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Humoral and Cellular Defense against Intestinal Murine Infection with *Yersinia enterocolitica*

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The role of phagocytes and the complement system as potential host defense mechanisms against bacterial infection were studied in mice with two isogenic strains of *Yersinia enterocolitica* serotype O8 differing in pathogenicity because of differences in plasmid content. Complement depletion in mice by intraperitoneal injection of cobra venom factor did not affect the course of colonization of the intestinal tissue by each strain, indicating that in mice complement is not essential for the elimination of these bacteria. This conclusion is supported by the fact that fresh murine serum had no bactericidal effect in vitro either on the pathogenic or on the nonpathogenic strain. However, in the intestinal tissue as well as in the peritoneal cavity, only the pathogenic, plasmid-bearing *Y. enterocolitica* strain survived, while the nonpathogenic, plasmidless strain was rapidly eliminated. Since elimination from the peritoneal cavity is due to phagocytosis by polymorphonuclear leukocytes and macrophages, resistance to phagocytosis in vivo seems to be the decisive factor determining the virulence of pathogenic *Y. enterocolitica* strains.

The mouse model of yersiniosis, established by Carter (3), is still the most convenient and widely used tool for the investigation of the interaction of yersiniae with the host, under conditions closely resembling those in humans. In mice and humans, yersiniae initially proliferate in the tissue of the terminal ileum, predominantly in the Peyer's patches. Recent studies in vivo demonstrated that the extent of initial binding and uptake of yersiniae by the Peyer's patch epithelium is similar for pathogenic and nonpathogenic, plasmidless strains (9). The immediate host defense against an infection includes the opsonic bactericidal action of complement as well as phagocytosis by macrophages and/or polymorphonuclear leukocytes. The relative importance of these mechanisms to yersinia infections has so far not been investigated.

The effect of serum on *Yersinia enterocolitica* has been studied in detail in vitro. Diluted human or rabbit serum is bactericidal for plasmidless but not for plasmid-bearing *Yersinia* strains (12, 16, 23). Resistance to the bactericidal action of human serum is temperature dependent, as is the expression of plasmid-coded *Yersinia* outer membrane proteins (YOPs) (23). The role of plasmid-coded properties is further supported by data showing that the non-immunoglobulin-mediated binding of human C3b to plasmid-bearing *Y. enterocolitica* is less efficient than that to plasmidless strains (32). The consumption of C3b from guinea pig serum may, however, be due to lipopolysaccharides and not to YOPs expressed by these strains (37). How far these in vitro findings apply to the in vivo situation, particularly after alterations in bacterial surface components during in vivo growth (22, 28), is not known.

Similarly, the interaction of phagocytes with *Y. enterocolitica* has been investigated mainly in vitro. The ability of yersiniae to inhibit phagocytosis and to induce a cytotoxic effect on cultured cells is plasmid coded (11, 18, 24). The inhibition of phagocytosis and of the metabolic burst in human polymorphonuclear leukocytes in vitro correlated with the expression of YOPs (16). Lian et al. (18) showed also that plasmidless *Y. enterocolitica* serotype O3 injected intradermally into a rabbit was phagocytosed, while the plasmid-bearing strain grew extracellularly.

The purpose of the present work was to elucidate the role of the innate immune mechanism in vivo during infection with *Y. enterocolitica* serotype O8. The protective effect of complement was assessed by extensive depletion of the complement system and monitoring of the course of the infection with *Y. enterocolitica*. The cellular defense mechanism in vivo was monitored after intraperitoneal (i.p.) infection with pathogenic bacteria.

MATERIALS AND METHODS

Animals. Six- to 8-week-old female and male CD-1 mice weighing about 30 g were used. The animals were given commercial pellets and water ad libitum. Twenty four h before oral infection they were deprived of water but had free access to food. To prevent reinfection by coprophagy, we placed the animals in separate cages with grid bottoms.

Bacterial strains and growth conditions. The plasmidless strain NCTC 10598 (here named $O8^-$) and the isogenic plasmid-bearing strain NCTC 10938 (here named $O8^+$) of *Y. enterocolitica* serotype O8 were obtained from the National Collection of Type Cultures, Central Public Health Laboratory (London, England). Bacteria were grown overnight at 24°C in brain heart infusion broth (Oxoid Ltd., London, England). This preculture was diluted to 1/15 in fresh brain heart infusion broth and incubated on a shaker at 37°C for 3 h. Prior to use the bacterial cells were collected by centrifugation, washed twice, and appropriately diluted with saline.

