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Electron Microscopic Evidence for In Vivo Extracellular Localization of *Yersinia pseudotuberculosis* Harboring the pYV Plasmid

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Electron microscopic evidence is presented that bacteria harboring the virulence plasmid pYV from *Yersinia pseudotuberculosis* are localized in extracellular sites during the course of infection in mice, often unambiguously undergoing active replication. Virulent pYV$^+$ bacteria, often seen adherent to platelets, severely restricted granuloma formation, creating necrotic microabscesses poorly populated with inflammatory cells. This contrasts with granulomas produced by pYV$^-$ bacteria, which appear to be composed mainly of polymorphonuclear and mononuclear cells. Our results therefore strongly suggest that active replication of pYV$^+$ bacteria predominantly, if not exclusively, occurs in vivo in extracellular sites.

The gram-negative bacterium *Yersinia pseudotuberculosis* is responsible for gastrointestinal infections in humans and many animal species, causing acute ileitis and mesenteric lymphadenitis, sometimes complicated by septicemia (3, 12). As for the two other pathogenic species of the genus *Yersinia*, *Y. pestis* and *Y. enterocolitica*, it is generally assumed that *Y. pseudotuberculosis* is a facultative intracellular pathogen. This is based on convergent reports showing that bacteria of the three species multiply in macrophages or epithelial cells in vitro (4, 5, 8, 13, 20, 21, 23). It is also established that the expression of virulence in *Y. pseudotuberculosis* requires, as in *Y. pestis* and *Y. enterocolitica*, the presence of an ~70-kilobase plasmid, called pYV, which is associated with temperature-inducible properties of virulent bacteria, including Ca^{2+}-dependent growth at 37°C, production of V and W antigens, and expression of outer membrane proteins (reviewed in references 1, 2, and 6). Virulence of *Y. pseudotuberculosis* also involved a chromosomal gene, designated *inv* (7), responsible for invasion of epithelial cells in vitro and facilitating the translocation of bacteria across the intestinal epithelium (reviewed in reference 11). However, little is known about the plasmid-mediated events occurring during infection and ultimately killing the infected host. In this work, a histopathological study was performed to evaluate the in vivo impact of the virulence plasmid pYV during a systemic infection by *Y. pseudotuberculosis*.

We used a highly virulent strain of *Y. pseudotuberculosis* serogroup I, designated IP2780, isolated from stools of a patient and kindly provided by H. H. Mollaret (Institut Pasteur, Paris). This strain harbors a pYV plasmid as determined by the alkaline lysis method (10), and bacterial growth was restricted at 37°C but not at room temperature on magnesium-oxalate agar (15). A plasmid-cured derivative, strain IP2780c, was obtained by five subcultures at 37°C in tryptocasein-soy broth (Diagnostics Pasteur, Marnes-la-Coquette, France) as previously described (17). The virulence of these two strains was studied by intravenously (i.v.) infecting 6- to 8-week-old female ICR Swiss mice (Charles River Breeding Laboratories, Saint-Aubin-lès-Elboeuf, France). The 50% lethal dose was estimated in groups of five mice to be 10 to 10^2 bacteria per mouse for strain IP2780 and $10^{5.8}$ bacteria per mouse for strain IP2780c, indicating that loss of plasmid pYV was associated with a significant decrease in the level of virulence. Bacterial growth was then observed in the organs (spleen, liver, lungs) of mice infected i.v. with 1×10^3 to 2×10^3 bacteria. For this purpose, groups of four mice were killed by chloroform at progressive time intervals and their organs were aseptically removed and homogenized in 0.15 M NaCl. Serial 10-fold dilutions were spread on tryptocasein-soy agar, and colonies were counted after 48 h of incubation at room temperature. pYV$^+$ bacteria multiplied very rapidly in host tissues over the first 2 days of infection, reaching ~10^8 bacteria per organ by day 4 and ultimately killing infected mice by day 5 (Fig. 1). In contrast, pYV$^-$ bacteria grew at a slower rate in host tissues, reaching ~10^4 to 10^5 bacteria per organ by day 4 (Fig. 1). Then bacterial replication ceased, and the microorganisms were completely eliminated in the organs by day 14 of infection (data not shown). Therefore, plasmid pYV strongly stimulates in vivo bacterial growth, although pYV$^-$ bacteria are still capable of multiplying in host tissues during the early phase of infection. These results confirm a previous report showing that chromosomal genes also promote the in vivo replication of *Y. pseudotuberculosis* (17).

