INFECTION AND IMMUNITY, July 1985, p. 145–151
0019-9567/85/070145-07$02.00/0

Vol. 49, No. 1

Inhibition of Human Neutrophil Chemiluminescence by Plasmid-Mediated Outer Membrane Proteins of *Yersinia enterocolitica*

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Received 3 December 1984/Accepted 27 March 1985

Recent studies have shown that the cell surface properties of *Yersinia enterocolitica* are altered by the presence of the virulence plasmid, which mediates temperature-inducible outer membrane proteins (OMP). We investigated the interaction of *Y. enterocolitica* with human polymorphonuclear leukocytes by monitoring luminol-enhanced chemiluminescence (CL) responses. A plasmid-bearing strain grown at 37°C induced four- to sixfold less CL than did the same strain grown at 25°C or a plasmidless, isogenic strain grown at either temperature. Inhibition of CL responses by whole cells was related to plasmid-mediated expression of OMP. The OMP alone could inhibit the CL response of polymorphonuclear leukocytes stimulated by either opsonized zymosan or whole cells of *Y. enterocolitica*. Pronase treatment of whole cells, which removed the plasmid-mediated OMP, resulted in partial but significant elimination of CL inhibition by whole cells and by OMP derived from them. Incubation with *Y. enterocolitica* for 60 min did not affect the viability of polymorphonuclear leukocytes. Our results suggest that the interaction of *Y. enterocolitica* with human polymorphonuclear leukocytes is directly affected by the plasmid-mediated OMP.

Since the discovery of a 42- to 48-megadalton plasmid in *Yersinia enterocolitica* that is associated with virulence in experimental infections (18, 37, 45), attention has been focused on the characterization of plasmid-mediated determinants and their role in pathogenesis. The in vitro properties of plasmid-bearing strains include calcium dependence (18, 34, 36), V- and W-antigen production (12, 34), autoagglutination (28, 40), toxicity for tissue culture cells (37, 44), altered outer membrane proteins (OMP) (4, 13, 30, 37, 38), and serum resistance (32, 34). The plasmid-mediated properties are expressed at 37°C but not at 25°C. Interestingly, the previously known virulence properties of *Yersinia pestis* and *Yersinia pseudotuberculosis*, such as calcium dependence, V-antigen production, and autoagglutination, are also mediated by virulence plasmids, and a high degree of homology has been demonstrated among the virulence plasmids from the three species (2, 5, 10, 16, 19, 35, 36, 38, 40) (for a review, see reference 9). These observations suggest that similar mechanisms of pathogenicity may exist among the pathogenic species of *Yersinia*. However, the role of the plasmid-mediated determinants is not known.

The ability of *Y. enterocolitica* to invade the intestinal mucosa has been shown to be of primary importance in pathogenesis by histopathological studies of human cases (8) and experimental enteritis (11, 33, 42). After invasion, the organism can presumably resist the natural defenses of the host to establish infection in the Peyer's patches and lamina propria (11, 33, 42). Recent studies have demonstrated that altered cell surface properties, such as unique OMP (4, 13, 30, 37, 38, 40) and hydrophobic fibrillar structure (23, 26), are the characteristics of *Y. enterocolitica* which express plasmid-mediated determinants. Attachment to the intestinal epithelial cells or invasion of these cells or both may be promoted by these plasmid-mediated cell surface properties (22, 30, 44). Studies with other bacteria have shown that surface properties play an important role in their interaction with mammalian cells, including phagocytic cells (29, 43). In this report, we describe the role of plasmid-mediated OMP in the interaction of *Y. enterocolitica* with human neutrophils, as monitored by luminol-enhanced chemiluminescence (CL) responses.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The two isogenic strains of *Y. enterocolitica* serotype 0:3 used in this study (strains MCH700S and MCH700L) were derived from a strain that was originally isolated from a patient with diarrhea (32). These isogenic strains were isolated from the parent strain by using magnesium oxalate agar (32). Strain MCH700S exhibits calcium dependency and is virulent in rabbits and mice (32, 33). Strain MCH700L is calcium independent and avirulent. The strains were stored at −70°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with 20% glycerol.

Bacteria were inoculated from frozen stock cultures into 10% BHI broth and grown overnight at room temperature (25°C) in a rotary shaker (150 rpm). Fresh BHI broth was inoculated with a 10% inoculum, and the culture was then incubated at 25 or 37°C. Each culture was designated by the strain number followed by the growth temperature (e.g., cultures MCH700S-37°C and MCH700S-25°C). Bacterial cultures used for CL assays were pelleted by centrifugation at 10,000 × *g* for 10 min, washed twice with 0.1 M phosphate-buffered saline (PBS) (pH 7.2), and suspended in Hanks balanced salt solution (HBSS) (pH 7.2) (GIBCO Diagnostics, Madison, Wis.) lacking phenol red indicator but containing 0.1% gelatin.