Serum sensitivity test. Fresh mouse or human serum (900 μl) was mixed with saline (100 μl) containing 10^7 bacteria. Tubes, including controls with saline instead of serum, were incubated at 37°C under gentle agitation. Bacterial viability

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was determined at various intervals by plating of serial dilutions in duplicate onto Endo agar and incubation for 48 h at 24°C.

Depletion of complement in vivo by CVF. Mice were injected i.p. with 400 μg of cobra venom factor (CVF; Sigma, Dreieich, Federal Republic of Germany) per kg of body weight 24 h prior to challenge with bacteria and then daily for the duration of the experiment (3 days). CVF-induced complement depletion was monitored by hemolysis assays with sheep erythrocytes sensitized with a rabbit anti-sheep erythrocyte antiserum diluted 1:50 with saline (20). Sera from the CVF-treated as well as untreated animals were serially diluted with 3.5 mM Veronal-acetate buffer containing 0.1% gelatin, and the dilutions were incubated in microtiter plates with approximately 10^8 sensitized erythrocytes per well for 30 min at 37°C. Nonlysed erythrocytes were pelleted by centrifugation, and the released hemoglobin in the supernatant was measured spectrophotometrically at 405 nm in an ELISA-reader (Dynatech, Denkendorf, Federal Republic of Germany).

Infection of complement-depleted mice. Eight CVF-pretreated and eight nontreated male mice were orally infected with 10^7 plasmid-bearing bacteria in 5 ml of saline as described previously (8). At specified times (19.5, 27.5, 43.5, and 63.5 h) after infection, groups of two mice were killed; the ileum from each mouse was removed, washed, and homogenized; and the number of bacteria colonizing the tissue was determined after plating on Endo agar as described previously (9).

Determination of the i.p. survival of bacteria. Bacteria of the two investigated *Y. enterocolitica* strains were grown and prepared as described previously (4). Groups of four mice for each strain were injected i.p. with approximately 5×10^6 bacteria in 250 μl of saline. Two mice in each group were killed after specified times, the peritoneal cavities were lavaged with 5 ml of saline, and the number of viable bacteria was determined. The recovery of the bacteria during the procedure was determined by lavage of the peritoneal cavities of three mice 5 min after the injection of pathogenic bacteria and by the determination of the CFU in the fluid. All calculations were corrected with the obtained recovery factor of 57%.

Electron microscopy. The bacteria were injected i.p. into groups of four mice as described above. Two mice in each group were killed after specified times, and the peritoneal cavities were lavaged with 5 ml of cold saline. The suspension was centrifuged for 30 min at $800 \times g$, and the pellet was fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The cells were postfixed in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2) for 1 h at 4°C, washed in 0.1 M cacodylate buffer, and dehydrated in a graded series of ethanol solutions at room temperature. The dehydrated cells were transferred to propylene oxide and embedded in Epon 812 resin (Serva, Heidelberg, Federal Republic of Germany). Semithin sections (0.5 μm) were mounted on glass slides and stained with toluidine blue for quick evaluation. Ultrathin sections (50 nm) were picked up on copper grids and stained with uranyl acetate and lead citrate. Specimens were examined with a Philips 410 transmission electron microscope at 80 kV. Phagocytosis was determined through evaluation of microphotographs of randomly chosen fields with a total of 100 to 200 bacteria.

Recovery of bacteria from Peyer's patches. Ten CD-1 mice were infected with the $O8^+$ strain as described previously (8). After 72 h, the animals were killed and the Peyer's patches were excised from the small intestines (5 to 10 Peyer's patches per mouse). The pooled Peyer's patches were cut into small pieces with a razor blade, vortexed, and decanted. The supernatants were collected, and the washing procedure was repeated twice. The supernatants were pooled and centrifuged at $5,000 \times g$ for 10 min. The bacterial pellet was resuspended in 1 ml of saline and injected into the peritoneum of four mice. An aliquot was plated to quantify the injected dose, which was approximately 5×10^6 bacteria per mouse.

