknown.

PMNs kill bacteria by oxygen-dependent and oxygen-independent mechanisms (14). Bacteria induce a respiratory burst in the leukocytes, whereby oxygen is converted into such toxic products as superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen (3, 6, 14, 22, 36). Hydrogen peroxide may be used by the lysosomal myeloperoxidase to oxidate chloride into the highly bactericidal agent hypochlorous acid (12, 21, 28, 36). In the oxygen-independent bactericidal mechanisms of the PMNs, cationic lysosomal proteins seem to play an important role (11).

The efficient killing of the attenuated strain of *F. tularensis* by PMNs may be related to the ability of PMNs to elicit a respiratory burst when phagocytosing this strain (4, 25). The aim of the present study was to find out whether the killing of the attenuated strain is oxygen dependent and whether the wild strain evades being killed by a failure to trigger the respiratory burst in the PMNs or by a higher resistance than the attenuated strain to the bactericidal products formed from oxygen by the PMNs.

MATERIALS AND METHODS

Bacteria and growth conditions. An attenuated strain of *F. tularensis* was supplied by the U.S. Army Medical Research Institute of Infectious Diseases (10). A wild strain of *F. tularensis* var. *palaearctica* (strain SBL R45) was supplied by R. Möllby, National Bacteriological Laboratory, Stockholm, Sweden (26). Both strains were catalase negative, as indicated by a slide test with 3% hydrogen peroxide. *Streptococcus faecalis* group D strain C3 was obtained from S. E. Holm, Clinical Bacteriological Laboratory, Umeå, Sweden. The bacteria were kept frozen in RPMI 1640 with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (RPMI-HEPES, GIBCO Bio-Cult, Glasgow, Scotland) at −70°C. At intervals of 1 month, samples were thawed, and the bacteria were cultivated on modified Thayer-Martin agar (26) at 37°C in 5% CO_2 in air. For each experiment the *F. tularensis* strains were grown for 2 days and the *S. faecalis* strain was grown overnight on the surface of the agar medium. They were then harvested and suspended in various fluids. All the experiments with the wild strain of *F. tularensis* were performed in a safety P3 laboratory.

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). Serum was also obtained from blood of a man who had suffered from tularemia 1 month previously. A serum pool was also prepared from five persons who denied that they had either tularemia or a tularemia vaccination (nonimmune serum). The agglutinin titers of these three sera against *F. tularensis* were 320, 1,280, and less than 20, respectively. The sera were stored at −70°C.

Preparation of human PMNs. Venous blood was obtained from healthy volunteers with no history of tularemia or tularemia vaccination and no demonstrable serum agglutinins against *F. tularensis*. The PMNs were prepared as previously described (25), with sedimentation of the erythrocytes in the presence of dextran (35) and lysis of remaining erythrocytes with 0.87% ammonium chloride (8). The PMNs were then washed twice and suspended in RPMI-HEPES at a density of 5×10^7/ml. Giemsa-stained preparations of the suspensions contained 75 ± 5% PMNs, 20 ± 5% lymphocytes, and 5 ± 3% monocytes (mean ± standard deviation).

Assay of phagocytosis and killing. Phagocytosis and killing of the bacteria by PMNs were simultaneously assayed in air and in a glove box with an atmosphere of 10% hydrogen and 5% carbon dioxide in nitrogen (38). To estimate the number killed, the total number of viable bacteria in sonicated samples was calculated (26). To separate phagocytosed from nonphagocytosed bacteria, the cells were sedimented by centrifugation at 110 × *g* for 10 min at 4°C (39), and the number of nonphagocytosed viable bacteria remaining in the supernatant was determined. For control purposes, the number of bacteria associated with PMNs but still viable was also estimated. The sediment was resuspended and sonicated (26), and the number of viable bacteria was calculated. The number of viable bacteria in the resuspended sediment equaled roughly the difference between the number of bacteria phagocytosed and the number killed.

Estimation of respiratory burst. Bacteria of the wild and attenuated strain of *F. tularensis* were preincubated at 37°C for 30 min in the presence of 20% serum or in the absence of serum. The bacteria were washed and suspended in Hanks

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balanced salt solution at a density of 6.25×10^9/ml. This composition of the preincubation mixture resulted in a maximal reduction of cytochrome *c*, as well as Nitro Blue Tetrazolium, in experiments in which the serum concentration was varied from 0 to 20% and the bacterial density was varied from 0 to 2.5×10^{10}/ml.

