INFECTION AND IMMUNITY, May 1987, p. 1176–1183
0019-9567/87/051176-08$02.00/0

Vol. 55, No. 5

Plasmid-Mediated Resistance to Phagocytosis in *Yersinia enterocolitica*

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Received 8 October 1986/Accepted 17 January 1987

Results of our previous studies have shown that the chemiluminescence response of human neutrophils (polymorphonuclear leukocytes [PMNs]) is inhibited by plasmid-mediated cell surface components from *Yersinia enterocolitica*. In this study we examined the susceptibility to phagocytosis of *Y. enterocolitica* cells with or without plasmid-mediated surface structure and the effect of isolated outer membrane fragments on phagocytosis of *Escherichia coli* by PMNs in vitro. *Y. enterocolitica* cells with expressed plasmid-mediated surface structure were much less sensitive to ingestion by PMNs than those without it, and the resistance to phagocytosis was readily eliminated in a dose-dependent fashion by pronase treatment of whole cells, which was shown to remove plasmid-encoded outer membrane proteins. Ingestion and intracellular killing of *E. coli* were inhibited significantly in the presence of isolated outer membrane fragments derived from plasmid-bearing *Y. enterocolitica* cells. To assess the interaction of *Y. enterocolitica* with phagocytic cells in vivo, two isogenic strains of *Y. enterocolitica*, differing only in the presence or absence of the virulence plasmid, were inoculated intradermally into the backs of rabbits; and tissue sections obtained at 12 h postinoculation were examined by light and electron microscopy. The plasmidless strain was found almost entirely in PMNs or mononuclear cells. In contrast, the plasmid-bearing strain was found to be surrounded by, or interspersed with, PMNs and mononuclear cells; but most bacteria were extracellular, with little evidence of phagocytosis. These results suggest that plasmid-mediated cell surface components of *Y. enterocolitica* act as antiphagocytic factors, thus facilitating the survival and proliferation of the organism in the host tissue.

The virulence of *Yersinia enterocolitica* is associated with the presence of a 42- to 48-megadalton plasmid (9, 27, 28, 37). Various plasmid-associated properties are expresed in vitro at 37°C, including novel outer membrane proteins (4, 7, 20, 28, 29) and fibril structure (15). However, the role of plasmid-encoded determinant(s) in pathogenesis is yet to be elucidated.

Results of recent studies in our laboratory (18) have shown that the chemiluminescence (CL) response of human neutrophils (polymorphonuclear leukocytes [PMNs]) to a plasmid-bearing strain of *Y. enterocolitica* grown at 37°C is significantly lower compared with the CL response to the same strain grown at 25°C or to a plasmidless isogenic strain grown at either temperature. Furthermore, outer membrane fragments prepared from the plasmid-bearing strain grown at 37°C were shown to inhibit PMN CL responses to opsonized zymosan or whole cells of *Y. enterocolitica*, suggesting an antiphagocytic role of plasmid-mediated cell surface components.

This study was designed to examine the effects of the plasmid-mediated cell surface components on phagocytosis in vitro and the interaction of *Y. enterocolitica* with phagocytic cells in vivo. An *Escherichia coli* strain was used in vitro in phagocytosis experiments because of technical problems associated with clumping or autoagglutination of plasmid-bearing strains of *Y. enterocolitica* grown at 37°C (16) and with serum sensitivity of strains lacking the virulence plasmid (24, 25).

(This study was presented in part at the 86th Annual Meeting of the American Society for Microbiology, 23 to 28 March 1986, in Washington, D.C. [C.-J. Lian and C. H. Pai, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B164, p. 51].)

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Two isogenic strains of *Y. enterocolitica* serotype O:3, biotype 4, MCH700S (plasmid-bearing) and MCH700L (plasmidless), were used in this study. Virulence in experimental infection and plasmid profiles of these strains have ben described previously (18, 24). *E. coli* 68308, a serum-resistant clinical isolate, was used in phagocytosis experiments. *Y. enterocolitica* strains from frozen stock cultures were inoculated into 10% brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and grown overnight at 25°C in a rotary shaker (150 rpm). Fresh brain heart infusion broth was inoculated with a 10% inoculum, and the culture was then incubated at 25 or 37°C for 3 to 4 h. Each culture is designated by the strain number followed by the growth temperature (e.g., MCH700S-37°C and MCH700S-25°C). The *E. coli* strain, maintained as a frozen stock, was grown in the same manner in 10% brain heart infusion broth at 37°C for 3 to 4 h. For CL or phagocytosis assays, bacteria were pelleted by centrifugation, washed twice with 0.1 M phosphate-buffered saline (PBS; pH 7.2), and suspended in Hanks balanced salt solution (HBSS; GIBCO Diagnostics, Madison, Wis.; pH 7.2) without phenol red indicator but containing 0.1% gelatin.

Preparation of outer membranes. Outer membranes were prepared by sucrose density gradient centrifugation by the procedure described previously (18).

