INFECTION AND IMMUNITY, Jan. 1985, p. 234–241
0019-9567/85/010234-08$02.00/0

Vol. 47, No. 1

Survival and Growth of *Yersinia pestis* Within Macrophages and an Effect of the Loss of the 47-Megadalton Plasmid on Growth in Macrophages

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Received 2 July 1984/Accepted 28 September 1984

The survival and growth of *Yersinia pestis* cells within mouse peritoneal cavities and within mouse peritoneal macrophages maintained in vitro was examined. Two strains were used which differed only in that one (KIM) contained the 47-megadalton plasmid associated with virulence and the second (KIM1) lacked this plasmid. The KIM cells, but not the KIM1 cells, acquired some resistance to phagocytosis during growth at 37°C which was not evident when cells were grown at 26°C. Whether previously grown at 26 or 37°C, however, a substantial portion of the cells of either strain which were phagocytized were apparently killed after phagocytosis in vivo, although this was not observed in vitro. KIM cells which survived phagocytosis proliferated within macrophages in vivo, but no increase in viable cells was seen with the KIM1 cells. Growth of the KIM1 cells within macrophages in vitro required that a complex supportive medium be used in which the bacteria could have grown if extracellular. This was not the case for the KIM cells which proliferated within macrophages supported in medium not permissive to bacterial growth. After phagocytosis of cells of either strain by macrophages maintained in vitro, phagolysosome formation occurred normally, as shown by the acridine orange dye staining technique. KIM and KIM1 cells were equally sensitive to hydrogen peroxide and superoxide anion, although the sensitivity in each case varied with growth temperature. The oxidative burst, as determined by the luminol chemiluminescence assay, was low when compared with that seen after phagocytosis of *Escherichia coli* cells. Chemiluminescence after phagocytosis of yeast cells by macrophages which had engulfed KIM or KIM1 was also low. We conclude that survival within macrophages is substantially independent of the 47-megadalton plasmid and may be a consequence, as least in part, of blockage of the oxidative burst or rapid removal of the oxidizing compounds formed. The 47-megadalton plasmid is apparently required for subsequent proliferation within the macrophage.

Yersinia pestis is the causative agent of bubonic plague. After subcutaneous injection into rodents and primates, the same general disease progression occurs. Extensive multiplication of bacteria occurs at the site of infection, and the bacteria enter the lymphatic system and spread to the regional lymph nodes, thoracic duct, and bloodstream. The resulting bacteremia allows the bacteria to spread to the liver, spleen, and bone marrow. After extensive multiplication of bacteria in these areas, septicemia develops (25). This progression, like those seen after alveolar or intravenous inoculation (10, 36), is consistent with both extracellular growth and growth within phagocytes. Accordingly, *Y. pestis* is considered to be a facultative intracellular parasite.

Like some extracellular parasites, *Y. pestis* produces antiphagocytic factors (3). An antiphagocytic capsule, the fraction 1 antigen, is produced at 37°C and confers resistance to phagocytosis by macrophages and neutrophils (20, 21). In addition, calcium-dependent strains (Cal^+ or Vwa^+), which require Ca^{2+} for growth at 37°C and produce V and W antigens under defined conditions, can develop a capsule-independent resistance to phagocytosis by macrophages (5–7). The inability of Cal^- cells to develop capsule-independent resistance to macrophage phagocytosis does not appear sufficient to account for their loss of virulence, since these cells still develop capsule-mediated resistance. Unlike Cal^+ cells, Cal^- cells fail to proliferate freely in the host, although both are equally resistant to killing by normal and immune serum factors (33). This suggests that differences in the ability to survive and proliferate within phagocytes may exist. Two previous studies of this possibility have produced contradictory results (7, 22).

In 1981, Ferber and Brubaker showed that calcium dependence correlates with the presence of a 47-megadalton plasmid (9). A similar relationship involving a 42-megadalton plasmid has been reported in *Y. pseudotuberculosis* and *Y. enterocolitica* (12, 13, 30). To determine whether any 47-megadalton plasmid-encoded factor was involved in survival after phagocytosis, we examined the survival and growth of *Y. pestis* cells within mouse peritoneal cavities and within normal mouse peritoneal macrophages cultivated in vitro. Two strains were used which differed only in that one strain, KIM1, lacked the 47-megadalton plasmid associated with calcium dependence. We show that cells from either strain can survive phagocytosis by macrophages maintained in vitro, although equivalent but incomplete killing of both cell types occurs in vivo. Evidence that both KIM and KIM1 cells block the oxidative burst but do not block degranulation after phagocytosis is presented. We also show that cells with the plasmid proliferate within macrophages in vivo and in vitro, whereas cells lacking the plasmid fail to increase in number in vivo and can multiply within macrophages in vitro only when the macrophages are supported in a medium which would have permitted extracellular growth.