Plasmid screening. Plasmids were analyzed by agarose gel electrophoresis, using the method of Portnoy et al. (37).

Preparation of human PMN. Human polymorphonuclear leukocytes (PMN) were prepared from pooled citrated pe-

ripheral blood obtained from 5 to 10 healthy individuals by a modification of the method of Boyum (6). Pooled blood was diluted 1:1 with PBS containing 0.1 mM glucose (PBS-glucose) and layered onto Ficoll-Paque (Pharmacia, Uppsala, Sweden) in 50-ml conical tubes. The tubes were centrifuged at 480 × *g* for 30 min at 15°C, after which the upper layers containing lymphocytes, monocytes, platelets, and serum were discarded. The bottom layer was mixed 1:1 with PBS-glucose containing 5% dextran (molecular weight, 500,000; Pharmacia) and 1.5% EDTA. After 60 min at room temperature, the upper phase was collected and washed twice with PBS-glucose. The remaining erythrocytes were removed by hypotonic lysis with a 0.84% NH_4Cl solution at 37°C for 10 min. The PMN were then pelleted by centrifugation at 480 × *g* for 5 min and washed three times with PBS-glucose. Final suspension of the PMN was in HBSS lacking phenol red indicator but containing 0.1% gelatin. Cell viability, as determined by trypan blue dye (0.2%) exclusion, was greater than 98%. PMN were counted with a hemacytometer and suspended in HBSS to a final concentration of 2×10^6 cells per ml.

Preparation of normal human serum for opsonization. Blood from at least five healthy individuals was allowed to clot for 1 h at room temperature. Pooled sera were divided into portions and frozen at −70°C. Frozen serum was thawed and used immediately.

Zymosan preparation. Zymosan A (Sigma Chemical Co., St. Louis, Mo.) suspended in PBS at a concentration of 20 mg/ml was sonicated with a model 1510 Sonicator (B. Braun Instrument Co., San Francisco, Calif.) until the suspension appeared homogeneous. This stock solution was stored at 4°C.

CL assay. Luminol-enhanced CL was measured with a luminometer (model 1251; LKB, Stockholm, Sweden) by using a modification of the procedure of Easmon et al. (15). To each cuvette were added 500 μl of bacterial suspension (5×10^7 CFU), 50 μl of normal human serum, 100 μl of luminol (5-amino-2,3-dihydro-1,4-phtalazine-dione; 10^{-5} M; Sigma), 50 μl of HBSS, and 500 μl of PMN (10^6 cells). Cuvettes containing the reaction mixture were placed immediately in the luminometer, which was preheated to 37°C. The CL response was measured automatically at 2-s intervals on each of eight cuvettes over a 90-min period, with a constant amount of agitation between measurements. In other experiments, the bacterial suspension was replaced by suspensions of OMP, zymosan A, OMP plus zymosan A, or OMP plus bacteria, and the amount of HBSS added was adjusted to a final volume of 1.2 ml in the reaction mixture.

Isolation of OMP and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. OMP were prepared by sucrose density gradient centrifugation, using a modification of the procedure of Hancock and Nikaido (21). Cells grown in 2 liters of BHI broth for 3 h were collected by centrifugation at 10,000 × *g* for 20 min at 4°C. The bacterial cells were washed in 30 mM Tris-hydrochloride (pH 8.0) and suspended in 15 ml of 20% (wt/vol) sucrose in 50 mM Tris-hydrochloride (pH 7.9). The cells were disrupted by sonication with a Braun model 1510 sonicator for 20 s four times in an ice bath. The cell debris was then removed by centrifugation at 3,000 × *g* for 10 min, and the supernatant was layered onto a sucrose step gradient comprised of 1 ml of 70% (wt/vol) sucrose, 2 ml of 64% (wt/vol) sucrose, 2 ml of 58% (wt/vol) sucrose, and 2 ml of 52% (wt/vol) sucrose in Tris buffer. The loaded gradients were centrifuged at 183,000 × *g* for 14 h in a Ti50 rotor by using a model L8-55 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The band in the 64% sucrose fraction was removed and diluted in 30 mM Tris buffer; this was followed by centrifugation at 177,000 × *g* for 1 h. The resulting pellets were washed once and finally suspended in a small volume of 0.1 M PBS (pH 7.2) and frozen at −70°C in portions.