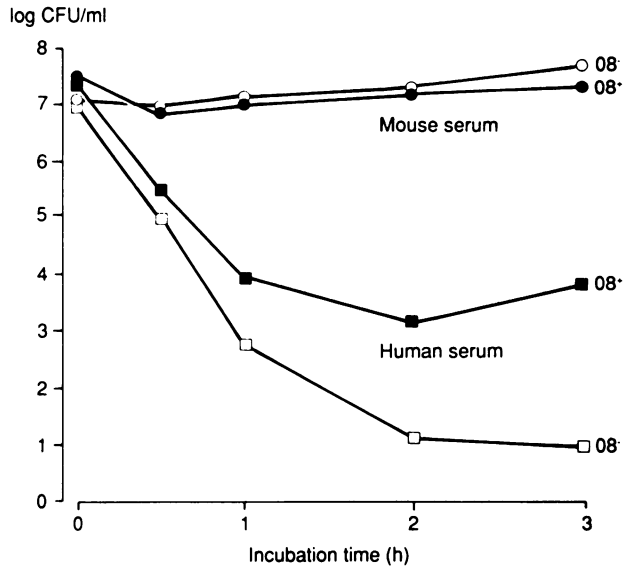


FIG. 1. Effect of fresh murine or human serum (90%) on the survival of the pathogenic ($O8^+$) or the nonpathogenic ($O8^-$) strain of *Y. enterocolitica*. Average values for three independent experiments differed by less than ±5%.

Immunoblotting of outer membranes. The outer membranes were isolated as described by Bölin et al. (1), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11.5% polyacrylamide) as described by Laemmli (15), and transferred to polyvinylidene difluoride (PVDF)-membrane (Millipore, Bedford, Mass.) as described by Towbin et al. (33). The antigens were detected with orally induced rabbit antiserum against the $O8^+$ strain preadsorbed with the $O8^-$ strain as described previously (9). Visualization was carried out with immunogold stain, and the signal was enhanced with silver salt solution (Janssen, Kaldenkirchen, Federal Republic of Germany) as described previously (21).

RESULTS

Survival of *Y. enterocolitica* in fresh murine serum. Both the pathogenic, plasmid-bearing $O8^+$ strain and the nonpathogenic, plasmidless $O8^-$ strain survived equally well in fresh mouse serum. By contrast, fresh human serum had a bactericidal effect, which was more pronounced for the $O8^-$ strain than for the $O8^+$ strain (Fig. 1).

Influence of complement depletion on the survival of *Y. enterocolitica* in vivo. For investigation of the effect of complement on bacterial survival in vivo, mice were depleted of complement by repeated i.p. administration of CVF. The extent of complement depletion was assessed by a hemolysis test, which showed that the treated mice had less than 1% normal complement activity (Fig. 2A). Irre-