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MATERIALS AND METHODS

Animals. Outbred Swiss Webster mice, 6 to 12 weeks old, were obtained from the Washington State University mouse colony. The animals were given commercial pellets and water ad libitum. Iron-compromised mice were obtained by injecting (intraperitoneally) 0.1 ml of a fresh filter-sterilized solution containing 0.4% $FeSO_4 \cdot 7H_2O$ in 0.87% NaCl 30 min before infection.

Bacterial strains and growth conditions. *Y. pestis* KIM was obtained from R. R. Brubaker (Michigan State University, East Lansing). This strain is Pgm^- (pigmentation; refers to ability to bind heme and related planar compounds) and is therefore avirulent for humans and mice but phenotypically virulent for iron-compromised mice (18). The Pgm factor is not related to the 47-megadalton plasmid associated with calcium dependence and virulence in *Y. pestis* (9). To ensure high virulence, the strain was passed through an iron-compromised mouse. A calcium-independent (Cal^-) isolate was selected from the animal-passed isolate by using magnesium oxalate agar as described by Higuchi and Smith (17). This strain, designated KIM1, lacks the 47-megadalton plasmid. *Escherichia coli* K-12 was obtained from the departmental stock culture collection. Strains were stored at −10°C in 60% glycerol and 40% 0.1 M sodium phosphate buffer (pH 7.0).

Bacteria cultured on blood agar base medium (BAB) (BBL Microbiology Systems, Cockeysville, Md.) at 26°C were used to inoculate a defined liquid minimal medium (2). After incubation at 26°C for 8 to 16 h, cells were transferred to fresh defined liquid medium. These cultures were incubated for 10 to 16 h before collection of the bacterial cells. All liquid cultures were grown aerobically (gyratory shaker bath at 200 rpm) at 26 or 37°C as specified, with ca. 2.5 mM $CaCl_2$. Generation times were 2 h ± 15 min in all cases. The logarithmically growing cells, with an optical density at 620 nm of ca. 1.5, were collected by centrifugation at 8,000 × *g* for 10 min, washed once with Hanks balanced salt solution (HBSS) and resuspended to ca. 10^7 cells per ml. Dilutions of this bacterial suspension were prepared in the tissue culture medium or solution specified for each experiment.

Intraperitoneal bacterial survival and localization. Bacteria suspended in saline were injected intraperitoneally in 0.1- to 0.2-ml volumes. At the indicated times, the peritoneal cavities were lavaged with 6 ml of cold Dulbecco phosphate-buffered saline (PBS), and intracellular, extracellular, and total viable counts were determined. The peritoneal lavage fluid was thoroughly mixed and diluted at least 1:10 in 0.1% tryptic soy broth to lyse the phagocytes, and total bacterial numbers were determined by plating on blood agar base plates. A 1-ml portion of the undiluted lavage fluid was centrifuged for 1 min at 200 × *g* (4°C) to sediment the phagocytes. The resulting supernatant fluid containing the free bacteria was carefully removed, and viable bacteria were determined by the plate count method. The pellet was resuspended in 1 ml of tryptic soy broth of 4°C to lyse the phagocytes, and viable macrophage-associated bacteria were determined by the plate count method. Colonies were scored after incubation at 26°C for 48 h. Smears of the pellet suspensions were stained with May-Grunwald Giesma stain for differential counts (8).

To test for osmotic sensitivity of *Y. pestis* cells, some mice were sacrificed at intervals and the peritoneal cavities were lavaged with 6 ml of cold HBSS containing 0.2 M sucrose. Bacteria were diluted in HBSS with 0.2 M sucrose and enumerated by the pour plate method with blood agar base containing 0.2 M sucrose (24). Bacteria were also enumerated by the same procedures, but without sucrose in the diluents or medium.

Tissue culture medium. The tissue culture medium used for this study contained 10.63 g of Eagle minimal essential medium (MEM), Hanks salts without glutamine (GIBCO Laboratories, Grand Island, N.Y.), and 0.35 g of sodium bicarbonate in 1 liter of distilled water. When necessary, the pH was adjusted to 7.3 with NaOH. The medium was filter sterilized under positive pressure, using a 0.22-μm-pore-size Millipore filter and stored at 4°C. Fetal calf serum (FCS; GIBCO) was inactivated before use by heating at 56°C for 30 min. A stock of penicillin G (Eli Lilly & Co., Indianapolis, Ind.) (10^4 U/ml) and streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) (10^4 μg/ml) was sterilized by filtration and stored in small batches at −10°C.

