Intracellular *Pasteurella pseudotuberculosis:* Multiplication in Cultured Spleen and Kidney Cells[1]

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Pasteurella pseudotuberculosis multiplied within rabbit cells in primary culture. Spleen cells from rabbits were either dispersed mechanically, exposed to the organism immediately after cell preparation, and grown as a pellet or the cells were dispersed enzymatically and grown as a monolayer for 4 to 6 days before the bacteria were introduced. Intracellular multiplication proceeded at a logarithmic rate for 1 to 2 days, with a generation time of about 70 min in pelleted cells and 4 to 5 hr in monolayered cells. Under the conditions employed, the wild-type virulent strain and an avirulent mutant multiplied at similar rates. Atmosphere, constituents of the medium, and multiplicity of infection influenced intracellular proliferation. The organism also proliferated in kidney cells. Microscopic observation of stained cells indicated limited growth of the pathogen in spleen cells at 37 C. In kidney cells, the pasteurellae localized in compartments; frequently, a single compartment with masses of proliferating organisms almost filled the cytoplasm.

Little is known of the pathogenesis of pseudotuberculosis, primarily a disease of rodents and birds but occasionally of man (14, 16, 22). Since 1953, over 1,000 human cases with mesenteric lymphadenitis and other forms of the disease have been identified as pseudotuberculosis. *Pasteurella pseudotuberculosis*, the causative agent, has not been identified as an intracellular pathogen, but the close taxonomic relationship to *Pasteurella pestis* suggests that it also may be a facultative intracellular parasite. At least 13 antigens are shared with *P. pestis*, among them the V and W antigens which are considered the major virulence antigens of both species (5, 15, 21). Although *P. pseudotuberculosis* lacks capsular antigen and the coagulase and fibrinolysin associated with invasion by *P. pestis*, other mechanisms of pathogenicity may be analogous.

In a classic study of the virulence of *P. pestis* from fleas, Cavanaugh and Randall (6) showed that the bacilli were ingested by peritoneal monocytes in guinea pigs and multiplied within them. Moreover, they found that monocytes exposed in vitro to *P. pestis* supported intracellular multiplication of virulent (VW+) but not avirulent (VW−) strains. In contrast, Janssen and Surgalla (12), using cultured peritoneal cells infected in vivo, found that both VW+ and VW− *P. pestis* survived in monocytes. From this and other data (11), they concluded that the ability to multiply

in free phagocytes is not a determining factor in the virulence of *P. pestis*. They hypothesized that a major determinant is the ability to multiply in fixed macrophages of the reticuloendothelieal system. No direct evidence was obtained.

The work reported here was undertaken (i) to determine if fully VW+ *P. pseudotuberculosis* multiplied in cultured spleen cells from rabbits, and, if so, (ii) to establish intracellular growth curves, and (iii) to compare the growth of a VW+ strain and a VW− strain known to differ from its parent only by the absence of V and W antigens. Multiplication of the organism within cultured kidney cells was examined also since more than one host cell type may be involved in pathogenesis.

MATERIALS AND METHODS

Pasteurellae. Fully VW+ *P. pseudotuberculosis* strain PB1 was obtained from Robert R. Brubaker. The prototroph was VW+ and calcium-dependent. The intravenous LD_{50} was about 40 cells in white mice (3).

Two VW− mutants of strain PB1 were used. One was obtained from R. R. Brubaker, and one was derived by us and characterized as VW− on the basis of its ability to grow at 37 C on magnesium oxalate agar (2, 9).

Stock cultures of *P. pseudotuberculosis* were stored in 60% glycerol-40% 0.06 M phosphate buffer (*p*H 7.0) at −20 C.

Subcultures on Blood Agar Base (Baltimore Biological Laboratories) were prepared from the glycerol stock cultures and incubated overnight at room temperature with an additional 4 hr at 37 C to develop

the potential to synthesize the VW antigens. The bacteria were suspended in 0.05% Tryptose (Difco) in 0.5% NaCl and standardized by a Libby photronreflectometer to contain 5×10^8 viable pasteurellae. Appropriate dilutions for infection of tissue cells were made in Eagle's minimal essential medium (MEM).

Preparation and infection of cell cultures. Spleen cells from normal rabbits were either dispersed, exposed to the organisms immediately after cell preparation, and grown as a pellet of dispersed cells or the cells were grown as a monolayer for 4 to 5 days before the bacteria were introduced.