In temperature shift experiments in which small volumes of cultures were used, OMP profiles were determined by using Sarkosyl extracts, as described by Bölin et al. (4). OMP preparations were solubilized by boiling for 4 min in 2% sodium dodecyl sulfate–12.5% glycerol–1% 2-mercaptoethanol–62.5 mM Tris-hydrochloride (pH 6.8). Samples containing 30 to 40 μg of proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the discontinuous buffer system of Laemmli (27), using 10% acrylamide and 0.27% bisacrylamide in the running gel. The protein content of each sample was determined by the Bradford method (7), using protein assay reagents (Bio-Rad Laboratories, Richmond, Calif.) and bovine serum albumin as the standard. Molecular weight markers and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories; the markers used were myosin (molecular weight, 200,000), β-galactosidase (116,000), phosphorylase B (92,500), bovine serum albumin (66,200), and ovalbumin (45,000). Proteins were fixed by immersing the gel for 20 min in a mixture containing 20% methanol and 10% trichloroacetic acid. The protein bands were then stained for 30 min at 56°C with 0.25% Coomassie brilliant blue R250 in 25% methanol–10% acetic acid. Destaining was carried out in a mixture containing 10% methanol and 10% acetic acid, with several changes. The staining intensity of the OMP profile was recorded with a scanning densitometer (Bromma-220Z Ultrascan laser densitometer; LKB Stockholm, Sweden).

Pronase treatment of whole cells. Bacterial cells were treated with pronase (protease type XIV; Sigma) by the method of Martinez (30). Washed cultures of *Y. enterocolitica* were suspended to one-tenth of their original volume in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2) supplemented with 5 mM Ca^{2+}. These cultures were then incubated with pronase (1 mg/ml) for 30 min at 37°C. Control bacterial cultures were suspended in the same buffer but without pronase. Bovine serum albumin (0.1%; Sigma) was added to the incubation mixtures to quench the reaction. The cultures were then pelleted by centrifugation at 10,000 × *g* for 10 min and washed three times with PBS.

PMN viability. PMN (8.3×10^5 cells per ml) and bacteria (4.2×10^7 CFU/ml) were incubated at 37°C, and samples were examined for PMN viability at 0, 30, and 60 min by using the trypan blue exclusion technique. A total of 140 to 210 cells were counted in each sample.

RESULTS

Plasmid profile. Strain MCH700S contained plasmid DNA with a molecular weight of approximately 42×10^6, whereas such a plasmid was lacking in strain MCH700L (Fig. 1). The presence of a plasmid in strain MCH700S was consistent with the following previously reported properties of this strain: calcium dependence, autoagglutination, serum resistance, and virulence in mice and rabbits (32, 33).

CL response to whole cells of *Y. enterocolitica*. Strains MCH700S and MCH700L were grown at either 25 or 37°C, and the luminol-enhanced CL responses of human PMN to these strains were examined (Fig. 2). A significant response was observed when PMN were incubated with culture

MCH700S-25°C, MCH700L-37°C, or MCH700L-25°C in the presence of 4.2% normal human serum. No CL response was generated in the absence of serum (data not shown). The CL response to culture MCH700S-37°C was four- to sixfold lower than the responses observed for the other cultures. This suggested that the depression of the PMN CL response was mediated by a plasmid-mediated determinant(s), since temperature inducibility is one of the characteristics of plasmid-mediated properties in *Y. enterocolitica* (4, 13, 30, 37).

The temperature inducibility of the CL-depressing property in the plasmid-bearing strain was further demonstrated in temperature shift experiments. Strain MCH700S and MCH700L cultures growing logarithmically at 25°C were shifted to 37°C, and at different times after the temperature shift the cultures were examined for the ability to stimulate CL. Control cultures were also examined without a temperature shift. Figure 3 shows that the CL response of PMN was depressed significantly by strain MCH700S within 30 min after the temperature shift to 37°C, indicating that the factor(s) associated with CL depression was expressed rapidly at 37°C. The temperature shift had no effect on strain MCH700L.

Effect of temperature shift on expression of plasmid-mediated OMP. The fact that the surface properties of *Y. enterocolitica* are altered by the presence of plasmid-mediated OMP (4, 30, 37) and the fact that the CL response of PMN can be affected by the surface properties of stimulants (24, 25, 31) suggested that the plasmid-mediated OMP might be responsible, at least in part, for the depression of the CL response. Figure 4 shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of the OMP of strain MCH700S after a temperature shift with time, which indicated that the expression of the CL-depressing factor(s) after the temperature shift coincided approximately with the appearance of an OMP with a molecular weight of about 200,000, similar to protein I of Bölin et al. (4). A parallel experiment with the plasmidless strain revealed no such changes in the outer membrane profile (data not shown).