Harvesting and maintenance of macrophages. Mice were killed by carbon dioxide suffocation, and the peritoneal cells were harvested by lavage with 6 ml of cold HBSS containing 100 μg of streptomycin and 100 U of penicillin G per ml. The peritoneal cells from several animals were pooled, collected by centrifugation at 200 × *g* for 10 min, and suspended to a concentration of 10^6 cells per ml in tissue culture medium consisting of MEM, 10% FCS, and antibiotics as specified above. Portions (2 or 0.4 ml) were used to seed culture dishes (3031 Falcon) or glass cover slips placed in the bottom of the dishes, respectively. After 10 min at ambient temperature, the dishes with cover slips were very gently flooded with 2 ml of tissue culture medium. Before use, the cover slips were placed in concentrated HCl overnight, washed exhaustively in distilled water, and autoclaved. After incubation for 2 to 3 h at 37°C in a humidified atmosphere of 5% CO_2 in air, the cell cultures were washed extensively with either warm PBS or HBSS to remove nonadherent cells. The adherent cells were then covered with warm culture medium without antibiotics and incubated for 12 to 20 h before infection. The resultant macrophages were not "angry" and were not as actively phagocytic as thioglycollate-stimulated macrophages.

Infection of the macrophage cultures. Bacteria were suspended in tissue culture medium at 37°C to a concentration of 10^6 cells per ml. The supportive medium was decanted from the macrophage culture and replaced with 3 ml of bacterial suspension. After 30 min of stationary incubation at 37°C in 5% CO_2 in air, the bacterial suspension was removed and the macrophage culture was washed extensively but gently at least five times with warm PBS to remove extracellular bacteria. Fewer than 5% of the viable bacteria were recovered in the final wash. The macrophage culture was then covered with fresh, warm tissue culture medium, and incubation was continued in 5% CO_2 in air at 37°C. In preliminary studies, scanning electron microscopy of samples fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), dehydrated with alcohol, and coated with gold in a high-vacuum evaporator indicated that more than 95% of the remaining bacteria were either in, or completely under, the macrophages. The average number of bacteria per macrophage and the percentage of macrophages infected were determined in subsequent experiments by fixing the cover slip cultures at the indicated times and then staining with May-Grunwald Giemsa stain.

Macrophage viability was determined by using cell adherence and Nigrosin exclusion. Nigrosin exclusion was determined as described by Mishell and Shiigi (27) on 10 randomly selected fields (40× objective) for each cover slip.

The percent viable macrophages was calculated from the zero time sample, since uninfected cultures showed no decline in viable numbers over the assay period.

Viable extracellular bacteria were enumerated by removing the culture medium and washing the adherent macrophages once with PBS at 37°C. The medium and PBS wash were pooled, diluted in tryptic soy broth, and plated on blood agar base plates. To determine viable intracellular bacteria, the washed macrophage culture resulting from the earlier procedure was overlaid with 1 ml of tryptic soy broth, adherent cells were dislodged with a rubber policeman, and the resulting suspension was pipetted vigorously for 30 s and then diluted and plated as described above. In both cases, colonies were scored after incubation at 26°C for 48 h. In some cases, acridine orange (AO) was used as a vital stain to evaluate the viability of bacteria within the macrophages, using the procedure of Smith and Rommel (32) except for the substitution of HBSS for Greys balanced salt solution. AO was dissolved in HBSS to 50 μg/ml, filter sterilized, stored at −10°C, and thawed just before use. These solutions were never exposed to direct light. Cover slips were observed by a Zeiss fluorescence microscope within 20 min of staining.

Phagosome-lysosome fusion. To monitor phagosome-lysosome fusion, the macrophage lysosomes were labeled with AO before phagocytosis, and fusion was followed by observing the changes in AO distribution by using dark-field vital fluorescence microscopy as described in protocol A of Gordon et al. (14); 10 mM NH_4Cl was used to inhibit fusion for the negative control and bakers' yeast was used as a positive control. Transfer of the AO to form rims of stain around the bacteria or staining of the bacteria was considered evidence of fusion.

CL assays. The HBSS used in the chemiluminescence (CL) experiments was supplemented with 10% FCS and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4). Luminol (Eastman Kodak Co., Rochester, N.Y.) was dissolved in dimethyl sulfoxide to 100 mg/ml and stored at room temperature for up to 2 months. The working stock was made by diluting this solution 1:400 in HBSS on the day of the experiment. These solutions were protected from light at all times.

Peritoneal cells, harvested by lavage as described earlier, were suspended in HBSS to a concentration of 5×10^6 cells per ml and stored at 4°C. Logarithmically growing yeast (aerobic in tryptic soy broth) or bacterial (growth as described above) cells were harvested and washed at least three times in PBS at 4°C, resuspended to 10^8 cells per ml in HBSS, and stored at 4°C. The remaining procedures were performed under indirect red illumination.

The assay was initiated by adding 100 μl of the peritoneal exudate cell solution, 100 μl of luminol working solution, and 500 to 800 μl of HBSS to dark-adapted 5-ml scintillation vials which were then placed in a water bath at 37°C and shaken at 60 rpm. CL was measured with a Beckman scintillation counter at ambient temperature, set out of coincidence with a 0.5-min counting period and in continuous counting mode. Samples were removed from the water bath only for the counting period. Once a stable baseline CL had been established, target cells (0 to 300 μl) were added to give a final volume of 1 ml, and the sample was counted until the CL peaked. The results were reported as peak height above background unless otherwise noted (1, 35). Uptake of yeast and bacteria by peritoneal cells in these experiments was quantitated by using cover-slip cultures stained with May-Grunwald Giemsa stain.