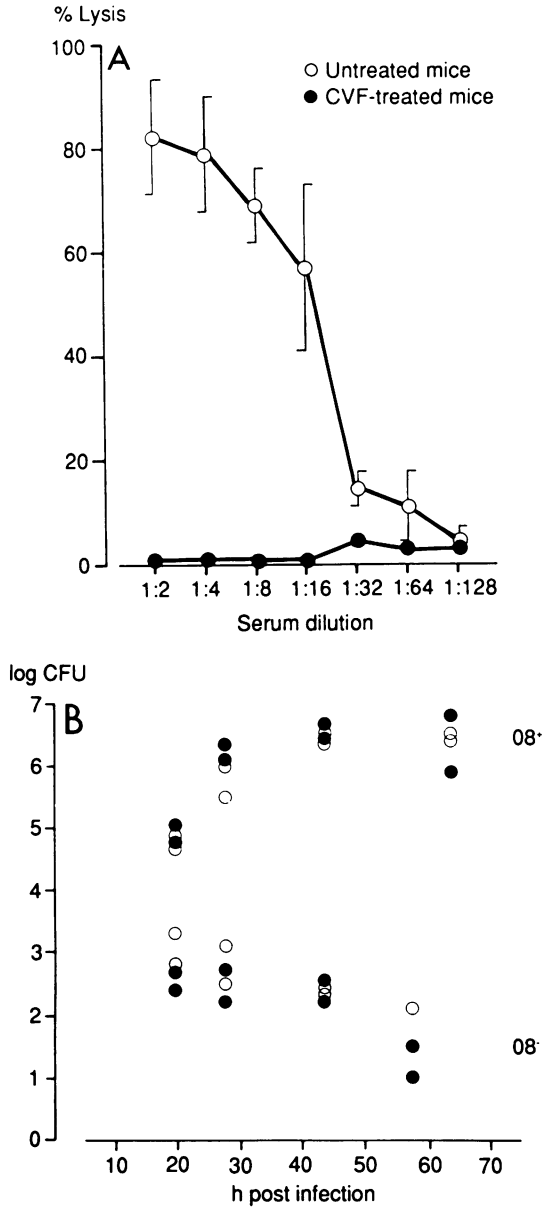


FIG. 2. (A) Hemolytic assays demonstrating the efficiency of complement depletion in mice 24 h after the administration of CVF. Values are means ± standard deviations for three mice for each curve. (B) Numbers of yersiniae persistent in murine ileal tissue at different times after oral infection of normal mice (○) or CVF-treated mice (●) with the pathogenic ($O8^+$) or the nonpathogenic ($O8^-$) strain of *Y. enterocolitica*. Each point represents one mouse.

spective of the treatment, pathogenic bacteria proliferated and the apathogenic ones were eliminated from the tissue (Fig. 2B); i.e., complement depletion had no significant influence on the course of infection of murine ileal tissue by the $O8^+$ or the $O8^-$ strain (Fig. 2B). Thus, both strains appear to be resistant to the murine complement system in vitro and in vivo. The inability of the $O8^-$ strain to multiply in the ileal tissue does not seem to be related to complement activation.

Interaction of *Y. enterocolitica* with phagocytic cells in vivo. While numerous studies have demonstrated the phagocytosis of nonpathogenic *Yersinia* strains in vitro (4, 13, 30, 31), phagocytosis of these strains in intestinal tissue has not yet been shown. Attempts to demonstrate the elimination of $O8^-$ bacteria by phagocytosis in the lamina propria were also unsuccessful in the present work. Indeed, neither extracellular nor intracellular plasmidless bacteria could be detected in tissue by transmission electron microscopy.

Therefore, an in vivo system which allows observation of the interaction between phagocytes and the injected bacteria was used. Phagocytosis of bacteria administered i.p. was assessed by enumeration of viable bacteria and electron photomicrographic analysis of cell suspensions obtained from the peritoneal cavity. The phagocytosis of the $O8^-$ strain was rapid, and the percentage of phagocytosed bacteria reached 96% within 0.5 h (Table 1 and Fig. 3A). The bacteria did not survive intracellularly but were killed within 2 to 3 h (Fig. 4). Thus, the extracellular proliferation of this strain in the peritoneal cavity is prevented by fast and complete phagocytosis.

By contrast, the percentages of intracellular $O8^+$ strain were only about 28% at 0.5 h and 47% at 1 h after administration (Table 1 and Fig. 3B). The less efficient phagocytosis allowed this strain to survive and, after a lag phase of more than 3 h (Fig. 4), to proliferate rapidly in the extracellular fluid (Fig. 3C) and to kill the mice within 24 h.