The generation of superoxide anion by PMNs was estimated as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* by a modification of the method of Babior et al. (1). In glass tubes (10 by 100 mm), 0.1 ml of PMN suspension (5×10^6 cells), 0.4 ml of preincubated bacteria (2.5×10^9 cells in Hanks balanced salt solution), 0.2 ml of 0.8 mM cytochrome *c* (Boehringer, Mannheim, Federal Republic of Germany), and 1.3 ml of Hanks balanced salt solution were mixed. Paired reaction mixtures with and without added superoxide dismutase (10 μg/ml; Sigma Chemical Company, St. Louis, Mo.) were employed. The reaction mixtures were incubated at 37°C for 15 min, after which the reactions were stopped at 0°C. The tubes were centrifuged at 4°C at 1,000 × *g* for 10 min, and the absorbances of the supernatants were determined at 550 nm by using 80 μM cytochrome *c* as a blank. The amounts of cytochrome *c* reduced were determined by using an extinction coefficient of 21.1 cm^{-1} mM^{-1} (40) (reduced-oxidized), and the difference in cytochrome *c* reduction between parallel supernatants with and without added superoxide dismutase was used as a measure of superoxide anion generation. The reduction of Nitro Blue Tetrazolium by human PMNs was estimated essentially as described by Baehner and Nathan (2). In glass tubes (10 by 100 mm), 0.1 ml of PMN suspension, 0.4 ml of preincubated bacteria, 0.4 ml of 0.1% Nitro Blue Tetrazolium (E. Merck AG, Darmstadt, Federal Republic of Germany) and 0.1 ml of Hanks balanced salt solution were mixed. The mixtures were incubated at 37°C for 30 min, after which the reactions were stopped by the addition of 5.0 ml of 0.5 N hydrochloric acid. The tubes were centrifuged at 400 × *g* for 10 min, the pellet was suspended in 4.0 ml of pyridine (Merck), and reduced Nitro Blue Tetrazolium was extracted for 30 min in a boiling water bath. The tubes were recentrifuged, and the optical densities of the supernatants were determined at 580 nm with pyridine as a blank.

Estimation of bactericidal effects of oxidants. Susceptibility to xanthine-xanthine oxidase was estimated essentially as described by Murray and Cohn (30). Xanthine (0.3 mM; BDH, Poole, England) and xanthine oxidase (0.2 mg/ml; Boehringer) were separately suspended in phosphate-buffered saline (pH 7.4). In capped plastic tubes (10 by 100 mm), 2×10^3 bacteria of the wild or the attenuated strain were mixed with 0.3 mM xanthine and 0.2 mg of xanthine oxidase in a final volume of 1 ml of phosphate-buffered saline. Immediately after mixing and after 60 min of incubation at 37°C, samples were drawn, and viable counts were made. Control incubations with excluded xanthine, xanthine oxidase, or both were simultaneously run.

Susceptibility to hydrogen peroxide was estimated essentially as described by Berglin et al. (3). Hydrogen peroxide (30% [wt/wt] Perhydrol; Merck) was diluted in distilled water to a concentration of 100 mM. Further dilution was performed in phosphate-buffered saline (pH 7.4) at the concentrations stated. The concentration of hydrogen peroxide was standardized by acid permanganate titration and was routinely checked by a horseradish peroxidase-catalyzed reaction with 2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid) (7). In plastic tubes (10 by 100 mm), bacteria (2×10^3/ml) of the wild or attenuated strain of *F. tularensis* were mixed with various concentrations of hydrogen peroxide in a final volume of 5 ml and incubated at 37°C. Samples were drawn immediately after mixing and at various intervals thereafter. Viable counts were made.

Susceptibility to hypochlorous acid was estimated essentially as described by Seyfried and Fraser (32). Sodium hypochlorite solution (5% available chloride; KEBO, Spånga, Sweden) was diluted in phosphate buffer (pH 7.4) (6.7 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) or in 0.1 M sodium acetate buffer (pH 5.0) to the concentrations stated. In plastic tubes, 5×10^6 bacteria of the wild or the attenuated strain were incubated in 5.0 ml of buffer at 37°C in the absence or the presence of various concentrations of hypochlorous acid. After 1 min of incubation, 320 μg of sodium thiosulfate (Merck) was added to stop the reaction, and viable counts were made.