Bacterial sensitivity to hydrogen peroxide and superoxide anion. H_2O_2 (final concentration, 2.2×10^{-3} M) was added to bacteria suspended to a cell density of 10^5 cells per ml in 0.05 M potassium phosphate (pH 7.0), or bacteria were suspended to 10^5 cells per ml in a reaction mixture containing 10^{-5} M riboflavin, 0.15 M methionine, 10^{-5} M EDTA, and 0.05 M potassium phosphate buffer (pH 7.0) (37, 38), and superoxide generation was initiated by using a 15-W fluorescent light to photoactivate the riboflavin. Samples were removed at intervals for enumeration of viable bacteria. Five samples were always run simultaneously: *E. coli* K-12 (37°C), *Y. pestis* KIM (26 and 37°C), and *Y. pestis* KIM1 (26 and 37°C). Cells were grown at the indicated temperatures, but sensitivities were measured at 26°C in each case. A parallel set of cultures not exposed to light or without H_2O_2 served as controls.

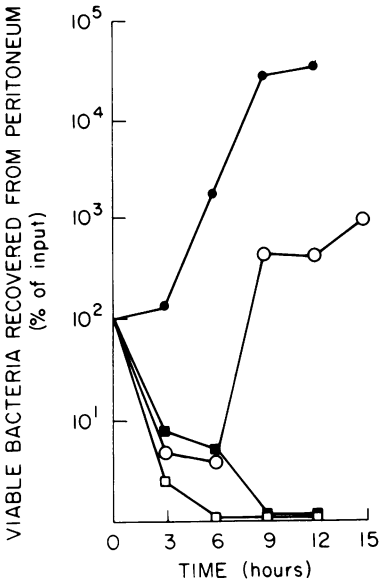

FIG. 1. Intraperitoneal survival and growth of *Y. pestis* in iron-compromised mice. Percent survival was used to combine data from experiments with different numbers of viable bacteria as inocula. Symbols: ●, KIM cells pregrown at 37°C; ○, KIM cells pregrown at 26°C; ■, KIM1 cells pregrown at 37°C; □, KIM1 cells pregrown at 26°C. Inocula were 4×10^4, 6×10^4, 9×10^5 and 1.2×10^6, respectively. Inocula of 10^4 to 10^7 in each case yielded similar results.

RESULTS

Bacterial survival and growth in vivo. The intraperitoneal 50% lethal doses of strains KIM and KIM1 were less than 50 and greater than 10^6, respectively, in iron-compromised mice and greater than 10^6 for both strains in normal mice. Intraperitoneal growth was followed by injecting bacteria into the peritoneal cavities of iron-compromised mice and subsequently lavaging, diluting, and plating the contents of the peritoneal cavities. Although iron had no appreciable effect during an initial period of bactericidal activity, it appeared necessary for subsequent bacterial growth (data not presented). Iron-compromised mice were used for all further in vivo studies.

The intraperitoneal survival and growth of KIM and KIM1 cells grown at 26 or 37°C before infection were compared (Fig. 1). The KIM1 cells grown at either temperature responded similarly; the viability declined rapidly in the first few hours and then continued to decline at a much slower rate. The viability of KIM cells grown at 26°C initially declined, as for the KIM1 cells, but began to increase after ca. 6 h. The viability of KIM cells grown at 37°C did not

decline in the same manner and after a short lag period increased exponentially.

Since the observed increase in KIM (26°C) cells between 6 and 9 h was more rapid than *Y. pestis* is known to grow in vitro, attempts were made to determine whether the initial drop in viable counts was an artifact. In attempts to increase the yield of phagocytic cells and bacteria, changes in the lavage fluid and lavage procedure were tested. These included addition of 0.2% EDTA to the PBS lavage fluid, use of lavage fluid prewarmed to 37°C, and lavaging for extended times (up to 5 min). All failed to yield more viable bacteria than were obtained by the original procedure. Harvesting, diluting, and pour plating the peritoneal contents in the presence of 0.2 M sucrose, which is effective in stabilizing osmotically sensitive *Brucella* strains formed within the macrophages (24), also gave the same results as the original procedure.

The intraperitoneal locations of the viable bacteria were determined. The peritoneal lavage fluids were subjected to low-speed centrifugation to separate the phagocytes from uningested bacteria. The strains which declined in viable counts were the ones associated with phagocytic cells (Fig. 2). The KIM1 (26 or 37°C) cells were intracellular throughout the observed period. The KIM (26°C) cells were phagocytized initially, and later released, whereas the KIM (37°C) cells were predominantly extracellular. This procedure enumerated only viable bacteria. Therefore, although it accounted for ca. 100% of the KIM (37°C) cells, it accounted for only 1 to 5% of the inocula of the other cell types. The total number of bacterial cells (alive and dead) within peritoneal exudate cells from infected mice was determined after May-Grunwald Giemsa staining. This technique, although only roughly quantitative, indicated that the killed bacteria were associated with mononuclear cells at 3 h (data not presented).