The preparation of dispersed spleen cells has been described (18). In brief, immediately after removal from a normal rabbit the spleen was lightly minced with scissors and forced through a fine steel sieve, and the sieve was rinsed with phosphate-buffered saline. The cell suspension was centrifuged at 600 rev/min for 10 min. The cells were suspended in Eagle's MEM (Earle's salts) containing 15% normal inactivated rabbit serum. Nucleated cells were counted on a hemocytometer, and the number was adjusted to 10^7 per ml. Pasteurellae in Eagle's medium were added to give 10^2 to 10^6 per ml of cell suspension. The cells were dispensed at 1 ml per tube (16 by 125 mm), gassed with 5% CO_2 plus 95% O_2, and incubated in an upright position for 2 hr at 37 C. After phagocytosis, the medium above the cells was carefully aspirated off and replaced with one containing 5 μg of streptomycin per ml. Medium was renewed at 2- to 3-day intervals.

Monolayer cultures of spleen cells were prepared as previously described (20). Briefly, 2-mm fragments of spleen in 0.25% trypsin were incubated overnight at 4 C in an atmosphere of 5% CO_2 plus 95% air. The trypsin was replaced with 50 ml of 0.01% collagenase, and the tissue was incubated for 45 min on a magnetic stirrer at 37 C. After centrifugation at 600 rev/min for 10 min, the cells were suspended at 10^7 per ml of Eagle's medium, dispensed at 1 ml per Leighton tube (with or without cover slips), gassed with 5% CO_2 plus 95% air, and incubated. The following day the tubes were drained carefully to remove the maximum number of cells not attached to the glass, and fresh medium was added. On day 4 to 5, the medium was replaced with one containing 10^2 to 10^6 P. pseudotuberculosis. Phagocytosis, etc., proceeded as above.

Monolayer cultures of kidney cells were prepared by trypsinization with 0.25% trypsin (19). The separated cells were suspended at 10^6 per ml of Eagle's medium and then dispensed, grown, and infected as with the spleen cell monolayers.

Intracellular multiplication. The initiation of phagocytosis was designated as zero hour. To determine the number of viable intracellular bacteria, the medium was removed and the cells were washed once with 5 ml of 0.5% NaCl containing 0.05% Tryptose. The tissue cells were lysed by mechanical shaking for 1 hr with 5 ml of distilled water containing 0.05% Tryptose. Pasteurellae were enumerated by serial dilution and count of colonies on Blood Agar Base. The number of viable organisms per tube was expressed as the geometric mean of three or more tubes.

For microscopic observation of intracellular multiplication, monolayer cells on cover slips were stained by a modification of the May-Grünwald-Giemsa method (13) and by acridine orange (1).

RESULTS

Growth in dispersed cells. Figure 1 shows typical growth curves of VW^+ P. pseudotuberculosis in dispersed rabbit spleen cells. In an atmosphere of 5% CO_2 plus 95% O_2 and with 10^2 organisms initially present, logarithmic growth occurred between 6 and 30 hr. The number of intracellular pasteurellae reached 10^8 by 42 hr and remained at this level through 72 hr. During the log phase of growth, the generation time was approximately 70 min. Similar rates and maxima were obtained with cells from two other rabbits initially containing 10^2 and 10^3 intracellular organisms; pasteurellae multiplied more slowly in cells from a fourth rabbit attaining a maximum of 7×10^5 at 48 hr (data not shown). With an atmosphere of 5% CO_2 plus 95% air, little or no multiplication occurred in cells pelleted under 1 ml of medium. Since the rabbits were not syngenic and spleen cells could not be pooled, the variables and points in time tested were limited. To examine persistence of the organisms in parasitized cells, pasteurellae were enumerated at 2- to 3-day intervals. After maxima, the number declined
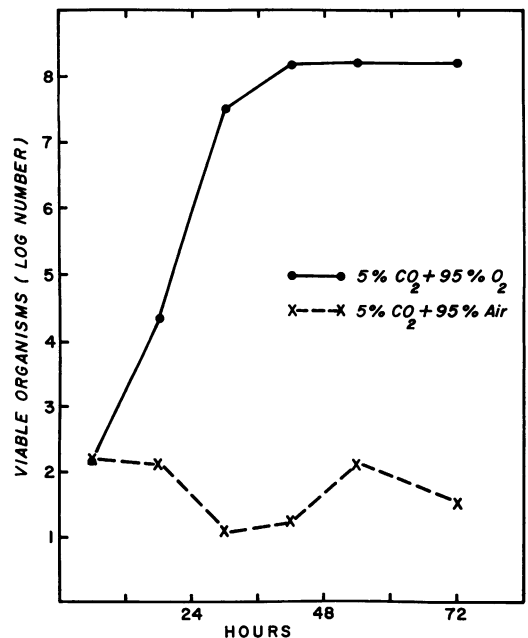