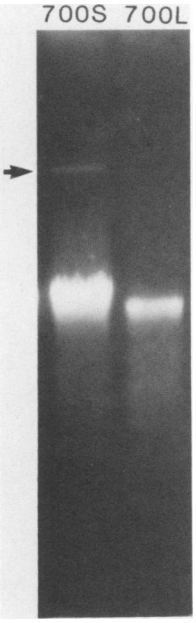

FIG. 1. Agarose gel electrophoresis of plasmid DNA isolated from *Y. enterocolitica* strains MCH700S (700S) and MCH700L (700L).

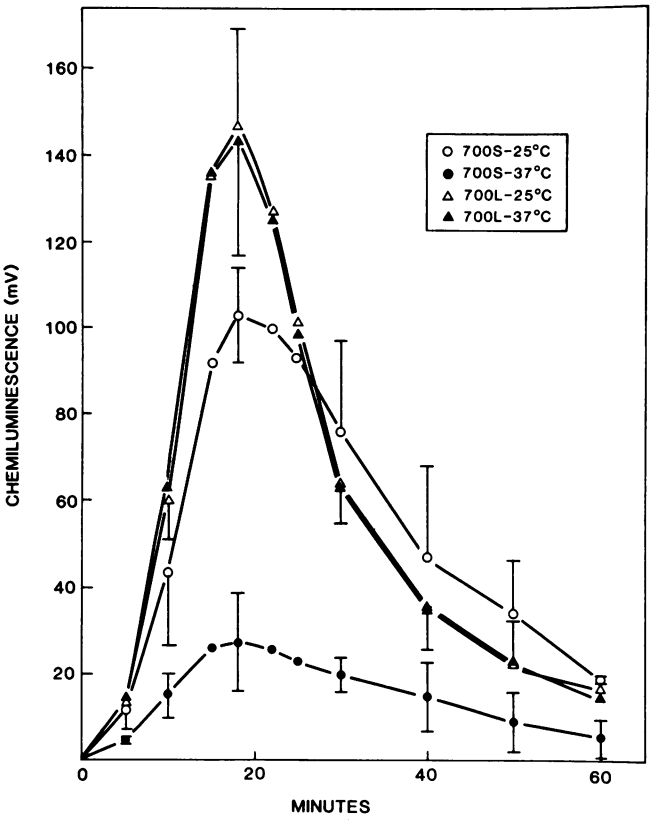

FIG. 2. CL responses of human PMN to *Y. enterocolitica* strains. Strains MCH700S and MCH700L were grown for 3 h at 25 or 37°C. The reaction mixtures contained PMN (8.3×10^5 cells per ml), bacteria (4.2×10^7 CFU/ml), pooled normal human serum (4.2%), and luminol (8.3×10^{-7} M). Each point represents the mean of CL responses ± standard deviation from four separate experiments.

Effect of plasmid-mediated OMP on the CL response. To test the possibility that the plasmid-mediated OMP were responsible for the observed depression of the CL response by culture MCH700S-37°C, we examined the CL responses of PMN to OMP isolated from *Y. enterocolitica* strains grown at two different temperatures (Fig. 5). OMP derived from the plasmidless strain or from the plasmid-bearing strain grown at 25°C stimulated the CL response, whereas the CL response was not observed with the OMP from the plasmid-bearing cells grown at 37°C. The inhibitory effect of the plasmid-mediated OMP on the CL response was further demonstrated in experiments (Fig. 6) in which isolated OMP were added to reaction mixtures containing PMN and opsonized zymosan. Opsonized zymosan at a concentration of 0.5 mg/ml elicited a CL response very similar to that observed for whole cells of the plasmidless strain of *Y. enterocolitica* (Fig. 2). Addition of OMP derived from strain MCH700L or culture MCH700S-25°C resulted in a slight but insignificant inhibition of the zymosan-induced CL response. However, OMP from culture MCH700S-37°C inhibited the CL response significantly (Fig. 6). Furthermore, this inhibition was dose related (Table 1). A similar pattern of CL inhibition was observed when the OMP preparation was incubated with PMN in the presence of whole cells from culture MCH700S-25°C or strain MCH700L (data not shown).