Bacterial survival and growth within macrophages in vitro. Consistent with in vivo studies, the KIM (37°C) cells were significantly less susceptible to phagocytosis by macrophages maintained in vitro than the KIM (26°C) or KIM1 (26 or 37°C) cells (Table 1). Bacterial survival and growth within the cultured macrophages were followed by using AO vital staining. The apparent decrease in the number of viable *Y. pestis* cells observed in some cases in the first hours in vivo was not observed in any case in vitro, even though the macrophages were capable of killing *E. coli* (data not presented). However, when HBSS and 10% FCS was used as maintenance medium, as in vivo, KIM1 cells were unable to grow significantly, whereas the KIM cells grew. In contrast, however, both KIM and KIM1 cells survived and grew within the macrophages in vitro when MEM with 10% FCS was used as maintenance medium (Fig. 3). MEM with 10% FCS supports growth of *Y. pestis* cells, whereas HBSS with 10% FCS does not. The numbers of extracellular bacteria, however, were too low initially to attribute the net increase in bacteria to extracellular growth and subsequent phagocytosis in any case. The effect of MEM with 10% FCS on KIM1 cells was apparently an effect on the growth of the bacteria within the macrophages.

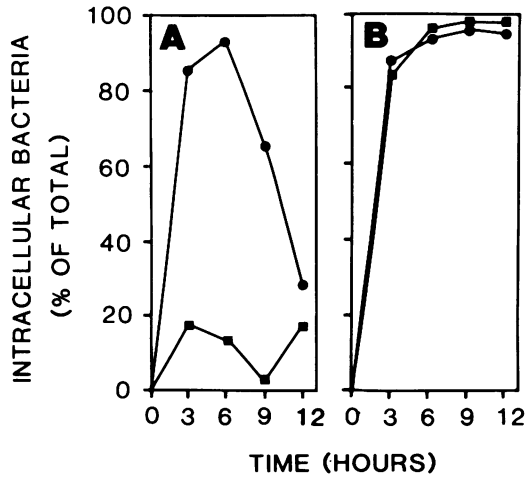

FIG. 2. Localization of viable bacteria within the peritoneal cavities of infected mice. Values are the mean number of cells harvested from two to four mice. Panels: A, KIM cells; B, KIM1 cells. Symbols: ●, Cells pregrown at 26°C; ■, cells pregrown at 37°C.

TABLE 1. In vitro macrophage phagocytosis of KIM and KIM1 cells

Bacterium (temp [°C])[a]	No. of bacteria ingested per macrophage[b] ± SD	% of macrophages infected[b] ± SD
Y. pestis KIM (37)	1.1 ± 0.5	32 ± 8
Y. pestis KIM (26)	5.5 ± 2	79 ± 4
Y. pestis KIM1 (37)	4.3 ± 1	72 ± 9
Y. pestis KIM1 (26)	5.1 ± 2	85 ± 5

[a] The bacteria were used at a multiplicity of infection of 100.
[b] Values are the mean value of triplicate samples harvested after 30 min of incubation. Between 150 and 300 macrophages were observed and counted for each sample.

Bacterial resistance to H_2O_2 and O_2^-. The relative sensitivities of KIM, KIM1,and *E. coli* K-12 cells to two bactericidal macrophage products, hydrogen peroxide and superoxide anion, were determined. No significant difference in the sensitivities of KIM and KIM1 cells was seen in any case. Both KIM and KIM1 cells grown at 37°C were more sensitive to hydrogen peroxide but less sensitive to superoxide anions than were cells grown at 26°C (Fig. 4).

Macrophage functions after phagocytosis. Phagosome-lysosome fusion after bacterial uptake was examined by using macrophages with lysosomes prelabeled with AO and observing the transfer of the stain to the bacteria after phagocytosis. Phagosome-lysosome fusion after phagocytosis of *E. coli* or either KIM or KIM1 cells was equivalent (Table 2). The kinetics of the CL response after phagocytosis of *E. coli* was typical of that reported previously (26). All the *Y. pestis* strains triggered less CL than did the *E. coli* K-12, and each triggered less CL per phagocytized bacterium as the averaged number of bacteria phagocytized per macrophage was increased. The KIM (37°C) cells triggered the lowest CL response and the KIM1 (37°C) cells triggered the highest (Table 3). When macrophages were infected so that the average macrophage ingested one *Y. pestis* cell and the macrophages were subsequently challenged with a second target, bakers' yeast, the resulting CL was inhibited, suggesting suppression of the CL response rather than the lack of stimulation of the response (Table 4).

DISCUSSION

Our in vivo and in vitro studies indicate that loss of the 47-megadalton plasmid, which is associated with the Cal^+ phenotype, results in increased susceptibility of cells grown at 37°C to phagocytosis by macrophages. This is consistent with the conclusions of earlier workers who compared virulent (Cal^+) and avirulent (Cal^-) cells (3).