FIG. 1. *Effect of atmosphere on the intracellular multiplication of Pasteurella pseudotuberculosis (VW^+) in dispersed rabbit spleen cells.*

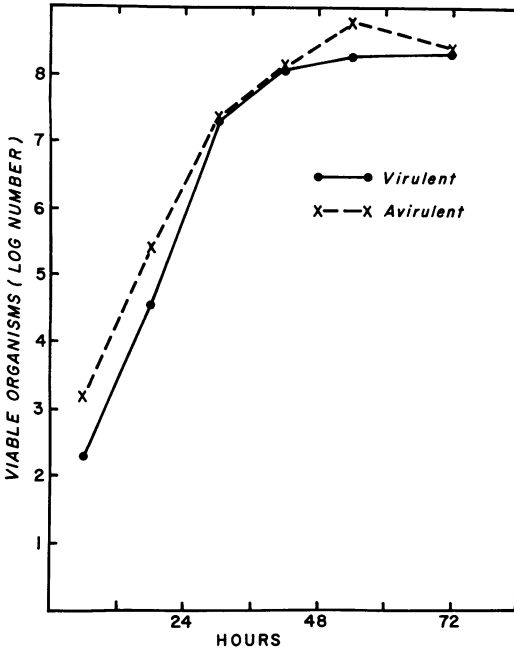FIG. 2. *Comparison of the intracellular growth rates of virulent* (VW^+) *and avirulent* (VW^-) *strains in dispersed rabbit spleen cells.*

multiplied at similar rates, with a generation time of 4 to 5 hr (data not presented). The effect of fresh serum during the phogocytosis period on subsequent intracellular multiplication of VW^+ and VW^- organisms was investigated with spleen and kidney cells. Serum obtained from rabbits the day before the test was pooled and stored at -20 C. Before use, a sample was heated at 56 C for 30 min. VW^+ and VW^- cells in Eagle's MEM without serum were prepared to contain equal numbers of pasteurellae. Immediately before use for phagocytosis, each was supplemented with 15% heated serum for group A or fresh serum for groups B and C. With heated serum, spleen and kidney cells phagocytosed equal numbers of organisms, 10^4 (group A, Fig. 4). As found with other monolayer spleen cells, VW^- organisms multiplied as rapidly or more rapidly than VW^+ ones. With fresh serum and concomitant bactericidal action, spleen cells phagocytosed 10^3 organisms (group B) as compared to 10^1 by kidney cells (group C). The spleen cells contained almost equal numbers of VW^+ and VW^- organisms at 8 hr, and intracellular multiplication proceeded at the same rate. The kidney cells also contained equal numbers of VW^+ and VW^- organisms. In them, the VW^- mutant multiplied more rapidly than the VW^+ mutant. Thus, the
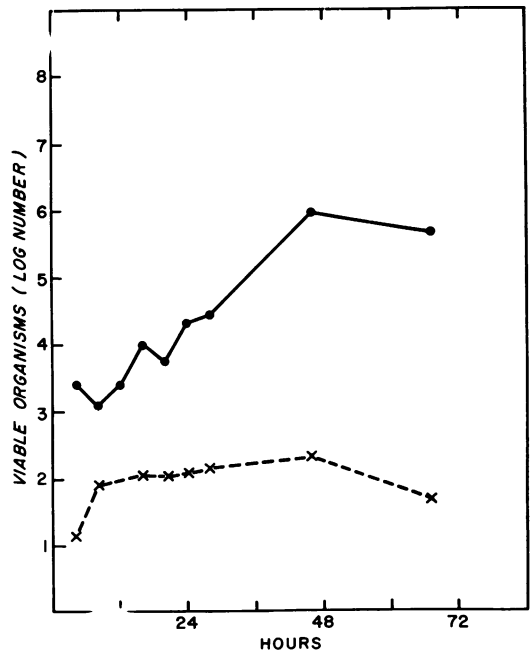
rapidly; i.e., with cells containing 7×10^5 bacteria at 2 days, only 3×10^2 and <10 were viable at 6 and 9 days, respectively. As Fig. 2 shows, VW^+ and VW^- strains multiplied within cells at the same rate.

Growth in monolayer cells. The curves in Fig. 3 to 6 were obtained with monolayer cells exposed to *P. pseudotuberculosis* after 5 days in vitro. When spleen cells were exposed to 10^2 to 10^6 pasteurellae, the cells phagocytosed approximately 1 log fewer organisms than the extracellular number. Little multiplication occurred in cells that contained 14 organisms at 4 hr; 218 were viable at 46 hr (Fig. 3). With 2.5×10^3 organisms in cells at 4 hr, 10^6 were present at 46 hr, a generation time of 4 to 5 hr. Multiplication proceeded at a similar rate in other spleen cells initially infected with 10^3 to 10^4 organisms (Fig. 4 to 6). Thus, the pasteurellae proliferated more slowly in monolayer spleen cells infected after 5 days than in dispersed spleen cells infected immediately after cell preparation. Growth of *P. pseudotuberculosis* proceeded also in kidney cells. Growth curves with spleen and kidney cells from the same rabbit infected with the same number of VW^+ organisms closely paralleled each other (group A of Fig. 4).