Human PMNs. PMNs were prepared from pooled citrated peripheral blood obtained from 5 to 10 healthy individuals by the procedures described previously (18). Isolated PMNs (about 98% viable, as determined by trypan blue dye exclusion) were suspended in HBSS (pH 7.2) containing 0.1% gelatin.

CL assay. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, Mo.)-enhanced CL was measured in a luminometer (model 1251; LKB, Stockholm, Sweden) as described previously (18). To each cuvette were added 500 μl of bacterial suspension (5×10^7 CFU) or outer membrane preparation (1 mg of protein per ml) plus bacterial suspension, 50 μl of normal human serum (NHS), 10 μl of 10^{-4} M luminol, and 500 μl of PMN suspension (1×10^6 cells). HBSS was added to adjust the final volume to 1.2 ml.

Phagocytosis assay. The uptake and intracellular killing of bacteria by human PMNs were determined microscopically (22) by the use of an acridine orange staining method (33) combined with the use of crystal violet to quench fluorescence of noningested bacteria (11). Isolated PMN suspension (4×10^5 cells in 0.2 ml) was placed on a series of sterile cover slips (22 by 22 mm) and incubated at 37°C in a humidified CO_2 (6%) incubator for 60 to 90 min. Nonattached PMNs were removed by rinsing the cover slips gently in warm (37°C) 0.1 M PBS (pH 7.2) containing 1 mM glucose. Bacterial suspensions (2×10^7 CFU in 0.2 ml), which were preopsonized in 5% NHS for 15 min at 37°C, were placed onto the PMN monolayer; and the cover slips were incubated at 37°C in the humidified CO_2 incubator. At various time intervals, cover slips were removed and rinsed in three washes of ice-cold PBS with 1 mM glucose. The cover slips were drip-dried and stained with acridine orange (0.1 mg/ml in 0.1 M PBS [pH 7.2]; color index no. 46005; Fisher Scientific Co., Fair Lawn, N.J.) for 1 min. Excess stain was removed; and the cover slips were drip-dried, counterstained with crystal violet (1 mg/ml in 0.1 M PBS [pH 7.2]; color index no. 42555); Hartman-Leddon Co., Philadelphia, Pa.) for 1 min, and washed in ice-cold PBS. The final wash was with ice-cold HBSS with 0.1% gelatin. Stained slides were kept in a refrigerator until microscopic examination. Slides were examined under a UV epifluorescent microscope (model BH-2; Olympus) with an oil immersion objective lens with an adjustable iris. In this assay, intracellular bacteria fluoresce green when viable and red if nonviable (33). All extracellular bacteria were not visible because of the counterstain with crystal violet. A total of 100 PMNs were counted, and phagocytosis was expresed as follows. Percent phagocytosis was the percentage of PMNs containing one or more bacteria. Both viable and nonviable bacteria were included in this estimate. Phagocytic index was the average number of bacteria ingested per participating PMN, i.e., phagocytic index = (total number of intracellular bacteria in 100 PMNs)/(number of PMNs containing at least one bacteria). Percent killed was the percentage of intracellular nonviable bacteria, i.e., percent killed = (total number of nonviable bacteria in 100 PMNs)/(total number of intracellular bacteria in 100 PMN) × 100.

Surface labeling of *Y. enterocolitica*. Surface labeling of *Y. enterocolitica* was carried out by using the Iodo-gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; Pierce Chemical Co., Rockford, Ill.) procedure (19, 35). Bacterial cultures were centrifuged, and the cells were washed and suspended in 0.1 M PBS (pH 7.2) to give an A_{420} of 5.0. The cell suspensions were incubated with 50 μl (0.1 mCi) of $Na^{125}I$ (specific activity; 17.4 Ci/mg; New England Nuclear Corp., Boston, Mass.) in a reaction vial coated with Iodo-gen. The procedure was carried out as described previously (35) for 1 min at room temperature. Labeled bacteria were centrifuged and washed twice with PBS containing 10 mM sodium iodide.

Pronase treatment of labeled bacteria. Surface-labeled bacteria were washed once and suspended in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer containing 5 mM Ca^{2+} (pH 7.3). Pronase (protease type XIV; Sigma) treatment was carried out by the methods described by Martinez (20).

TABLE 1. Phagocytosis of *Y. enterocolitica* by human PMN[a]

Y. enterocolitica strain	% Phagocytosis
MCH700L-25°C	94 ± 6
MCH700L-37°C	89 ± 7
MCH700S-25°C	86 ± 2
MCH700S-37°C	48 ± 18[b]

[a] PMN monolayers were incubated for 2 h with bacteria (2×10^7) preopsonized with 5% NHS. Data (percentage of PMNs that contained one or more intracellular bacteria) are means ± standard deviations from three separate experiments.

[b] Percent phagocytosis of *Y. enterocolitica* MCH700S-37°C versus that of other *Y. enterocolitica* cultures: $P < 0.05$ (Student's *t* test).