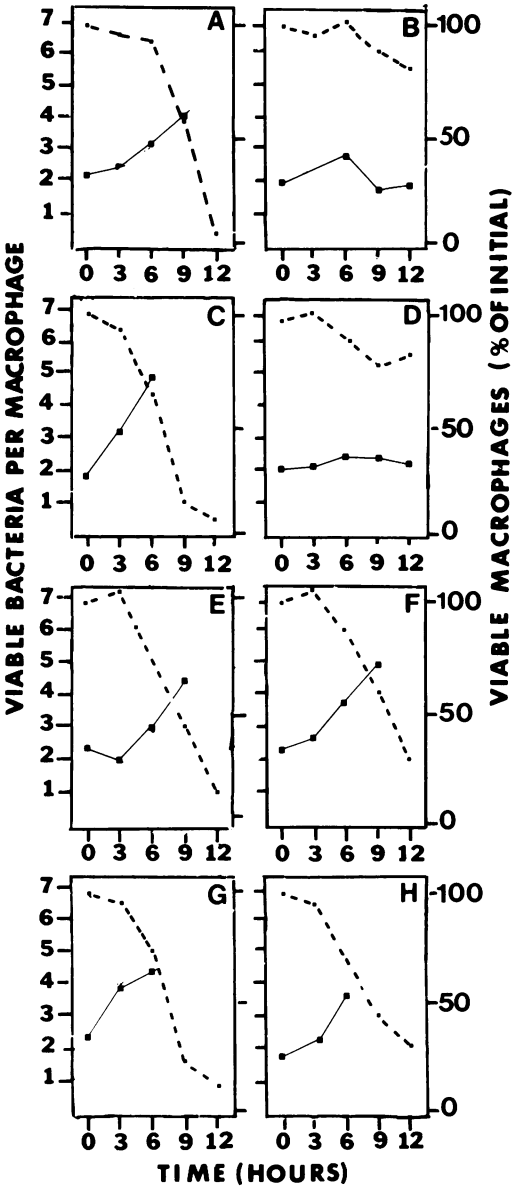

FIG. 3. In vitro interaction of *Y. pestis* and resident peritoneal macrophages with HBSS and 10% FCS (panels A to D) or MEM plus 10% FCS (panels E to H) used as maintenance medium (results of typical experiments). Average number of viable bacteria per macrophage (■): A and E, KIM (26°C); B and F, KIM1 (26°C); C and G, KIM (37°C); D and H, KIM1 (37°C). Macrophage viability is expressed as a percentage of viable macrophages at the time of infection (– – –). Resident mouse macrophages from a single pooled batch were used 1 day after harvest. After a 30-min infection period, the macrophage cultures were washed extensively to remove extracellular bacteria. Duplicate slides were examined, and at least 50 macrophages were scored per slide. Multiplicities of infection were adjusted so that in each case the average macrophage ingested the same number of bacteria. May-Grunwald Giemsa staining of the macrophages after uptake and washing revealed that ca. 50% of the macrophages were infected with an average of between 1 and 2 bacteria per macrophage. Intracellular numbers were no longer determined when more than 50% of the macrophages had been killed or after 12 h. Bacterial viability was determined by the AO vital staining procedure.

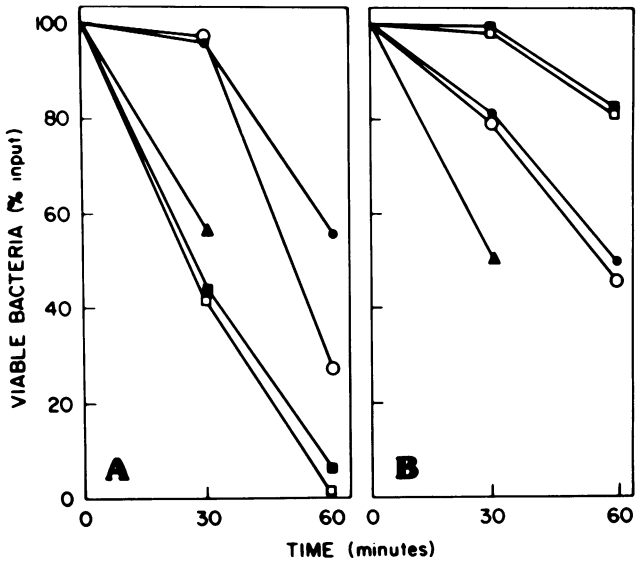

FIG. 4. (A) Sensitivity of *Y. pestis* to hydrogen peroxide. Percent survival of cells suspended in 2.2×10^{-3} M H_2O_2–0.05 M potassium phosphate buffer (pH 7.2) (mean of duplicate samples, variation less than 7% for all values) is shown. (B) Sensitivity of *Y. pestis* to superoxide anion. Percent survival of cells suspended in 10^{-5} M riboflavin–0.15 M methionine–0.05 M potassium phosphate buffer–10^{-5} M EDTA (pH 7.0) and subjected to illumination is shown (mean of duplicate samples, variation less than 9% for all values). Symbols: ■, KIM (37°C); ●, KIM (26°C); □, KIM1 (37°C); ○, KIM1 (26°C); ▲, *E. coli*. Control groups not exposed to light or H_2O_2 did not have reduced viability for any strain or culture condition.