Comparison of the growth of VW^+ and VW^- organisms in monolayer spleen cells from four rabbits showed that the parent and the mutant

FIG. 3. *Intracellular growth rate of Pasteurella pseudotuberculosis* (VW^+) *in rabbit spleen cells grown as a monolayer and initially infected with two numbers of organisms.*
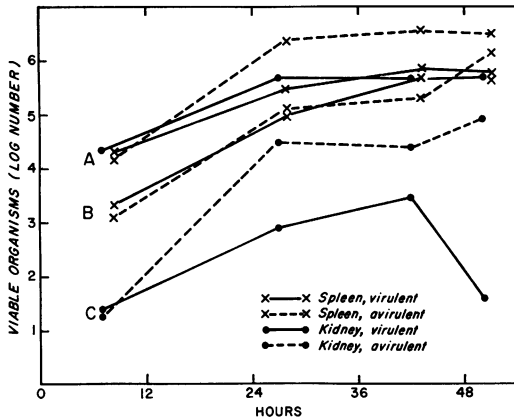
FIG. 4. *Effect of fresh serum in the medium during the phagocytosis period on the subsequent intracellular multiplication of virulent* (VW^+) *and avirulent* (VW^-) *strains in monolayer spleen and kidney cells. Fresh, normal rabbit serum: A, heated at 56 C for 30 min; B and C, unheated.*

presence of 15% fresh serum in the medium resulted in bactericidal reduction of the numbers of *P. pseudotuberculosis* in the extracellular medium and ultimately in the cells. It did not, however, affect intracellular growth rates or differentiate VW^+ from VW^- organisms.

Some experiments were designed to determine persistence of infection in monolayer spleen cells. Intracellular VW^+ pasteurellae persisted in spleen cells of one rabbit for as long as 17 days (Fig. 5). The persistence of VW^+ and VW^- strains was compared in cells from another rabbit (not shown). With 10^6 VW^+ and VW^- intracellular organisms on day 2, the number of VW^- organisms declined somewhat less rapidly than VW^+ ones. On day 10, there were 10^3 VW^- cells and 10^1 VW^+ cells per tube. None were viable on day 14, but most of the spleen cell monolayer in the tubes had disintegrated by this time.

Agamma bovine fetal serum was compared with normal rabbit serum in the growth medium (Fig. 5). Although the log phases of growth and maxima were similar, the intracellular bacteria died more rapidly with bovine fetal serum than with normal rabbit serum in the medium. Both curves in Fig. 5 peaked again at 5 days, indicating that a second cycle of intracellular growth may have occurred.

Since the early findings had demonstrated that *P. pseudotuberculosis* proliferated rapidly within dispersed cells in medium containing 5 µg of streptomycin per ml, the antibiotic was used routinely at this concentration. After repetitious testing confirmed the long generation time of the organism in monolayer cells, intracellular growth

rates were compared with 2.5, 5.0, and 10 µg of streptomycin per ml in the medium. Multiplication proceeded at similar rates, although at 8 hr more bacilli were viable with 2.5 than with 5.0 or 10.0 µg of streptomycin per ml (Fig. 6). Possibly antibiotic action continues in the phagosome with the higher concentrations of antibiotic prolonging the lag phase.

Microscopic observations. The appearance of *P. pseudotuberculosis* within Giemsa-stained cultured cells depended on the tissue of origin and temperature of incubation (Fig. 7 and 8). The duplicate preparations stained with acridine orange are not shown. Acridine orange fluorescence revealed morphological and cytochemical features of the cells and the bacteria which were not evident with Giemsa staining. Since ribonucleic acid stains red, proliferating bacteria appeared red; dead bacteria and latent organisms producing little protein stained green. Previous experiments with *Brucella* (M. Richardson, Bacteriol. Proc. p. 83, 1964) had shown that heat-killed brucellae could be seen in the juxtanuclear area of cultured lymphoid cells stained with acridine orange for 3 or more weeks after phagocytosis.