A histological study was then performed on the organs of infected mice. Tissues were fixed overnight in 3% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) at 4°C. Samples were then rinsed in phosphate buffer, postfixed for 1 h in 1% osmium tetroxide, dehydrated, and embedded in Epon (E. Merck AG, Darmstadt, Federal Republic of Germany). Semithin sections were stained with toluidine blue and observed with a light microscope. Ultrathin sections were collected on 150-mesh grids and then counterstained with uranyl acetate and lead citrate. In addition, some sections were treated by the periodic acid-thiocarbohydrazide-silver proteinate method (22) to better visualize the structures with high glucidic radical content. Ultrathin sections were observed with a Philips EM 300 transmission electron microscope at 80 kV. By day 4 of an i.v. infection with 10^3 pYV$^+$ bacteria, we observed multiple necrotic abscesses disseminated in the organs and associated with thrombosis of the surrounding capillaries. Strikingly, these infectious foci were

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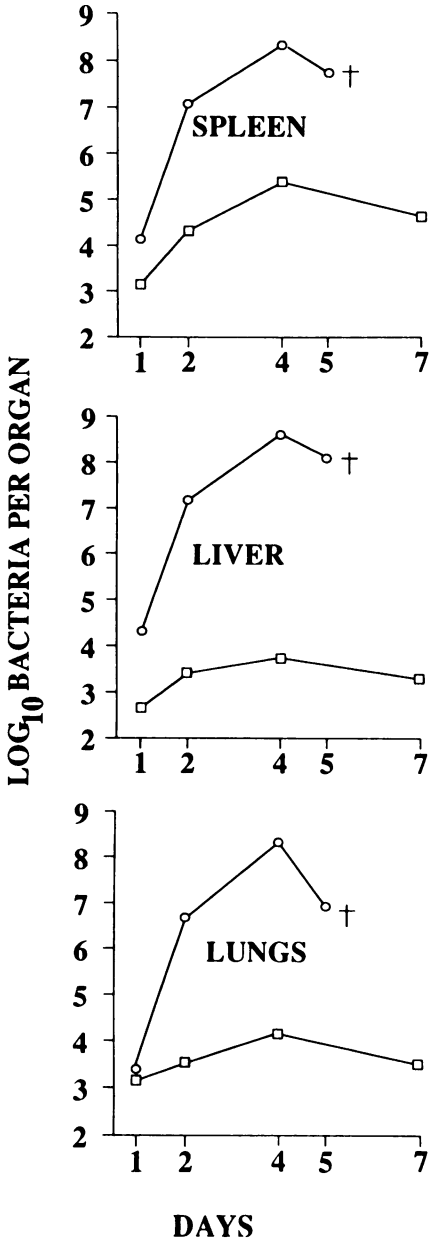