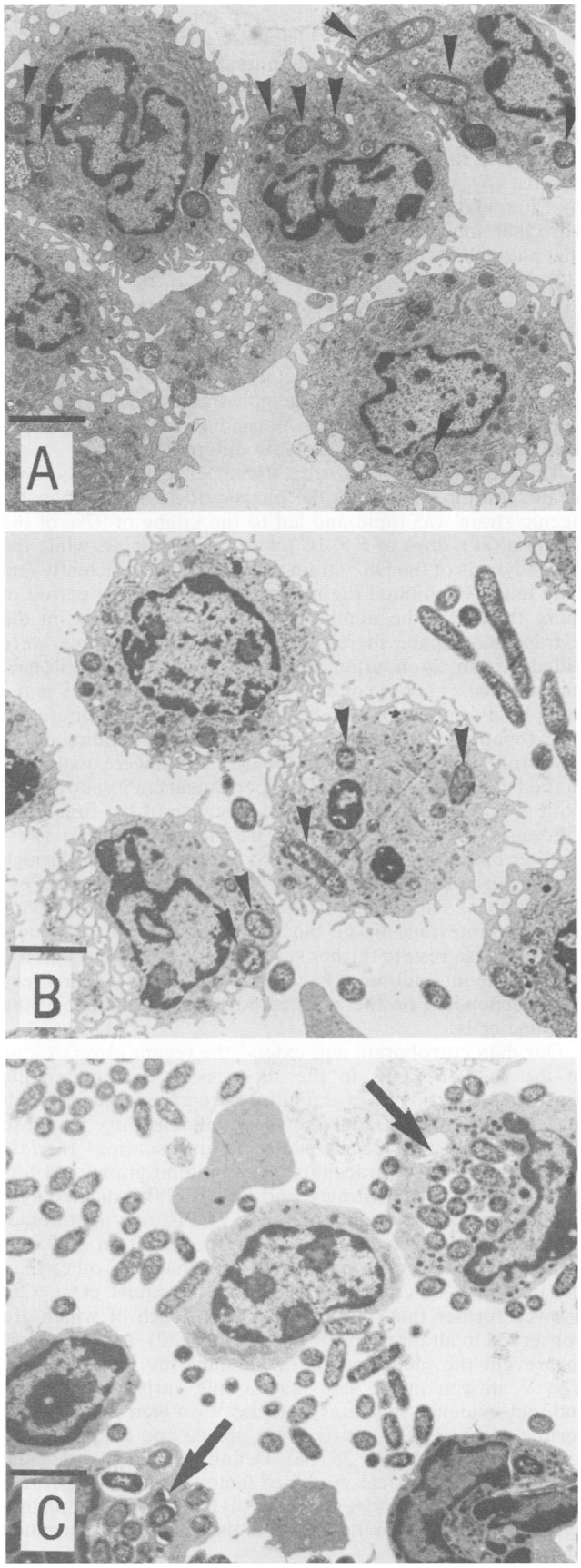
The difference in the initial uptake efficiency of the two strains was also reflected in the median number of intracellular bacteria, which tended to be lower for the pathogenic strain (Table 1). The cells involved in the phagocytic process included granulocytes and macrophages; the percentages of phagocytes which had ingested bacteria 1 h after administration were similar for the $O8^+$ strain (64% ± 6%) and the $O8^-$ strain (61% ± 16%) (mean ± standard deviation for four mice), probably reflecting the maximal uptake capacity of the peritoneal phagocytic system. Starting from the 2nd hour after infection, the percentage of phagocytes containing bacteria was about 40% for both strains, and this value was also found at the 6th hour for the $O8^+$ strain, i.e., this strain was still phagocytosed with comparable efficiency. Phagocytosis did not result in the destruction of phagocytes but in the lysis of bacteria (Fig. 3C).

i.p. survival of bacteria grown in vitro and in Peyer's patches. The susceptibility of the pathogenic strain to i.p. phagocytosis is in striking contrast to the almost complete resistance to phagocytosis of this strain in Peyer's patches. To test whether the bacteria grown in Peyer's patches possess properties different from those of bacteria grown i.p., we isolated $O8^+$ bacteria from infected murine Peyer's patches, appropriately diluted them, and injected them into the peritoneal cavities of the mice. In two experiments involving four mice each, the injected bacteria did not,

TABLE 1. Phagocytosis of bacteria after i.p. administration[a]

Strain	Time after administration (h)	No. of bacteria/ phagocyte	% Ingested bacteria
$O8^-$	0.5	4.0 ± 1.1	96.3 ± 2.8
	1.0	4.2 ± 1.1	89.8 ± 6.8
$O8^+$	0.5	2.1 ± 0.7	28.0 ± 10.0
	1.0	3.3 ± 0.9	46.5 ± 4.2

[a] For each value, exudates from four mice were separately evaluated by electron microscopy, and the standard deviation was calculated after the enumeration of 100 to 200 bacteria.



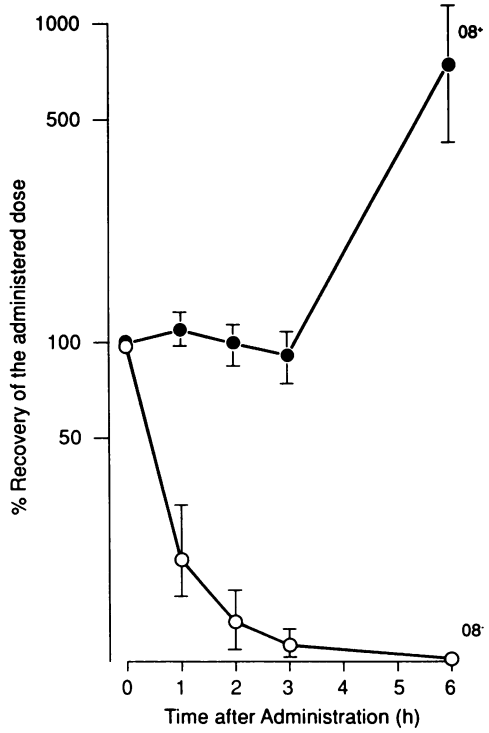


FIG. 4. Survival of the pathogenic ($O8^+$) (●) and the nonpathogenic ($O8^-$) (○) strains of *Y. enterocolitica* in the murine peritoneal cavity. Values are means ± standard deviations for six experiments with a total of four to eight mice per time point.