That temperature alters the delicate balance between parasites and mammalian cells was evident in stained cells. The cells pictured in Fig. 7 originated from the same rabbit spleen and were grown at 37 C until after infection when random cultures were incubated at 26 or at 37 C. At 37 C, the infection appeared self-limiting (A and D). By 2 days after parasitization, coccoid forms predominated. A few bacillary forms were evident which stained well with Giemsa; lightly stained organisms and bacterial ghosts abounded. Duplicate acridine-orange stained cells contained masses of green organisms with an occasional red bacterium. *P. pseudotuberculosis* proliferated extensively in the cytoplasm of spleen cells incubated at 26 C (B, C, E and F). Chaining occurred
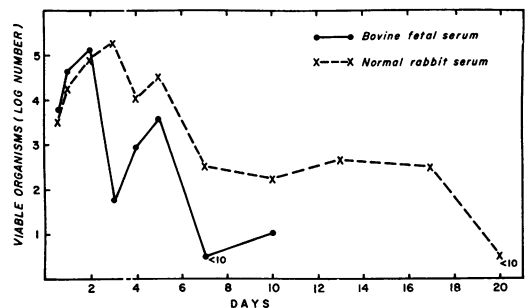

FIG. 5. *Multiplication of a virulent strain* (VW^+) *in monolayer spleen cells grown in medium supplemented with normal rabbit serum or bovine fetal serum.*
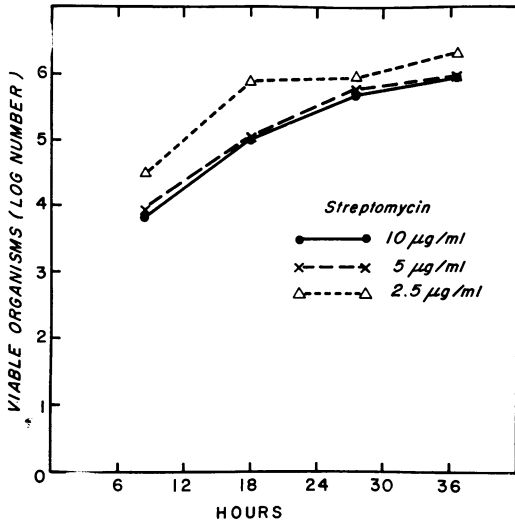
FIG. 6. *Effect of streptomycin at different concentrations on intracellular multiplication of Pasteurella pseudotuberculosis (VW+).*

and well-stained bacilli filled many cytoplasmic extensions, suggesting that spread of infection might occur by direct cell-to-cell transfer.

Bacillary forms predominated in kidney cells incubated at 37 C (Fig. 8). Occasionally bacilli spread throughout the cytoplasm (C, cell a). More often multiplication localized (A and C, cell b). In some cells, membrane-bound compartments developed which contained masses of bacilli; frequently a single compartment almost filled the cytoplasm (B and C, cell c). Empty holes in the cytoplasm were observed, as were extracellular bacilli, singly and in masses, with adherent cellular material. In Fig. 8B, a single coccoid organism with adherent material is evident below the cell. In preparations stained with acridine orange, the material around extracellular bacilli stained green. Viable, red-orange bacilli in the green material could be seen on the edge of or in cytoplasmic extensions of uninfected kidney cells, suggesting that another cycle of extracellular multiplication might ensue.

DISCUSSION

The data obtained in this investigation demonstrated that *P. pseudotuberculosis* proliferates intracellularly in vitro. Since the bacilli withstood hypotonic lysis of the tissue cells, permitting serial dilution with colony counts of viable organisms and replication, intracellular growth curves could be established. We found that *P. pseudotuberculosis* multiplied at a reproducible rate in rabbit cells in primary culture. In dispersed spleen cells of three or four rabbits infected immediately

after cell preparation, the pasteurellae multiplied during the log phase of growth with a generation time of about 70 min. This approximates the growth rate in culture media. In monolayer spleen and kidney cells derived by collagenase and trypsin treatment, respectively, and cultured for several days before the bacteria were introduced, the organisms multiplied with a generation time of 4 to 5 hr. Whether the higher host cell resistance manifested by monolayer cells was due to physiological conditions or to the greater resistance of a selected cell population is not known. Dispersed cells, with an unselected population, may contain the cells most susceptible to parasitization. On the other hand, the trauma of cell preparation with immediate exposure to the pathogen could lower cell resistance sufficiently to permit rapid intracellular multiplication. As cells establish themselves in culture, pinocytosis increases, possibly resulting in greater uptake of streptomycin into the cell. The difference observed in the multiplication rate of *P. pseudotuberculosis* in freshly prepared cells and in established cells conceivably could be due to intracellular concentration of streptomycin.