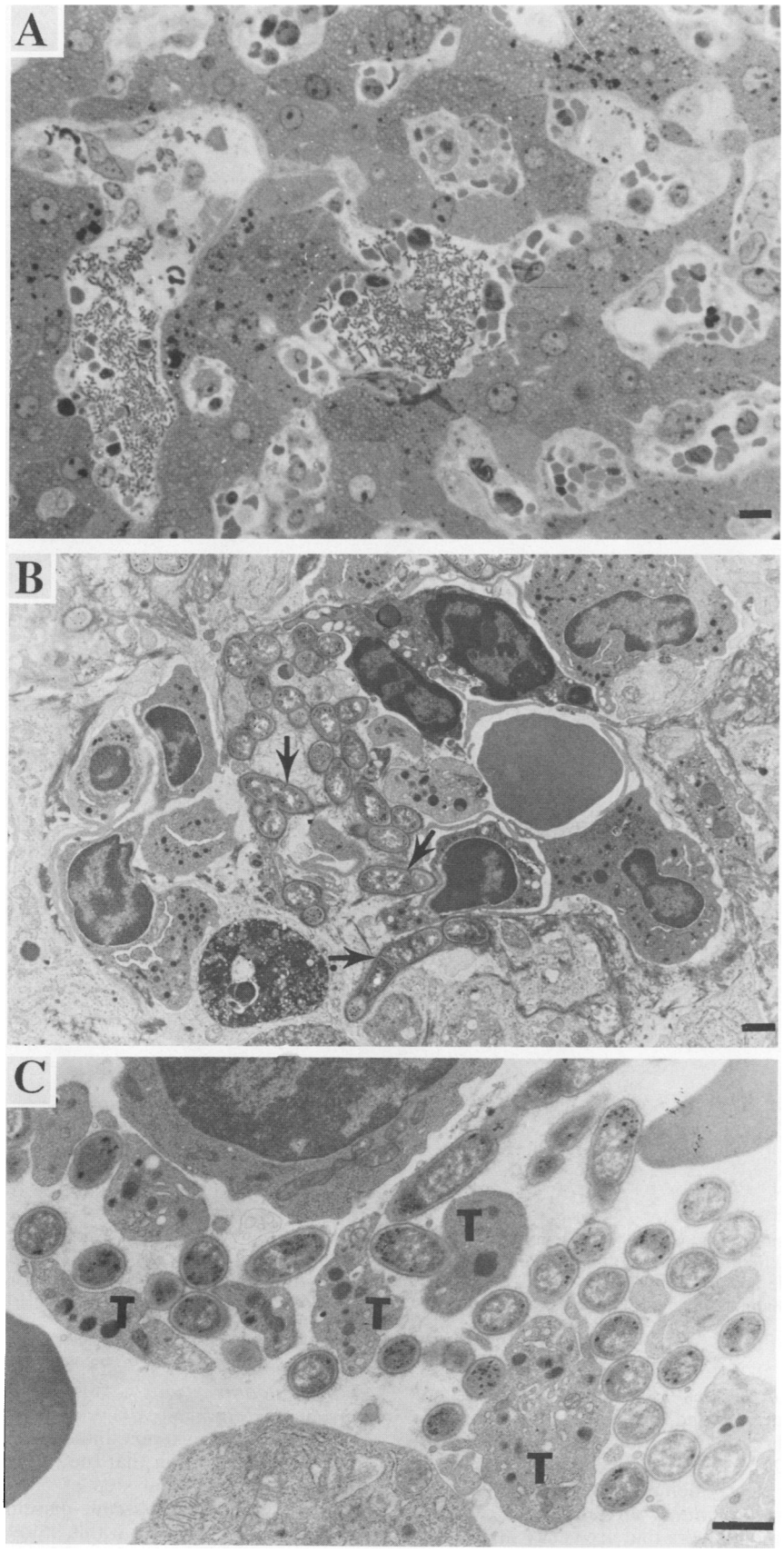
FIG. 1. Bacterial growth curves in organs of mice infected with *Y. pseudotuberculosis*. Mice were inoculated i.v. with 1×10^3 to 2×10^3 bacteria from strain IP2780 (pYV^+ bacteria) or its plasmid-free derivative (pYV^- bacteria). Bacterial survival was observed in organs in relation to time. pYV^+ bacteria induced rapid bacterial multiplication in host tissues, ultimately killing animals by day 5. In contrast, pYV^- bacteria produced a sublethal infection, although bacterial replication occurred in organs during the early phase of infection. Symbols: ○, pYV^+ bacteria; □, pYV^- bacteria. Each point is the mean value of groups of four mice; the standard deviation was less than 0.60 $\log_{10}$ bacteria.

poorly populated with inflammatory cells, and clusters of extracellular bacteria packed in capillaries were easily visible. Bacteria were never detected inside inflammatory or parenchymal cells on serial sections repeatedly performed on infected tissues (about 50 foci examined). A typical aspect seen in the liver is illustrated in Fig. 2A, with bacteria clumping in liver sinusoids. The extracellular localization of pYV^+ bacteria was further confirmed during the early phase of infection by increasing the bacterial challenge (~10^6 i.v.) to better visualize bacteria in host tissues. The early lesions induced in host tissues were examined by electron microscopy on day 1 of infection. Bacteria were visible in discrete foci scattered in organs, as illustrated in the liver shown in Fig. 2B and C. Again, these microabscesses were poorly populated with inflammatory cells, being mostly composed of free bacteria, diffuse cellular debris, and fibrin deposits. Microorganisms always appeared in extracellular sites; they were apparently intact and sometimes unambiguously undergoing active replication (Fig. 2B, arrows). Interestingly, bacteria were often seen apparently adherent to thrombocytes in the lumen of liver sinusoids (Fig. 2C). Platelets are recognized as small anucleate corpuscules with dense typical secretory granules and tubular invaginations from the plasmalemma (Fig. 2C). To our knowledge, this observation has never been mentioned before for this bacterial pathogen and might be relevant to the pathogenesis of *Y. pseudotuberculosis* infection. Indeed, this direct bacterium-thrombocyte interaction might activate platelet aggregation, thus inducing vascular thrombosis. We are currently investigating this phenomenon with an in vitro model.