Analysis of labeled surface proteins. Labeled surface proteins were identified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of whole-cell lysates and subsequent autoradiography. Labeled bacteria were suspended in 0.5% Triton X-100 in 0.5 M Tris hydrochloride (pH 6.8) and were disrupted by freezing and thawing. The lysate was solubilized by boiling for 5 min in 2% SDS–12.5% glycerol–1% mercaptoethanol–62.5 mM Tris hydrochloride (pH 6.8), and samples containing 2.5×10^5 cpm were analyzed by SDS-PAGE in a discontinuous buffer system as described previously (18). The gels were dried and exposed to X-Omat AR (XAR-5) film (Eastman Kodak Co., Rochester, N.Y.) for several hours to obtain autoradiograms.

In vivo experiments. To examine the interaction of *Y. enterocolitica* with phagocytes in vivo, the organism (CFU ranging from 10^5 to 10^9 in 0.1 ml of suspension) was inoculated intradermally into the clean, shaved backs of rabbits (New Zealand White; weight 1.5 kg). PBS was also injected as a control. Each site of inoculation was spaced 2 to 3 cm apart. At 12 h postinjection, rabbits were overdosed with sodium pentobarbital, and the whole cutaneous layer (1 cm^2 in size) around the inoculation site was removed for histological examination. One-half of each specimen was fixed in 10% phosphate-buffered Formalin and embedded in glycol methacrylate. Sections were stained with hematoxylin, eosin, and Giemsa stains. The other half was fixed in 2.5% cacodylate-buffered glutaraldehyde solution, postfixed in 2% cacodylate-buffered osmium tetroxide for 1 h, dehydrated through graded acetone, and embedded in Epon 812. Selected thin sections were stained with uranyl acetate and lead acetate and examined with an electron microscope (H-600; Hitachi).

RESULTS

Phagocytosis of *Y. enterocolitica*. The phagocytosis by human PMNs of *Y. enterocolitica* strains grown at 37 or 25°C was measured after 120 min of incubation (Table 1). Ingestion of bacteria, as expressed by percent phagocytosis (percentage of PMNs with one or more intracellular bacteria), was significantly ($P < 0.05$) lower for strain MCH700S-37°C compared with MCH700S-25°C or for strain MCH700L-37°C or MCH700L-25°C. These results were consistent with the previous finding (18) that the CL response of human PMNs is significantly lower when the cells are exposed to plasmid-bearing *Y. enterocolitica* cells grown at 37°C than when they are exposed to the same strain grown at 25°C or to a plasmidless strain grown at either temperature.

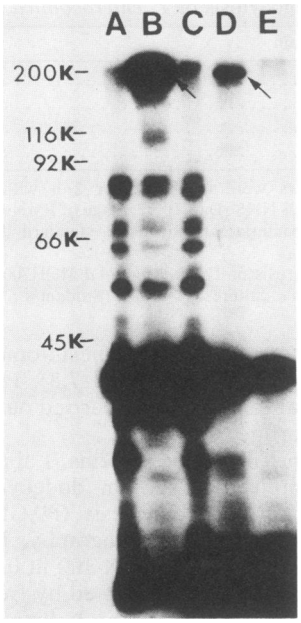

FIG. 1. Autoradiograph of SDS-PAGE of whole cell lysates of ^{125}I-surface-labeled *Y. enterocolitica* cells with or without pronase treatment. Lanes: A, strain MCH700L-37°C without pronase treatment; B, strain MCH700S-37°C without pronase treatment; C, strain MCH700S-25°C, without pronase treatment; D, strain MCH700S-37°C treated with pronase (0.1 mg/ml); E, strain MCH700S-37°C treated with pronase (1.0 mg/ml). Molecular mass markers (in kilodaltons) are listed to the left of the figure. Protein 1 described by Bölin et al. (4) is indicated by arrows.

Effect of pronase treatment on phagocytosis. To examine the hypothesis that resistance to phagocytosis is mediated by plasmid-mediated cell surface components, *Y. enterocolitica* cells were treated with a proteolytic enzyme at various concentrations to remove some surface-exposed proteins, and phagocytosis of treated cells was compared with that of untreated cells. Hydrolysis of surface-exposed proteins was monitored by autoradiography of iodinated surface proteins after separation by SDS-PAGE (Fig. 1). Pronase treatment (0.1 mg/ml; Fig. 1, lane D) resulted in the partial digestion of several surface-exposed proteins, including protein 1, a high-molecular-weight (ca. 200,000), plasmid-encoded outer membrane protein described by Bölin et al. (4). Digestion of protein 1 was virtually complete when whole cells were treated with pronase at a higher concentration (1.0 mg/ml; Fig. 1, lane E). We then compared percent phagocytosis of *Y. enterocolitica* cells possessing various amounts of plasmid-mediated cell surface components (Table 2). *Y. enterocolitica* MCH700S-37°C became susceptible to phagocytosis following pronase treatment, and the degree of susceptibility correlated with the extent of the hydrolysis of plasmid-mediated cell surface components. Pronase treatment had no effect on the ingestion of *Y. enterocolitica* without expressed plasmid-mediated surface structures.