RESULTS

The killing of the attenuated strain of *F. tularensis* was strictly oxygen dependent. In air, 90% of the bacteria were phagocytosed and killed within 60 min (Fig. 1a). In an anaerobic environment, the phagocytosis was of the same order of magnitude, although no killing occurred (Fig. 1b). The absence of a bactericidal effect of PMNs in the anaerobic environment might be due to uncontrolled cytotoxic effects of the environment. To exclude this possibility, phagocytosis and killing of a strain of *S. faecalis* were simultaneously studied by using leukocytes from the same suspension as used in the experiment with *F. tularensis*. Human PMNs have previously been shown to kill *S. faecalis* in an anaerobic environment (27). The function of the PMNs was found to be preserved insofar as they killed bacteria of *S. faecalis* equally well under anaerobic (Fig. 1d) and aerobic (Fig. 1c) conditions. Neither the attenuated strain of

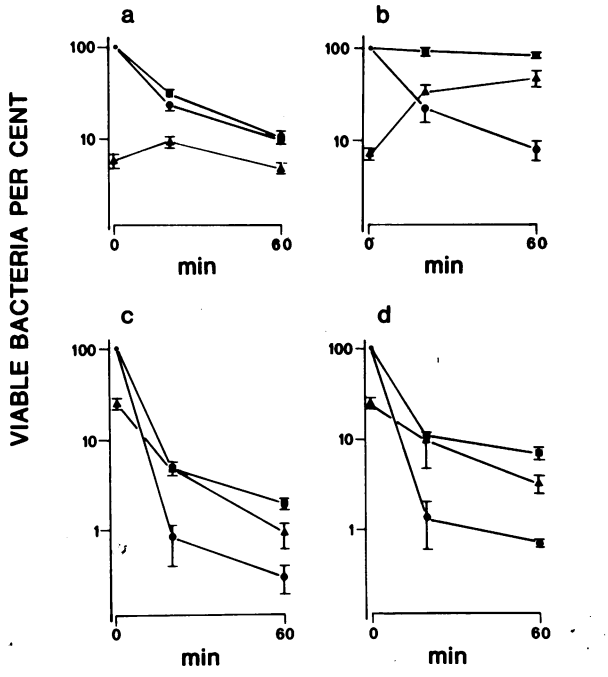

FIG. 1. Phagocytosis (●) and killing (■) of *F. tularensis* LVS (a and b) and *S. faecalis* C3 (c and d) in aerobic (a and c) and anaerobic (b and d) environments. The percentage of inoculum associated with PMNs but still viable is also shown (▲). The mean ± standard error of the mean of six (a, b) and three (c, d) experiments is shown.

TABLE 1. Induction of respiratory burst in human PMNs by wild and attenuated *F. tularensis* organisms

Serum used for preincubation[a]	Cytochrome *c* reduction (nmol/15 min per 5 × 10^6 PMNs)		Nitroblue tetrazolium reduction (OD_{580})[b]	
	Wild strain	Attenuated strain	Wild strain	Attenuated strain
Immune	28.3 ± 0.7 (5)[c]	25.3 ± 1.0 (5)	0.92 ± 0.03 (12)	0.89 ± 0.04 (12)
Nonimmune	4.6 ± 0.9 (4)	5.8 ± 1.3 (4)	0.18 ± 0.01 (5)	0.30 ± 0.02 (5)

[a] Bacteria were preincubated at 37°C for 30 min in 20% serum, washed, and resuspended.
[b] OD_{580}, Optical density at 580 nm.
[c] Mean ± standard error of the mean. The number of experiments is indicated in parentheses.

F. tularensis nor *S. faecalis* was killed by serum (data not shown).

Both the wild and the attenuated strains induced a respiratory burst in PMNs. When preopsonized with vaccinee serum, bacteria of the wild as well as the attenuated strain induced generation of superoxide anions disclosed by superoxide dismutase-inhibitable reduction of cytochrome *c* (Table 1). Similar results were obtained by using patient serum for preopsonization (data not shown). When the bacteria were preopsonized with nonimmune serum, only minor amounts of superoxide anions were generated (Table 1). In the absence of PMNs, there was no generation (data not shown). When the respiratory burst was assayed by using the Nitro Blue Tetrazolium test, the results were similar (Table 1).

The attenuated strain was only slightly more susceptible than the wild strain to hydrogen peroxide and to products of the xanthine-xanthine oxidase system. When the two strains were incubated for 60 min in the presence of hydrogen peroxide at concentrations ranging from 0.01 to 20 mM, there was a gradually decreasing survival rate, with 0.3% of the attenuated bacteria and 1.2% of the wild bacteria surviving a 20 mM solution (Fig. 2). When the bacteria were incubated for 60 min in the presence of an aerated mixture of xanthine and xanthine oxidase, 26% of attenuated bacteria survived, whereas 84 to 108% of the bacteria survived in the control solutions (Table 2). Sixty-one percent of the wild bacteria survived in the presence of the mixture, compared with 82 to 101% in control solutions (Table 2).