The histological damages induced by pYV^- bacteria were quite different. By day 4 of the same i.v. infection (~10^3), rare small granulomas were visible, mostly composed of inflammatory cells, without any necrosis or visible bacteria. With an increase in the infecting dose (~10^6), pYV^- bacteria induced multiple granulomas in host tissues by day 4 of infection (Fig. 3A). These infectious foci were mainly composed of mononuclear and polymorphonuclear cells, as evidenced by electron microscopy (Fig. 3B). No visible bacteria were observed in these granulomas, even with this high infecting dose, presumably owing to the low amounts of pYV^- bacteria in organs. Our results therefore indicate that pYV^+ bacteria severely inhibit granuloma formation in contrast to pYV^- bacteria, which produce a strong inflammatory response. Similar results have been recently reported with virulent strains of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, which showed a lack of inflammation associated with extensive necrosis contrasting with the granulomatous response induced by their plasmidless derivatives or plasmid mutants (19, 24). This inhibition of granuloma formation might explain the in vivo immunosuppression that we previously observed in mice infected with a strain of *Y. pseudotuberculosis* harboring a virulence plasmid (16).

The concept that reticuloendothelial cells serve as repli-

FIG. 2. Extracellular localization of pYV^+ bacteria in infectious foci of the liver. (A) Semithin section of liver observed with a light microscope (toluidine blue staining). Bacteria are packed in sinusoids by day 4 of an i.v. infection with 10^3 bacteria; bacteria were never seen inside hepatocytes or inflammatory cells (×500; bar, 10 μm). (B and C) Ultrathin sections of liver abscesses by day 1 of an i.v. infection with 10^6 bacteria observed by electron microscopy (periodic acid-thiocarbohydrazide-silver proteinate staining). (B) Bacteria are exclusively extracellular, sometimes unambiguously undergoing active replication (arrows) (×5,500; bar, 1 μm). (C) Extracellular bacteria adherent to thrombocytes (T) in the lumen of a liver sinusoid (×11,000; bar, 1 μm).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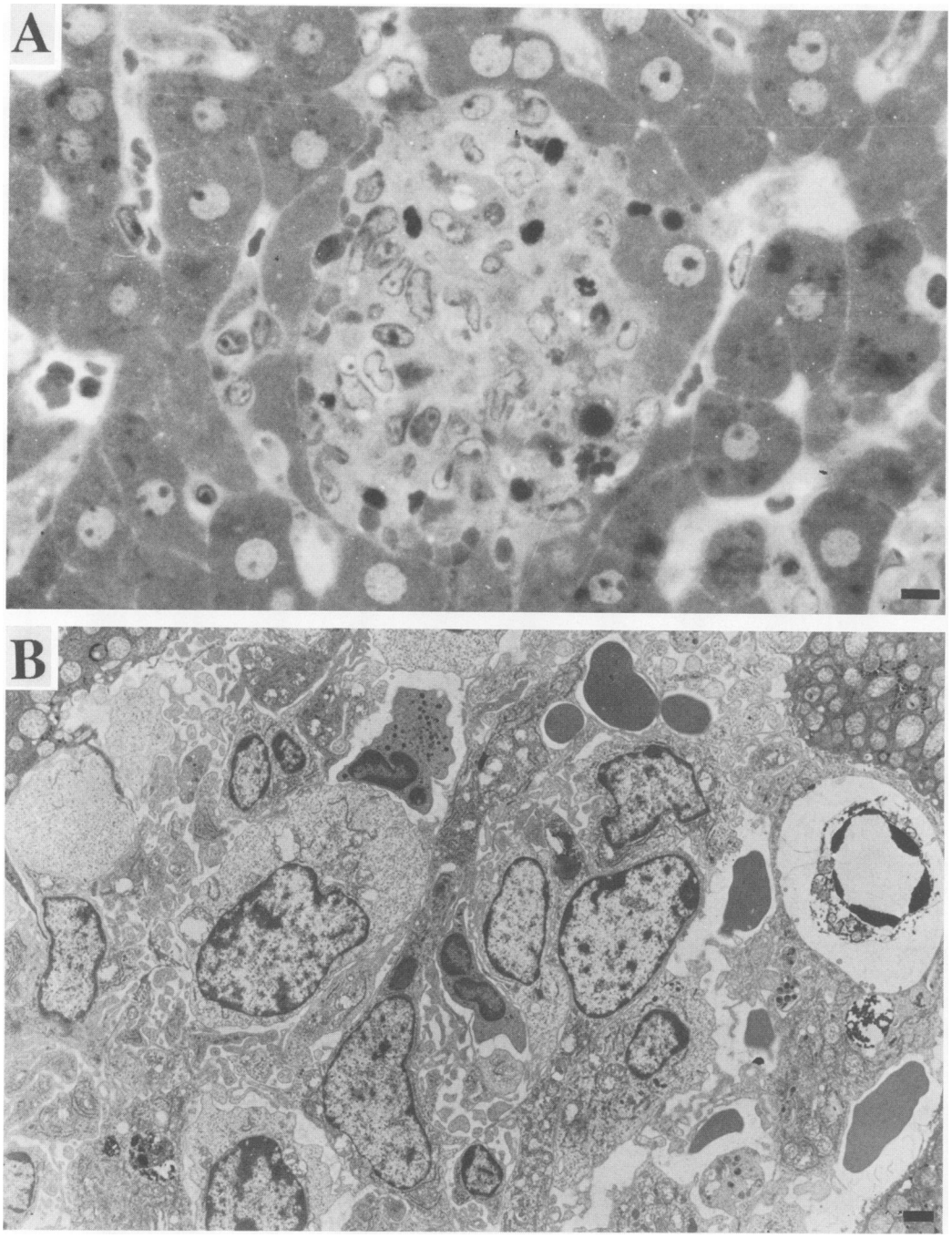