Previous studies designed to determine whether calcium dependence factors influence bacterial survival or growth within phagocytes, or both, are in conflict. The first in vitro experiments indicating intracellular survival and growth were reported by Cavanaugh and Randall, who concluded that Cal^-, but not Cal^+, cells were killed by macrophages and that both were killed by neutrophils (7). It should be noted, however, that they compared results of experiments with neutrophils and macrophages from different animal species and independent Cal^+ and Cal^- isolates. The Cal^- strain used (A1122) has since been shown to have low murine toxin levels, unusual growth requirements at 37°C, and an unusual plasmid content (9, 16, 34). In addition, they used a protocol in which the Cal^- cells were taken up more rapidly than the Cal^+ (195/P) cells and therefore they were

TABLE 2. Phagosome-lysosome fusion in resident peritoneal mouse macrophages 150 min after phagocytosis of various cells

Bacterium (temp [°C])	% Fusion[a] in:	
	Untreated macrophages	Ammonia-treated macrophages[b]
E. coli K-12	86	15
Y. pestis KIM (37)	83	20
Y. pestis KIM (26)	86	17
Y. pestis KIM1 (37)	91	13
Y. pestis KIM1 (26)	88	25

[a] Each value was determined by examining 50 to 80 randomly chosen cells 150 min after phagocytosis.

[b] Values obtained with ammonia-treated macrophages are provided as negative controls.

TABLE 3. Resident macrophage CL after phagocytosis of live bacteria under conditions of matched phagocytosis[a]

Bacterium (temp [°C])	CL[b] per indicated no. of phagocytized bacteria[c] ± SD		
	0.5	1.0	2.0
E. coli K-12	120.0 ± 30	83.0 ± 15	70.0 ± 18
Y. pestis KIM (37)	2.2 ± 0.2	1.9 ± 2	1.1 ± 0.5
Y. pestis KIM (26)	11.0 ± 6.0	9.7 ± 8	5.1 ± 4
Y. pestis KIM1 (37)	15.0 ± 10	19.0 ± 14	6.4 ± 4
Y. pestis KIM1 (26)	8.3 ± 6	7.4 ± 5	5.2 ± 3

[a] Results are representative of three experiments; baseline CL is 30,000 to 50,000 cpm, and maximum peak height is ca. 550,000 cpm at 0.5 *E. coli* cells ingested per macrophage.

[b] CL = [(peak cpm height − baseline cpm)/average number of bacteria per macrophage] × 100/number of peritoneal exudate cells.

[c] Average number of bacteria phagocytized per macrophage, determined by staining of smears and enumeration of macrophages and intracellular bacteria.

comparing different effective doses in their in vitro studies. In the second study, Janssen and Surgalla found no significant difference in the intra-phagocytic survival of independent Cal^+ (Alexander) and Cal^- (A1224) cells in vivo or in vitro (22). In these studies, heavy inocula were used for both in vivo and in vitro experiments. High numbers of *Y. pestis* cells can disrupt other host functions (4, 11, 36) and may have permitted growth of Cal^- cells which would have been otherwise prohibited. In addition, the validity of the in vitro conditions was questioned by the authors. We conclude that neither study alone nor the two together adequately addressed the question.

The most critical flaw in both studies is that otherwise isogenic strains were not used. In this study we used strains which differed only in the presence (strain KIM) or absence (strain KIM1) of the 47-megadalton plasmid; it was designed initially to determine whether the loss of the plasmid reduced the ability of the bacterium to survive or grow within mouse macrophages. For safety reasons, we used Pgm^- strains of *Y. pestis* in this study, necessitating the use of iron-compromised mice for in vivo studies. Although the general features of infections of Pgm^- strains in iron-compromised mice are quite similar to infections by wild-type strains in normal animals, it is likely that some features of the infection do not precisely duplicate infection of a normal animal by fully virulent cells (19, 23, 28). In this model system, both KIM and KIM1 cells grown at 26°C apparently were susceptible to a significant degree of killing after phagocytosis, but a significant number of cells survived. The surviving KIM1 cells remained viable over the next 12 h but no net bacterial multiplication was detected. In contrast, the surviving KIM cells proliferated within the macrophages. These results suggested that the presence of the 47-megadalton plasmid did not enhance survival but did allow proliferation within the macrophages and that an additional factor (or factors) was enhancing survival following phagocytosis.

TABLE 4. Effect of earlier uptake of *Y. pestis* on resident macrophage CL triggered by yeast ingestion

Bacterium (temp [°C])[a]	CL[b]	Inhibition of CL (%)
None	130	0
Y. pestis KIM (37)	−2.3	100
Y. pestis KIM (26)	10	92
Y. pestis KIM1 (37)	35	73
Y. pestis KIM1 (26)	8.3	94

[a] Bacterial concentrations were used to give an average of one bacterium ingested per macrophage.