however, survive better in the peritoneum than did the same strain grown in vitro at 37°C (data not shown), suggesting that the observed resistance to phagocytosis in Peyer's patches is not due to a different membrane composition of the bacteria. Immunoblot analysis of yersiniae grown in the peritoneal cavity with preadsorbed serum confirmed this hypothesis: the membranes of bacteria grown in the peritoneal cavity expressed the same YOPs (Fig. 5) as did the membranes of bacteria grown in Peyer's patches (22).

DISCUSSION

Recent data indicate that the interactions of *Y. enterocolitica* with epithelial cells as well as with phagocytes are qualitatively different in vitro and in vivo. The discrepancy is due to (i) modulation of the expression of YOPs by the environment, in particular, by in vivo growth conditions (22, 28), (ii) phenotypic and functional differences in cultured epithelial cells and cells with which the bacteria interact in vivo, and (iii) the in vivo interdependence of humoral and cellular defense mechanisms, usually considered separately in vitro.

In the present work, the relative roles of complement and phagocytes in the initial phase of *Y. enterocolitica* infection

FIG. 3. Electron photomicrographs demonstrating the interaction of mouse peritoneal phagocytes with *Y. enterocolitica* $O8^-$ (A) or $O8^+$ (B and C) 0.5 h (A and B) or 6 h (C) after i.p. administration of bacteria. Bar, 2 μm. Intracellular bacteria are indicated by arrowheads, and phagocytes containing several bacteria are indicated by arrows. Some intracellular bacteria in panel C show signs of lysis.

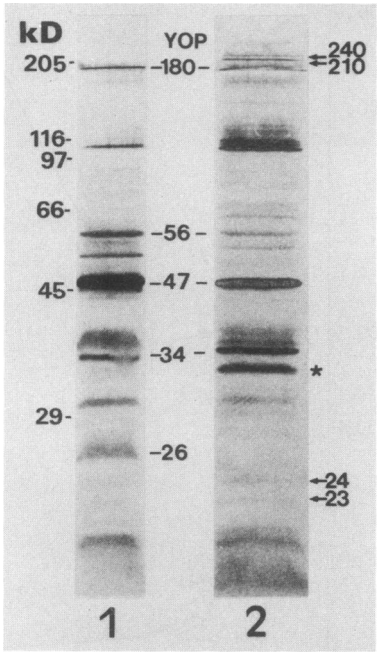


FIG. 5. Immunoblot of outer membranes isolated from bacteria grown in vitro (lane 1) or i.p. (lane 2) and analyzed with orally induced anti-O8$^+$ strain antiserum preadsorbed with the O8$^-$ strain. The main YOPs expressed in vitro and in vivo are indicated by their apparent molecular masses; arrows indicate new plasmid-coded membrane proteins. The prominent peptide with a mass of 30 kDa (asterisk) was present in the O8$^-$ strain in vivo, i.e., it was chromosomally coded.

in vivo were assessed in a murine model. Inhibition of the complement system with CVF did not influence the outcome of the infection after oral challenge with an O8$^+$ or an O8$^-$ strain. This observation, corroborated by the in vitro tests, suggests that in mice neither the O8$^+$ strain nor the O8$^-$ strain is affected by complement activity. Since both strains penetrate M cells in a similar manner (6) and persist in the ileum in the initial phase of infection to the same extent (9), the elimination of the O8$^-$ strain at the later stage must be accomplished solely by the activity of the phagocytic cells in the intestinal tissue.

Phagocytosis of intradermally injected yersiniae in rabbits has been demonstrated by Lian et al. (18). Rabbit complement has a bactericidal effect on *Y. enterocolitica* (34), and how far it contributes to phagocytosis has not been investigated in this animal. Repeated attempts to demonstrate phagocytosis in murine intestinal tissue by transmission electron microscopy were, however, not successful. Moreover, the inhibition of phagocytosis by repeated injection of dextran sulfate, which blocks the phagocytic system (7) by inhibiting the phagosome-lysosome function (14), did not affect the rate of elimination of the nonpathogenic strain from the ileal tissue (data not shown).