FIG. 3. Granulomas induced by pYV$^-$ bacteria in the liver. Mice were infected i.v. with 10^6 pYV$^-$ bacteria, and tissues were examined by day 4 of infection. (A) Semithin section of liver granuloma observed with a light microscope (toluidine blue staining); a typical aspect of granuloma without visible bacteria (×600; bar, 10 μm). (B) Ultrathin section of liver granuloma observed by electron microscopy (uranyl acetate and lead citrate staining). The granuloma is mostly composed of polymorphonuclear and mononuclear cells without visible bacteria (×4,500; bar, 1 μm).

cation sites for *Yersinia* species is based on in vitro experiments showing phagocytosis and intracellular multiplication of bacteria from the three main pathogenic species of *Yersinia* (4, 5, 8, 13, 20, 21, 23). However, with regard to the invasive process, it is clearly demonstrated that *Y. pseudotuberculosis* and *Y. enterocolitica* are unable to replicate extensively in HEp-2 monolayers that are easily invaded by bacteria (18). The *inv* gene might facilitate the translocation of *Y. pseudotuberculosis* after oral infection, but this chromosomal determinant would be insufficient to ensure bacterial survival in a systemic infection (14). It must be pointed out in this connection that the i.v. route used in our study bypasses the intestinal step of bacterial translocation and reflects the phase of systemic dissemination by highly virulent bacteria. We failed in this study to detect by a careful electron microscopic study any intracellular pYV$^+$ bacteria

in host tissues infected with *Y. pseudotuberculosis* either in hepatocytes or in professional phagocytes. Lian et al. (9) also found that, in vivo, most visible bacteria from *Y. enterocolitica* were extracellular, with little evidence of phagocytosis. Obviously, it cannot be ruled out that rare bacteria do multiply intracellularly, especially in the very early stage of infection. However, since the large majority of bacteria are localized outside cells, often undergoing active replication (Fig. 2B), we are forced to conclude that the in vivo bacterial multiplication occurs predominantly in extracellular sites. This discrepancy with the in vitro findings might be explained on the basis of the environmental conditions encountered by pYV^+ bacteria in host tissues.

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