Although ingestion of *Y. enterocolitica* could be measured semiquantitatively by estimating percent phagocytosis, we found that more quantitative measurements, such as phagocytic index, which requires the enumeration of individual bacteria, were difficult to perform and that data were not reproducible. The difficulty might have been due to the clumping of plasmid-bearing *Y. enterocolitica* cells with an altered cell surface. Plasmid-associated cell surface hydrophobicity and autoagglutination have been well documented (14, 16). Another potential problem in performing phagocytosis assays with *Y. enterocolitica* was that the organisms lacking the virulence plasmid were extremely susceptible to the bactericidal action of NHS (20, 24, 25), and therefore, it would not be possible to assess PMN-dependent killing in the presence of bactericidal action in serum. To solve these problems, we decided to use a serum-resistant strain of *E. coli* as a test organism and to determine the effect of isolated *Y. enterocolitica* outer membrane fragments on the phagocytosis of the test organism. A considerable amount of PMN CL response was induced by the *E. coli* strain, and that response was inhibited significantly by outer membrane

TABLE 2. Effect of pronase treatment on the phagocytosis of *Y. enterocolitica*

Strain	Pronase concn (mg/ml)[a]	% Phagocytosis[b]
MCH700S-37°C	0	29.0 ± 2.9
	0.1	64.7 ± 6.2[c]
	1.0	89.0 ± 2.9[d]
MCH700S-25°C	0	83.3 ± 8.5
	1.0	88.7 ± 4.5
MCH700L-37°C	0	93.7 ± 2.6
	1.0	93.3 ± 3.1

[a] *Y. enterocolitica* suspensions were treated with pronase for 30 min at 37°C before they were incubated with PMN monolayers.
[b] Percentage of PMNs that contained one or more intracellular bacteria. Data are means ± standard deviations from two separate experiments.
[c] $P < 0.02$ versus phagocytosis of untreated MCH700S-37°C (Student's t test).
[d] $P < 0.01$ versus phagocytosis of untreated MCH700S-37°C and $P < 0.05$ versus phagocytosis of MCH700S-37°C treated with 0.1 mg of pronase per ml (Student's t test).

TABLE 3. Effect of outer membrane fragments from *Y. enterocolitica* on CL response to whole cells of *E. coli*[a]

Outer membrane fragments derived from:	Peak-height CL response (mV)	P value[b]
—[c]	70.2 ± 27.3	
MCH700L-37°C[d]	53.8 ± 16.7	NS
MCH700S-37°C[d]	28.3 ± 4.0	<0.01

[a] The reaction mixture contained PMNs (10^6 cells), *E. coli* (5×10^7 CFU), normal human serum (5%), and luminol (10^{-5} M). Outer membrane fragments, when present, were at a concentration of 1 mg of protein per ml. Data were means ± standard deviations for three separate experiments.
[b] Versus CL response without outer membrane fragments (Student's t test). NS, Not significant.
[c] —, Without outer membrane fragments.
[d] *Y. enterocolitica* MCH700L-37°C and MCH700S-37°C.

TABLE 4. Effect of isolated outer membrane fragments from *Y. enterocolitica* on the ingestion of *E. coli* by human PMNs[a]

Outer membrane fragments[b]	No. of *E. coli* cells ingested/ 100 PMNs	% Phagocytosis
None	861 ± 11	99 ± 1
YE 700L-37°C	627 ± 108	97 ± 1
YE 700S-37°C	134 ± 29[d]	55 ± 5[e]

[a] For experimental conditions, see the text. Data are means ± standard deviations from three separate experiments.
[b] At a concentration of 1 mg of protein per ml.
[c] Outer membrane fragments prepared from YE MCH700L -37°C or MCH-700S-37°C.
[d] $P < 0.01$ versus YE 700L-37°C (Student's t test).
[e] $P < 0.001$ versus YE 700L-37°C (Student's t test).

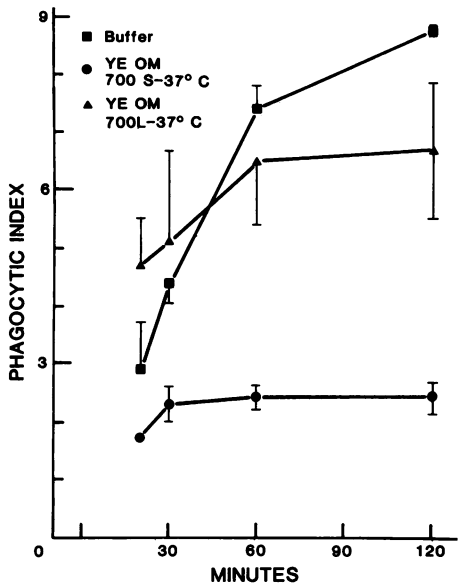

FIG. 2. Effect of outer membrane (OM) fragments from *Y. enterocolitica* on ingestion of *E. coli* by human PMNs. PMNs were incubated with *E. coli* in the presence or absence (buffer) of outer membrane fragments (1.0 mg/ml) derived from *Y. enterocolitica* MCH700S-37°C or MCH700L-37°C. The phagocytic index is defined as the number of ingested bacteria per participating PMN. Each point represents the mean ± standard deviation of three separate experiments.

fragments derived from plasmid-bearing *Y. enterocolitica* cells (Table 3). These results indicate the suitability of the *E. coli* strain as a test organism.