Similarly, the inhibition of leukocyte proliferation by treatment of mice with cyclophosphamide, as described by Cryz et al. (5), did not increase susceptibility to subsequent oral infection with the nonpathogenic *Y. enterocolitica* strain (data not shown). These treatments, however, may be not sufficiently effective in the intestinal microenvironment. Although resident macrophages in the Peyer's patches represent only 0.1 to 0.2% of the total nucleated cell population in the Peyer's patches (19), they are apparently sufficient to eliminate the nonpathogenic bacteria, even if the majority of the peripheral phagocytes are inhibited or eliminated.

These results, in combination with previous data obtained by electron microscopy (6), are consistent with the hypothesis that the penetration of M cells in the Peyer's patches is a relatively rare event. To monitor phagocytosis by electron microscopy, we used an alternative in vivo system described by Charnetzky and Shuford (4). It was previously shown in vitro that nonpathogenic *Yersinia* strains are susceptible and that pathogenic *Yersinia* strains are resistant to phagocytosis (13). However, as demonstrated by Charnetzky and Shuford (4) with *Y. pestis*, the ability of cultivated macrophages to kill bacteria may be impaired in vitro and is modulated by the medium used for growing macrophages, i.e., the in vitro and in vivo systems are not completely analogous. Furthermore, Hartiala et al. (10) recently demonstrated that the interactions of yersiniae with polymorphonuclear leukocytes in vivo and in vitro are qualitatively different.

The present work shows that in the peritoneal cavity, both strains are phagocytosed; the phagocytosis of the nonpathogenic strain was rapid and led to the killing of 97% of the bacteria (at a dose of 5×10^6) within 3 h (Fig. 4), while the phagocytosis of the O8$^+$ strain proceeded less efficiently and only initially inhibited the net growth. After a lag period of more than 3 h, the number of pathogenic bacteria in the extracellular space increased rapidly, and the mice were killed within 24 h after administration. After peritoneal phagocytosis, bacteria of both strains were lysed; even in the late phase of infection, no signs of intracellular proliferation or cytopathic effects were observed. The rapid killing of the O8$^-$ strain and the survival of the O8$^+$ strain were analogous in the Peyer's patches and in the peritoneal cavity; however, both processes differed, probably because of the functional differences between phagocytes resident in the two sites. Further extension of this observation was obtained through i.p. injection of O8$^+$ bacteria previously grown in the Peyer's patches. These organisms were resistant to phagocytosis in the intestinal tissue but not in the murine peritoneal cavity. These results further substantiate the caveat that the data on the interaction of bacteria with phagocytes are very much dependent on the source and the environment of the immune cells.

Our data corroborate and extend the recent observations of the role of YOPs in the suppression of the immune reaction of the host. Several authors reported that plasmidless *Yersinia* strains induce a strong inflammatory response in the liver and spleen, while plasmid-bearing *Yersinia* strains proliferate extracellularly and inhibit granuloma formation (27, 29, 36). Straley and Cibull (29) indicated that outer membrane proteins, notably, YOPE, may counteract the early defense reaction to *Y. pestis* infection and suggested that this protection is due to mechanisms other than the inhibition of complement binding. Rosquist et al. (26) showed further that YOPE and YOPH, both of which are conserved in all three species of yersiniae (2), act in concert to prevent the phagocytosis of yersiniae by macrophages. The V antigen might also play a role early in infection. Indirect evidence indicates that the V antigen is necessary for the survival of *Y. pestis* in the spleen and for prolonged growth in the liver (25, 35, 36). Definitive evidence that the V antigen is a classical virulence factor does not exist, and which of the other antigens are involved in inhibiting phagocytosis of yersiniae in the intestinal tissue can be determined only in vivo.

In conclusion, the comprehensive investigation of the

innate immunity to *Y. enterocolitica* indicates that in mice, as opposed to humans and rabbits, the phagocytic system is the decisive component for the elimination of the nonpathogenic strain. The lack of interaction with complement in the described model offers new possibilities for studying the resistance of *Y. enterocolitica* to phagocytosis in vivo.

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