Controversy continues concerning the use of antibiotics to control extracellular growth when studying intracellular multiplication of bacteria in vitro. Work in this laboratory with *Brucella abortus* has demonstrated the presence of streptomycin in tissue cells in vitro (20). Although low levels of streptomycin or tetracycline did not appear to inhibit intracellular growth of brucellae, a marked, synergistic, dose-dependent inhibitory action occurred with penicillin also in the medium. Hatten and Sulkin (8) elegantly confirmed this finding, demonstrating synergistic reduction of the number of *Brucella* L-forms in cultured hamster kidney cells. Patterson and Youmans (17) recently reported a dose-dependent inhibition of *Mycobacterium tuberculosis* in macrophages which was ascribed solely to streptomycin, although 50 units of penicillin and 50 units of nystatin were always present in the medium. With *M. lepraemurium*, an obligate intracellular parasite, Chang (7) showed a dose-dependent inhibition by low levels of streptomycin alone. After 4 weeks of culture in 5 µg of streptomycin per ml, growth was reduced 56%. In the work reported here, *P. pseudotuberculosis* multiplied rapidly for 2 days in dispersed spleen cells in the presence of 5 µg of streptomycin per ml. Proliferation proceeded more slowly in monolayer spleen and kidney cells but at similar rates in 2.5, 5.0, or 10.0 µg of streptomycin per ml.