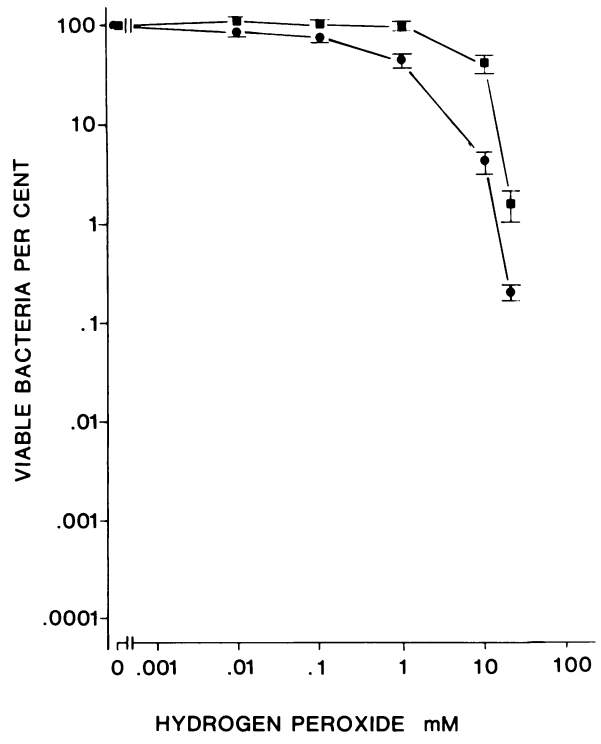

FIG. 2. Susceptibility of a wild (■) and an attenuated (●) strain of *F. tularensis* to hydrogen peroxide. Bacteria (2 × 10^3/ml) were incubated for 60 min in the presence of 0 to 20 mM hydrogen peroxide, and the number of surviving bacteria was estimated. The mean ± standard error of the mean of three experiments is shown.

The attenuated strain was efficiently killed by hypochlorous acid at concentrations to which the wild strain was resistant (Fig. 3). At pH 7.4 and 5.0, 0.1% and less than 0.001%, respectively, of the attenuated bacteria survived a 1-min exposure to 0.03 mM hypochlorous acid. The wild strain survived these treatments but was killed by exposure to 0.12 mM hypochlorous acid (Fig. 3).

DISCUSSION

The ability to survive in phagocytes is the key characteristic of intracellular parasites. *Mycobacterium tuberculosis* (16), *Brucella abortus* (23), *Listeria monocytogenes* (41), *Salmonella typhi* (29), *Legionella pneumophila* (24), and *Toxoplasma gondii* (30) all seem to be more or less susceptible to the oxygen products generated at the respiratory burst of the phagocytes. However, they may evade this host defense by preventing the phagocytes from activating their respiratory burst or by decomposing the hydrogen peroxide of the respiratory burst by catalase or both (9, 13, 16, 29, 42). It has also been suggested that intracellular parasites may evade the host defense by preventing phagolysosome fusion (15). Recent ultrastructural studies have not, however, confirmed this evasive mechanism (17).

No data have been published to explain how the intracellular parasite *F. tularensis* is protected from intracellular killing. An attenuated strain of this species is killed by human PMNs much more efficiently than a wild strain (26),

TABLE 2. Effect of xanthine-xanthine oxidase on wild and attenuated *F. tularensis* organisms

Additions[a]	% Survival[b]	
	Wild strain	Attenuated strain
None	90 ± 6 (5)[c]	103 ± 8 (4)
Xanthine	101 ± 2 (5)	84 ± 11 (4)
Xanthine oxidase	82 ± 10 (5)	90 ± 8 (4)
Xanthine + xanthine oxidase	61 ± 4 (5)	26 ± 4 (4)

[a] Bacteria (2 × 10^3/ml) were incubated for 60 min at 37°C.
[b] Percentage of the initial number of bacteria as disclosed by viable counts.
[c] Mean ± standard error of the mean. The number of experiments is indicated in parentheses.