[b] CL = [(peak cpm height − baseline cpm)/average number of yeast cells per macrophage] × 100/number of peritoneal exudate cells.

Since KIM and KIM1 cells differed in their ability to proliferate within macrophages in vivo, we examined the bacterium-macrophage interactions by using macrophages cultivated in vitro. These macrophages were unable to kill the large portion of the *Y. pestis* cells as was seen in the in vivo system, even though they were able to destroy *E. coli* cells (W. W. Shuford, M.S. thesis, Washington State University, Pullman, 1982). The reason for the impaired ability of the in vitro macrophages, relative to the in vivo macrophages, to kill *Y. pestis* is unknown, but this clearly shows that the in vivo and in vitro systems used are not completely analogous and that the in vitro results should be interpreted with caution. The cells with the 47-megadalton plasmid were able to proliferate freely within the macrophages, whereas the cells lacking this plasmid were not. The inability of the KIM1 cells to grow within the macrophages maintained in vitro was a function of the supportive medium used. Intracellular growth of KIM1 cells was seen only when the medium contained factors permitting bacterial growth, whereas KIM cells grew intracellularly in the absence of these factors in the support medium. Several studies have shown that host metabolic pools are not necessarily available to intracellular parasites and that essential growth factors may have to be provided for intracellular growth to occur (29). One explanation for the in vitro results presented here was that cells with the 47-megadalton plasmid, but not the cells lacking this plasmid, are able to grow on nutrients provided in the phagosome, independent of medium components provided by pinocytosis.

Janssen and Surgalla (22) also reported that the nature of the supportive medium affected intracellular growth. This may have contributed to the different results obtained by Cavanaugh and Randall (7) and Janssen and Surgalla (22). With medium composition as the only variable, we can confirm either in vitro study with our in vitro results. The combination of in vivo and in vitro studies, however, indicates that the 47-megadalton plasmid permits bacterial growth within the phagocyte but is not required for survival after phagocytosis.

The ability of *Y. pestis* cells to survive after phagocytosis was examined further, since this is a prerequisite for intracellular growth. There are at least three distinct environments which can be occupied by an intracellular parasite after phagocytosis: (i) they may multiply in phagolysosomes flooded with hydrolases; (ii) they may escape from the phagosomes into the cell cytoplasm of the cell; or (iii) they may block the phagosome-lysosome fusion, yet remain in the phagosome (15, 29). Previous studies indicated that *Y. pestis* remains within a vacuole (10); this study shows that phagosome-lysosome fusion occurs. Therefore, *Y. pestis* apparently multiplies, at least for a time, within a vacuole flooded with hydrolases. The 47-megadalton plasmid-encoded factors had no obvious effect on phagolysosome formation.

After phagocytosis, a chain of events occurs in macrophages which results in the production of oxidizing compounds such as H_2O_2 and O_2^-. These two compounds were found to be toxic to KIM, KIM1, and *E. coli* K-12 cells at pH 7.2. We have no probe to accurately measure the intraphagosomal levels of these compounds, and the action of the

acidic pH and hydrolytic enzymes of the phagolysosome on bacteria may combine to increase sensitivity. Nevertheless, the results suggest that *Y. pestis*, like *E. coli*, should be killed after phagocytosis. This was seen to some extent in vivo but not in vitro. This suggested that the oxidized compounds might not be produced after phagocytosis of *Y. pestis* cells.

This possibility was examined by measuring phagocytosis-triggered CL, which measures indirectly the production of the oxidizing compounds. CL was significantly lower after phagocytosis of KIM or KIM1 cells than after phagocytosis of *E. coli* K-12 cells. Inhibition of CL, as opposed to a lack of stimulation of the oxidative burst, was indicated with both KIM and KIM1 cells. Thus, if either the inhibition of the production of the reactive compounds or their destruction by scavengers occurs, it is independent of the 47-megadalton plasmid. Inhibition of the production of these compounds or rapid breakdown of the compounds dramatically decreases intraphagocytic killing of some parasites (26, 31) and may be an important factor in the survival of *Y. pestis* within macrophages. The elucidation of the molecular mechanisms used to accomplish this suppression requires further studies.

In conclusion, this study showed that a 47-megadalton plasmid-encoded factor or factors directly or indirectly allows the development at 37°C of a factor inhibiting phagocytosis by macrophages and facilitates bacterial proliferation within macrophages in vivo and, under some conditions, within macrophages maintained in vitro. This study also provides evidence that *Y. pestis* does not block phagosome-lysosome fusion but does either block the oxidative burst or allow for rapid removal of the oxidizing compounds formed. Since *Y. pestis* is sensitive to killing by H_2O_2 and O_2^- the latter characteristic would be expected to enhance bacterial survival after phagocytosis.

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