We propose to investigate cellular immunity in vitro by using cells from animals with chronic disease. *P. pseudotuberculosis* may serve as the
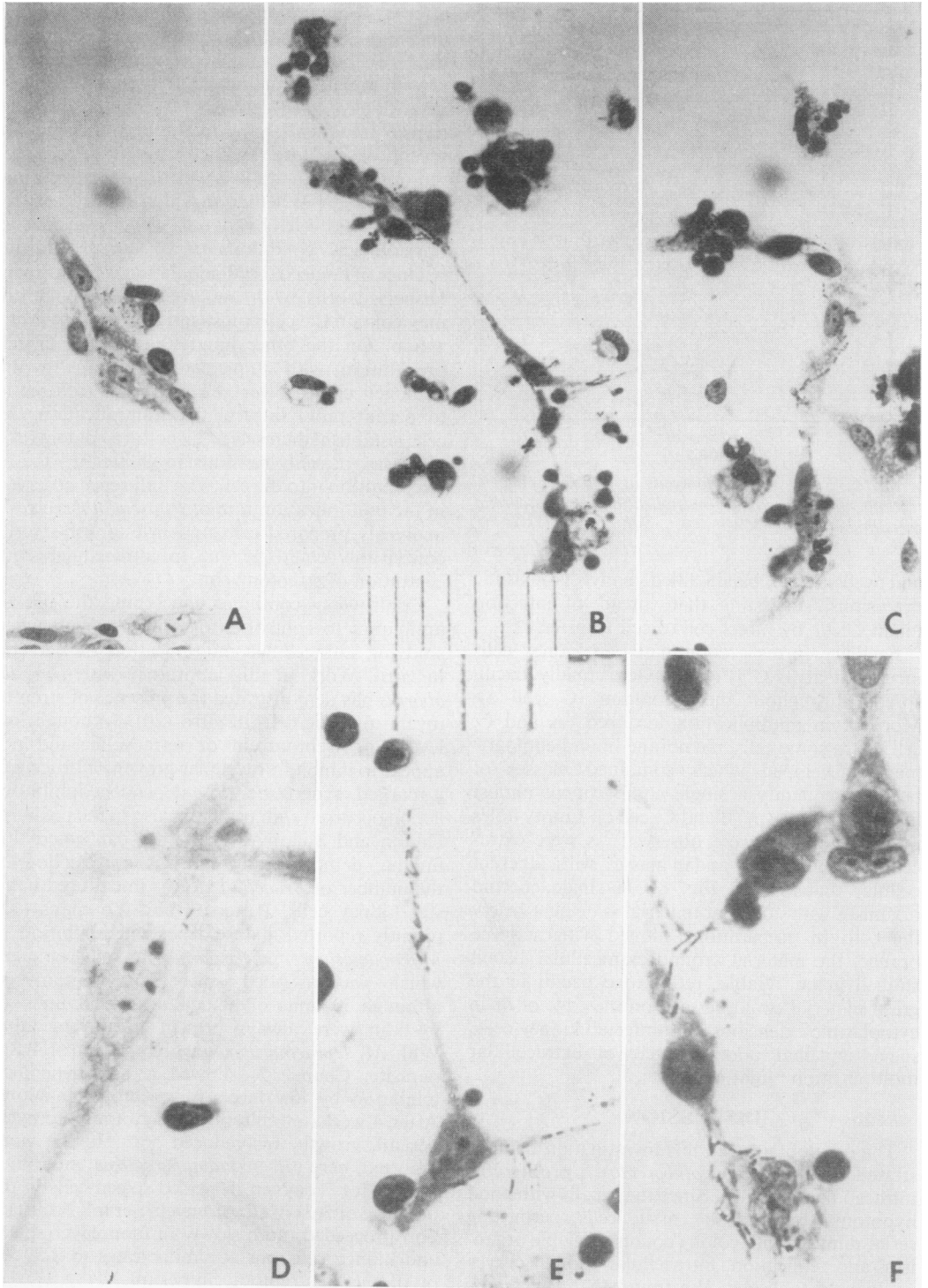
FIG. 7. *Photomicrographs of Pasteurella pseudotuberculosis in rabbit spleen cells 2 days after infection. Giemsa stain. A and D, incubated at 37 C; B, C, E, and F, incubated at 26 C. Distance between scale bars, 10 μm.*
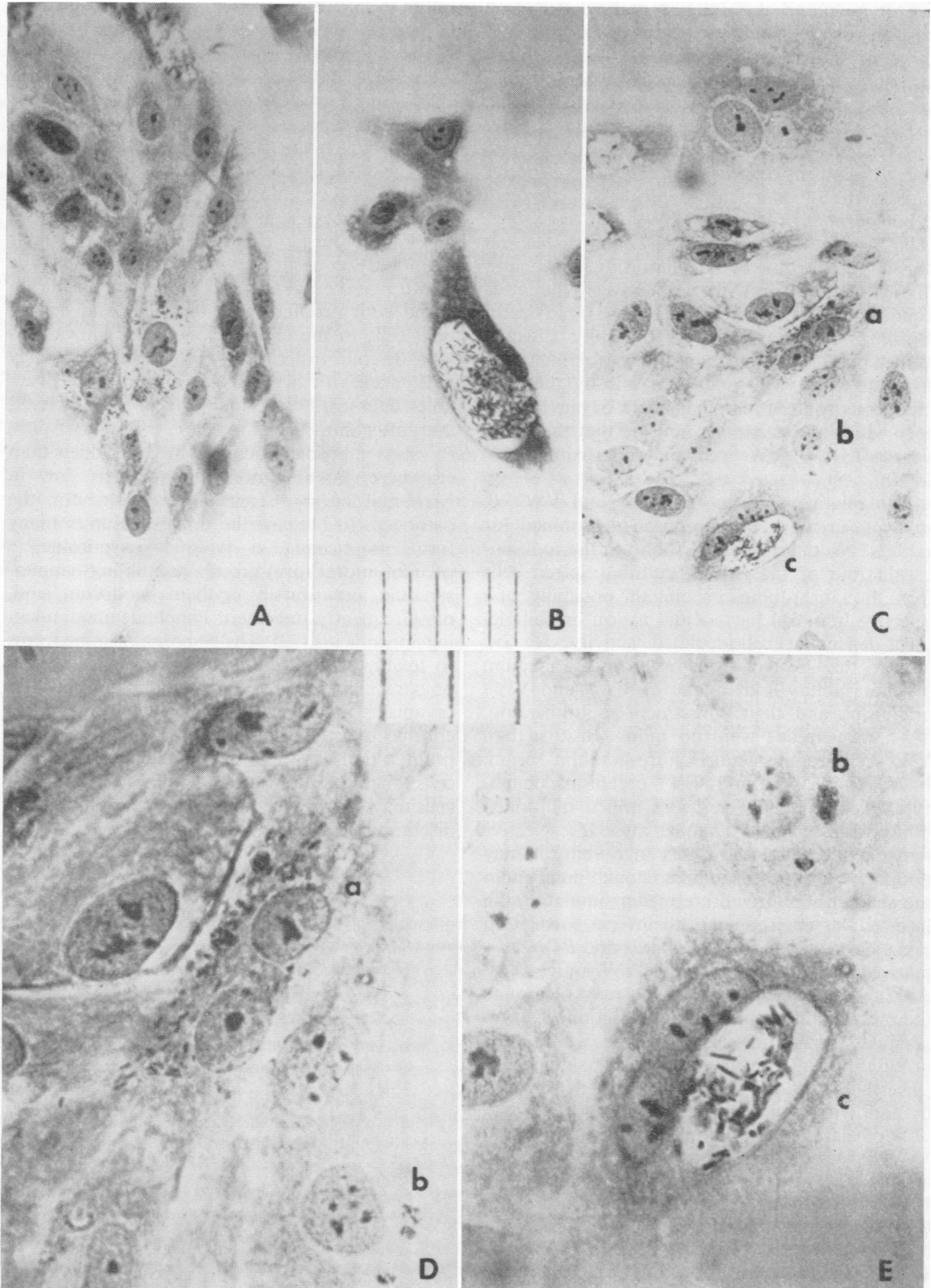
FIG. 8. *Photomicrographs of Pasteurella pseudotuberculosis in rabbit kidney cells. Giemsa stain; cells incubated at 37 C. A and B, 4 days after infection; C, 3 days after infection; D and F, higher magnification of cells in C. Distance between scale bars, 10 μm.*