Effect of plasmid-mediated cell surface components of *Y. enterocolitica* on the ingestion of *E. coli*. The number of *E. coli* cells ingested by PMNs, as well as percent phagocytosis, was significantly reduced in the presence of outer membrane fragments derived from strain MCH700S-37°C ($P < 0.01$) (Table 4). The uptake of *E. coli* was also reduced slightly in the presence of outer membrane fragments from the plasmidless strain of *Y. enterocolitica*, but not at a significant level. The average number of *E. coli* cells ingested per participating PMN (phagocytic index) is shown in Fig. 2. In the absence of outer membrane fragments, the number of ingested bacteria increased from about three at 20 min of incubation to almost nine at 120 min. In the presence of outer membrane fragments derived from strain MCH700S-37°C, phagocytosis of *E. coli* cells was inhibited, and the average number of ingested bacteria per participating PMN remained less than three at 120 min of incubation. The difference between the number of *E. coli* cells ingested in the presence and absence of the outer membrane fragments derived from strain MCH700S-37°C was highly significant ($P < 0.01$ to $P < 0.001$).

Intracellular killing of *E. coli*. The kinetics of intracellular killing of *E. coli* cells by human PMNs are shown in Fig. 3. Intracellular killing was inhibited significantly ($P < 0.01$) in the presence of outer membrane fragments derived from strain MCH700S-37°C. Intracellular killing was not affected by outer membrane preparations derived from the plasmidless strain, except at 120 min of incubation.

Phagocytosis of *Y. enterocolitica* in vivo. *Y. enterocolitica* strains were inoculated intradermally on the backs of rabbits, and biopsy specimens were obtained at 12 h postinoculation, at which time the area inoculated with 10^9 viable bacteria of strains MCH700S and MCH700L showed some redness. Sections of the biopsy specimens from both groups showed an established acute inflammatory response characterized by the presence of numerous PMNs (Fig. 4A and B). In rabbits inoculated with strain MCH700L, nearly all the bacteria were found in PMNs or mononuclear cells (Fig. 4B). By contrast, in sections taken from rabbits injected with strain MCH700S, most of the bacteria were found extracellularly in small colonies or in chains surrounded by or interspersed with inflammatory cells (Fig. 4A). These findings were confirmed by electron microscopy. Strain MCH700L was mostly found within phagocytic vacuoles (Fig. 5B), while most bacteria of strain MCH700S were not phagocytized but were seen extracellularly mixed with some fibrinous exudate (Fig. 5A).

DISCUSSION

Results of our previous studies have shown that luminol-enhanced CL responses of human PMNs are suppressed in a dose-related fashion by outer membrane fragments derived from plasmid-bearing *Y. enterocolitica* cells, suggesting that plasmid-mediated cell surface components play an antiphagocytic role (18). Because CL response is only an indirect measure of phagocytic activities (2, 10), further studies were desirable to obtain more direct evidence. However, some technical problems exist in quantitative phagocytosis assays involving *Y. enterocolitica*. The first problem is that plasmid-bearing cells tend to autoagglutinate at 37°C (16); autoagglutinated bacteria would be difficult to quantitate by either viable counts or microscopic examinations. The second problem is that plasmid-bearing cells grown at 37°C have increased surface charge and hydrophobicity (14); these altered surface properties may be associated with increased adherence with PMNs, and therefore, the accuracy of quan-

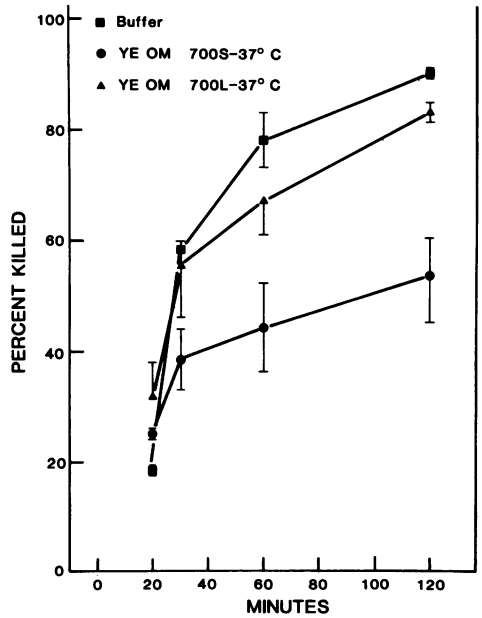

FIG. 3. Effect of outer membrane (OM) fragments from *Y. enterocolitica* on intracellular killing of *E. coli* by human PMNs. PMNs were incubated with *E. coli* in the presence or absence (buffer) of outer membrane fragments (1.0 mg/ml) derived from *Y. enterocolitica* MCH700S-37°C or MCH700L-37°C. Each point represents the mean ± standard deviation of three separate experiments.