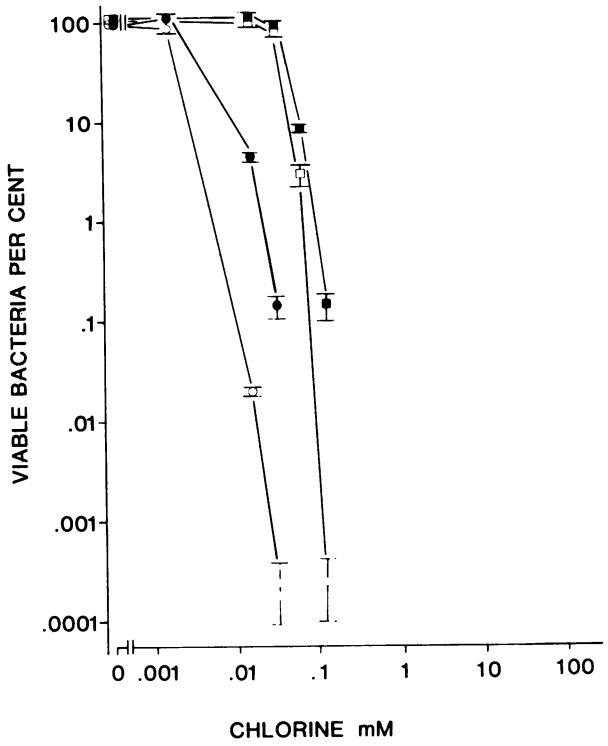

FIG. 3. Susceptibility of wild (□, ■) and an attenuated (○, ●) strain of *F. tularensis* to hypochlorous acid at pH 7.4 (■, ●) and pH 5.0 (□, ○). Bacteria (10^6/ml) were incubated for 1 min in the presence of 0 to 0.12 mM hypochlorous acid, and the number of surviving bacteria was estimated. The mean ± standard error of the mean of 8 to 12 experiments is shown.

and according to the present results, this killing occurred only in the presence of oxygen. The wild strain activated the respiratory burst of the PMNs as efficiently as did the attenuated strain. Neither strain produced catalase.

The wild strain was more resistant than the attenuated strain to bactericidal products known to be formed from oxygen by PMNs during phagocytosis. The wild strain survived an exposure to hydrogen peroxide or to a mixture of superoxide anions, hydrogen peroxide, and possible hydroxyl radicals from a xanthine-xanthine oxidase system (31) somewhat better than the attenuated strain. A more striking difference between the strains was found when they were exposed to hypochlorous acid. A concentration as low as 0.03 mM killed more than 99.9% of the attenuated bacteria at pH 7.4 within 1 min, whereas the wild organisms were completely resistant to this treatment. Hypochlorous acid is the product of the potent antimicrobial system of PMNs, hydrogen peroxide-myeloperoxide-chloride (21, 28, 36, 37), and as much as 28% of the oxygen consumed by PMNs has been shown to be converted into this product (12). The pH of the phagosome may decrease during phagocytosis (19, 20, 33). Hypochlorous acid would then be less dissociated (pK_a = 7.5) and its bactericidal activity would increase (32, 36). The present results also showed that hypochlorous acid was much more effective at pH 5.0 than at pH 7.4

The attenuated strain was much more susceptible to hypochlorous acid than to the other oxygen products. Exposure for 1 min to 0.03 mM hypochlorous acid at pH 7.4 killed more bacteria than 60 min of exposure to 10 mM hydrogen peroxide or to the xanthine-xanthine oxidase system. This result suggests that the attenuated strain is killed by PMNs mainly by the hydrogen peroxide-myeloperoxidase-chloride system. It was killed at a concentration of hypochlorous acid which increases the permeability of *Escherichia coli* so as to kill the organism (34). This is consistent with the view that this system is the most potent of the oxygen-dependent bactericidal systems of the PMNs (22).

The mechanism by which the wild strain of *F. tularensis* is protected against oxidants of PMNs is unknown. *Pseudomonas aeruginosa* has a capsule which, it has been suggested, protects it from the bactericidal effect of hypochlorous acid (32). *F. tularensis* possesses a capsule which contains lipid, protein, and carbohydrate (18) and whose removal renders the bacteria avirulent (17). It has been suggested that *F. tularensis* LVS possesses a lower amount of surface antigen than wild strains do (5), indicating that its capsule may be less abundant. The capsule might protect against hypochlorous acid. One possibility is that capsule protein reacts with hypochlorous acid, thereby scavenging the oxidizing power (37). In mycobacteria, a protective effect against mononuclear phagocytes has been ascribed to a glycolipid-rich envelope (9). It is not understood, however, in what way this envelope protects. An association between attenuation and susceptibility to hydrogen peroxide has been found in many, although not all, strains of *M. tuberculosis* (16). The susceptibility to hypochlorous acid was not tested.

In conclusion, a virulence mechanism of *F. tularensis* has been suggested that enables the organism to withstand oxidants produced by PMNs. The vaccine strain has lost this ability. The virulence factor remains to be identified.

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