prototype of an intracellular pathogen causing chronic disease in rabbits; deaths occur from 2 to 5 weeks and delayed hypersensitivity is demonstrable at 3 months in the survivors. The significance of the VW virulence antigen in chronic disease is unknown. In acute, rapidly fatal disease, differences in the median lethal dose of VW$^+$ and VW$^-$ organisms depend markedly on the species and route of inoculation (Brubaker, *personal communication*). In mice, VW$^+$ but not VW$^-$ cells produce an acute, rapidly fatal disease regardless of the route of inoculation. In guinea pigs, about 100 VW$^+$ or VW$^-$ organisms by the intravenous or intraperitoneal route cause rapid death. Chronic disease occurs in naturally infected guinea pigs (16) and in guinea pigs experimentally infected with VW$^+$ or VW$^-$ cells by the subcutaneous route. Preliminary data in our laboratory with only 15 rabbits indicate that the intravenous LD$_{50}$ of VW$^+$ cells may approximate 10^3 to 10^4.

The relative virulence of VW$^+$ and VW$^-$ P. pseudotuberculosis has not been determined for rabbits. No difference was found in the multiplication rate of the two in cultured spleen cells from the same rabbits. Probing the possibility of a selective humoral bactericidal action or complement-dependent cell-mediated activity, we examined the effect of fresh serum in the medium on the viability and uptake of VW$^+$ and VW$^-$ organisms and their subsequent multiplication. VW$^-$ pasteurellae were no more sensitive than VW$^+$ cells to the action of fresh rabbit serum. In addition, VW$^+$ and VW$^-$ organisms phagocytosed from the bactericidal milieu multiplied in rabbit spleen cells at similar rates. If a selective reticuloendothelial cell exists in rabbits, it may not have been present in high enough numbers in the cell cultures. In culture media simulating the intracellular environment, an inverse correlation exists between growth of *P. pestis* and *P. pseudotuberculosis* and production of VW antigen (4). Although intracellular residence may determine the outcome in host-parasite interaction, once an adequate population is established within cells proliferation per se may be of little significance since the VW antigens are synthesized primarily by nondividing organisms. The single demonstration of differential multiplication of VW$^+$ and VW$^-$ P. pestis within cells is that of Cavanaugh and Randall (6). VW$^+$ P. pestis increased from 10 at 2 hr to 10^6 at 48 hr in peritoneal monocytes from guinea pigs; in monocytes from mice, VW$^-$ organisms decreased from 10^5 at 2 hr to none at 48 hr. Cells from the same animals infected at the same multiplicity would provide a more valid comparison. In related work not presented here, we have observed erratic results when dispersed spleen cells were exposed to high numbers of P. pseudotuberculosis.

The significance of intracellular *P. pseudotuberculosis* in the pathogenesis of pseudotuberculosis is unknown. Cellular parasitism in vivo has not been reported. Unpublished studies by Janssen, which are cited by Surgalla, Andrews, and Cavanaugh (21), indicated that guinea pigs with pseudotuberculosis harbor viable organisms within phagocytes in the lesions and exudates developing during the disease. If intracellular multiplication occurs in vivo, more than one type of cell may be parasitized. Numerous bacilli are found with proliferating fibroblasts and epithelioid cells in the chronic lesions of guinea pigs (16). In our experiments with cultured kidney cells, masses of organisms localized in cell vacuoles. Janssen, Fukui, and Surgalla (10) observed that, after intracardial injection into guinea pigs, *P. pestis* reproduced earlier in the kidney than elsewhere. Possibly nonlymphoid cells, low in lysosomal enzymes, act as reservoirs for the pathogen. In humans the disease assumes many forms: septicemia, a typhoidal syndrome, a pseudotumoral form grossly resembling lymphosarcoma, occasionally erythema nodosum, and, most frequently, mesenteric lymphadenitis mimicking appendicitis (22). The response of some forms to treatment with antibiotics suggests inhibition of extracellular bacteria. Presumably intracellular organisms prevail in the typhoidal form and in chronic infection. Investigations of intracellular proliferation have focused on reticuloendothelial cells, but multiplication within nonlymphoid cells may be involved in the pathogenesis of acute or chronic disease, or both types of disease.

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