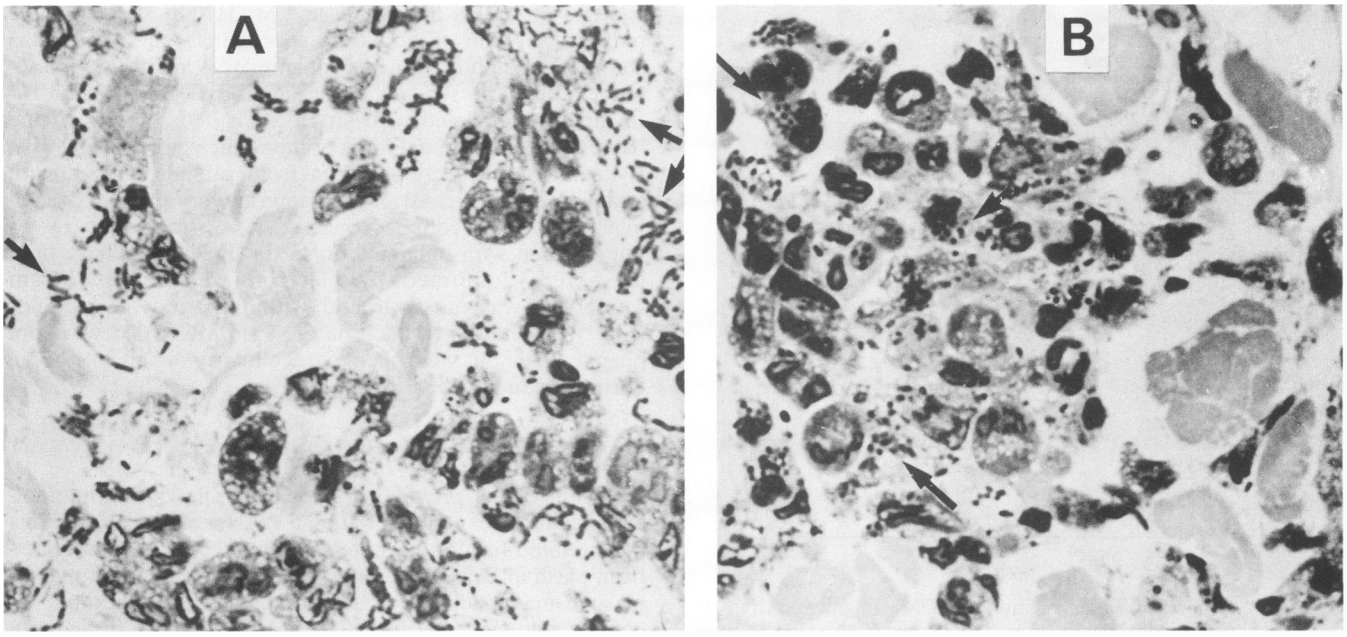

FIG. 4. Light micrograph of skin biopsies taken 12 h after intradermal inoculation of *Y. enterocolitica* cells on the backs of rabbits. (A) Strain MCH700S. Note the presence of inflammatory cells consisting of neutrophils and macrophages. Bacteria are mostly extracellular (arrows). (B) Strain MCH700L. Note the few extracellular bacteria. Large number of bacteria are located within neutrophils and macrophages (arrows). Hematoxylin and eosin stains were used. Magnification, ×1,250.

titative phagocytosis assays would depend on the effective differentiation of ingested and adherent bacteria. The third problem is that, except for plasmid-bearing cells grown at 37°C, *Y. enterocolitica* is extremely susceptible to the bactericidal action of NHS, which is required for opsonization (24, 25); intracellular killing by PMNs could not be assessed with serum-susceptible strains. To avoid these problems, we used a serum-resistant strain of *E. coli* as a test strain and examined the effect of outer membrane preparations derived from *Y. enterocolitica* on the phagocytosis of the test strain rather than *Y. enterocolitica* itself. The use of the acridine orange fluorescence method combined with a crystal violet staining method (11, 22, 33) allowed the differentiation of ingested bacteria from adherent ones; it also allowed a differential count of viable and nonviable bacteria.

The data presented in this study show that plasmid-mediated cell surface components render *Y. enterocolitica* cells resistant to phagocytosis by human PMNs (Tables 1 and 2). We were unable to document the fate of *Y. enterocolitica* cells within PMNs because of the sensitivity to serum and clumping of this organism. However, the antiphagocytic effect of the plasmid-mediated cell surface components was readily demonstrable when an *E. coli* strain was used as a test strain (Table 4 and Fig. 2 and 3); both ingestion and intracellular killing of *E. coli* were inhibited in the presence of an outer membrane preparation derived from plasmid-bearing *Y. enterocolitica* cells. Furthermore, that a plasmid-bearing strain of *Y. enterocolitica* is indeed resistant to phagocytosis in vivo is indicated by histological examinations of inflammatory lesions in rabbit skin induced by intradermal inoculation of the organism (Fig. 4 and 5). Numerous bacteria of the plasmidless strain were seen intracellularly in vacuoles of PMNs and mononuclear cells. In contrast, plasmid-bearing bacteria were surrounded by inflammatory cells without being phagocytized. Thus, this is the first study that presents evidence, both in vitro and in vivo, that plasmid-mediated cell surface components act as antiphagocytic factors, although such a role has been speculated before (27).

Cell surface components such as capsules, staphylococcal protein A, and the M protein of group A streptococci are known to contribute to the ability of bacterial cells to evade phagocytosis (12, 21, 26, 36). Although these surface structures are encoded by chromosomal genes, a recent report (1) testifies to the importance of plasmid-encoded surface properties in bacterial resistance to phagocytosis. *E. coli* plasmid R6-5 carries a gene that directs the synthesis of a highly exposed outer membrane protein, TraT (1). This protein has been shown to mediate resistance to both serum- and phagocyte-dependent killing. The antiphagocytic function of these surface structures appears to be related to their interference with opsonization by NHS (1, 12, 36).

In this study we did not attempt to identify the plasmid-mediated cell surface components that have an antiphagocytic function. We have shown previously (18) that the CL-depressing factor, which has now been shown to be identical to the antiphagocytic factor, is expressed rapidly when cultures of plasmid-bearing *Y. enterocolitica* are shifted from 25 to 37°C, with the timing of expression approximately coinciding with the appearance of plasmid-encoded outer membrane proteins. The presence of antiphagocytic activity in outer membrane preparations (Table 4 and Fig. 2 and 3) and the almost complete abolishment of the activity by pronase treatment of intact cells (Table 2) strongly suggest that plasmid-mediated, surface-exposed proteins, such as plasmid-encoded outer membrane proteins (4, 7, 20, 28, 29) or fibril structure (15), are the most probable candidates for the active components. Two other gene products described so far to be associated with the virulence plasmid are the V and W antigens (5). However, the V antigen is located specifically in the cytoplasm (34); the W antigen has been purified from spent culture medium (17), but its location is unknown. Although the structure of the lipopolysaccharide of *Y. enterocolitica* appears to be regu-

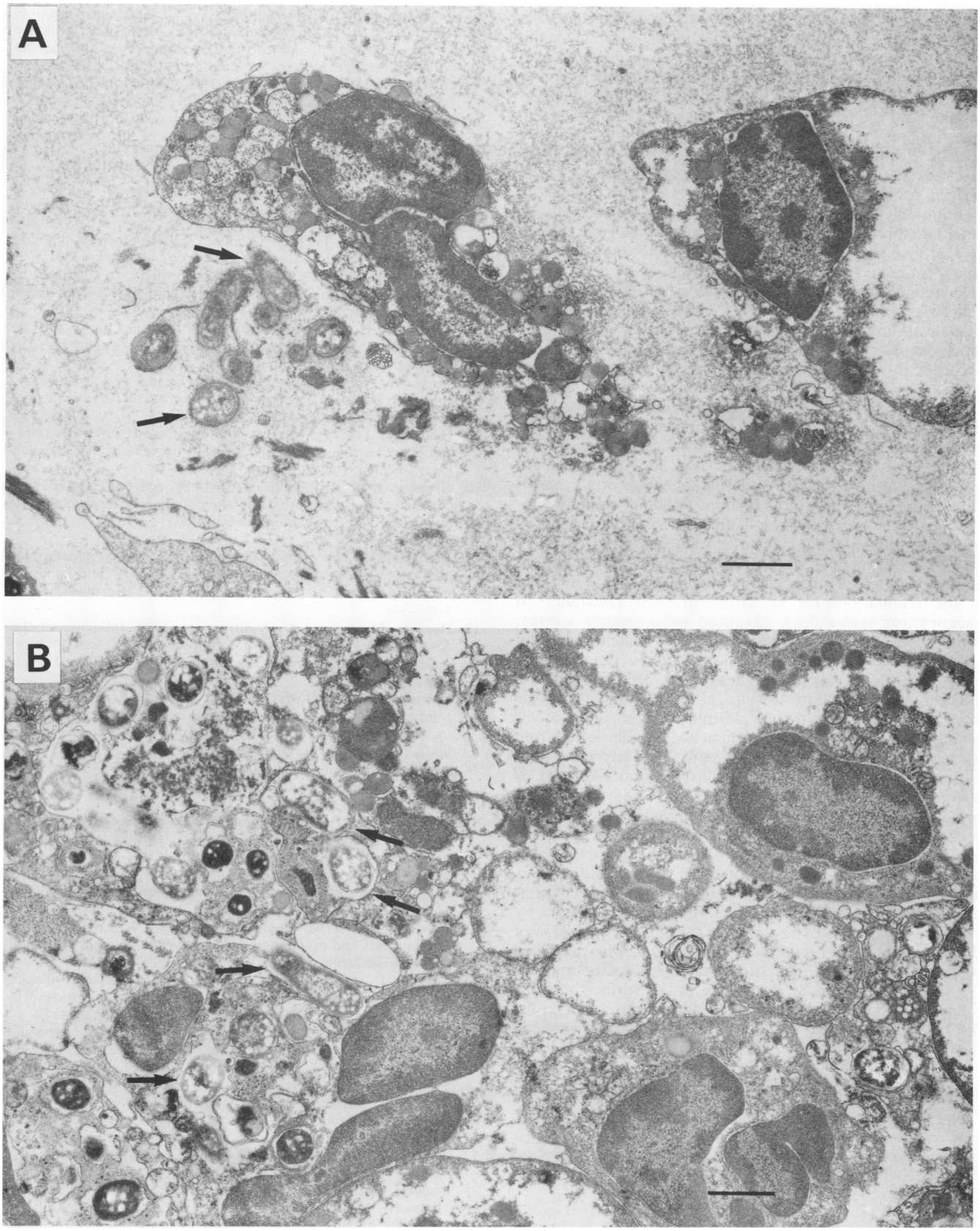

FIG. 5. Electron micrograph of skin biopsies taken 12 h after intradermal inoculation of *Y. enterocolitica* cells on the backs of rabbits. (A) Strain MCH700S. Bacteria are in the proximity of neutrophils but are not phagocytized (arrows). (B) Strain MCH700L. Note the bacteria within phagocytic vacuoles in neutrophils (arrows). Magnification, ×12,000; bar, 1 μm.

lated by growth temperature, irrespective of plasmid carriage (13, 23, 27), its possible role in the antiphagocytic function of the plasmid-mediated surface components cannot be eliminated completely. The outer membrane preparations used in this study undoubtedly contained lipopolysaccharide, and the activity of the pronase preparation used in this study may not be highly specific because it is not a pure protein. Furthermore, as was pointed out by Portnoy and Martinez (27), it is possible that a plasmid-encoded cell surface protein may be able to exert an antiphagocytic function only when the novel protein can be inserted into an outer membrane environment containing lipopolysaccharide of a proper structure. Identification of the antiphagocytic components must await genetic analysis.

Results of experiments to be reported elsewhere indicate that *Y. enterocolitica* lacking the virulence plasmid is also capable of invading the intestinal mucosa of rabbits, although clinical disease and advanced histological lesions are produced by plasmid-bearing strains only. These findings are consistent with the in vitro observation that the ability of *Y. enterocolitica* to invade tissue culture cells is independent of the virulence plasmid (24, 28, 31) and support the results presented in recent reports by Bakour et al. (3) and Robins-Browne et al. (30), which suggest that plasmidless strains are also invasive in vivo. Thus, the pathogenesis of *Y. enterocolitica* infections may be as follows. Food or water contaminated with *Y. enterocolitica* is ingested, and the organisms penetrate the epithelial layer of the intestinal mucosa. The invading organisms with expressed plasmid-mediated, antiphagocytic cell surface components are able to multiply in the lamina propria by virtue of their resistance to phagocytosis and induce advanced inflammatory lesions, while *Y. enterocolitica* cells lacking the virulence plasmid are eradicated by phagocytosis. The precise time that the plasmid-mediated cell surface components are synthesized is not known at present. Results of recent studies in our laboratory have shown that plasmid-encoded outer membrane proteins are fully expressed in bacteria harvested from the lumen of the small intestine of rabbits as early as 6 h after peroral challenge (C. H. Pai, unpublished data). Skurnik (32) has reported that the plasmid-encoded outer membrane proteins are synthesized by *Y. enterocolitica* grown in semipermeable capsules placed in the peritoneal cavity of guinea pigs. These observations suggest that the expression of plasmid-encoded determinants by *Y. enterocolitica* does not necessarily require the mammalian intracellular environment in vivo, which is low in free Ca^{2+}, as speculated by Brubaker (6). The precise time and the site of expression of the plasmid-encoded determinants in vivo can be examined by using plasmid-encoded surface antigen-specific antisera (8) to probe periodically the nature of the surface antigens following peroral challenge. This information is crucial for the elucidation of the pathogenic role of plasmid-encoded determinants.

ACKNOWLEDGMENTS

We thank Catherine Scott and Ibolya Gedeon for technical assistance and Shirley Eikerman for secretarial assistance.

This study was supported by grant MA7108 from the Medical Research Council of Canada. C.-J.L. was a recipient of an Alberta Heritage Foundation for Medical Research Studentship.

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