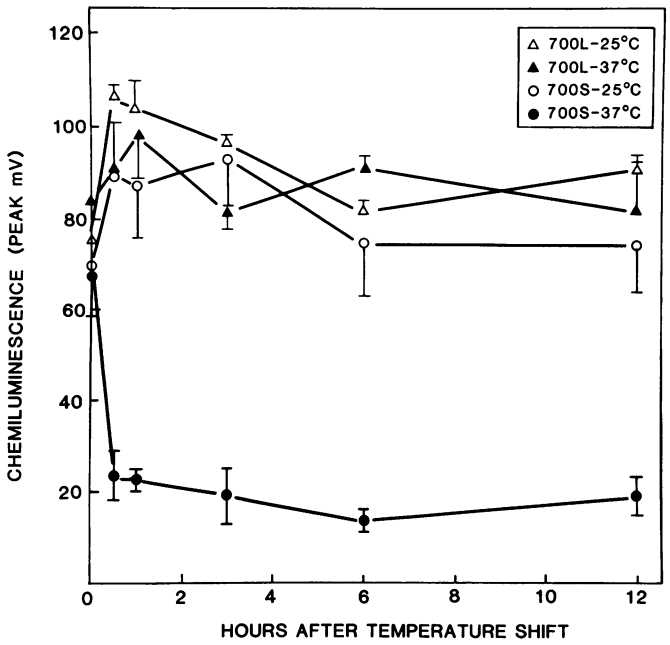

FIG. 3. CL responses of human PMN to *Y. enterocolitica* strains after shift of growth temperature. Two flasks containing fresh BHI broth were inoculated with a 10% inoculum of an overnight culture grown at 25°C. One culture flask was maintained at 25°C, and the other was incubated at 37°C. At the times indicated, a portion of each culture was removed and washed, and PMN were challenged for CL responses to these washed cultures. The peak height of the CL response of each determination is shown. Each point represents the mean peak height of the CL response ± the standard deviation of three separate experiments. For the contents of the reaction mixtures, see the legend to Fig. 2.

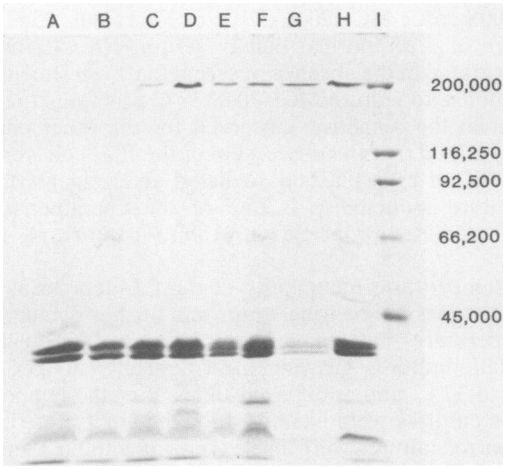

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *Y. enterocolitica* MCH700S OMP. A culture grown overnight at 25°C was inoculated into flasks containing fresh BHI broth prewarmed to 37°C. The flasks were incubated at 37°C. At timed intervals, the cultures were removed and analyzed for OMP. OMP were extracted by using Sarkosyl. Lane A, Overnight culture grown at 25°C; lane B, culture grown for 30 min at 37°C; lane C, culture grown for 1 h at 37°C; lane D, culture grown for 2 h at 37°C; lane E culture grown for 3 h at 37°C; lane F, culture grown for 6 H at 37°C; lane G, culture grown for 12 h at 37°C; lane H, culture grown for 24 h at 37°C; lane I, molecular weight standards.

Effect of pronase treatment. It has been shown that plasmid-mediated OMP are exposed to the cell surface and can be removed by treatment with proteases (30). Removal of the OMP is accompanied by alteration in serum resistance and cell surface hydrophobicity, which are characteristics of plasmid-bearing cells. These observations led us to examine the effect of pronase treatment on the CL response, which was altered by plasmid-mediated OMP. As Fig. 7 shows, the plasmid-mediated OMP were not detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after pronase treatment. The susceptibility of the OMP to pronase was further confirmed by a densitometer scan (data not shown).

The peak heights of the CL response to whole cells of culture MCH700S-37°C were elevated significantly ($P < 0.001$) after pronase treatment (Table 2). No difference was observed between the CL responses to pronase-treated and nontreated cells of culture MCH700S-25°C or MCH700L-37°C. These data further suggested that the plasmid-mediated OMP were involved in the inhibition of the CL response by PMN, although the inhibition by culture MCH700S-37°C was not completely eliminated by pronase treatment. The incomplete elimination of CL inhibition could have been due to the presence of residual OMP not removed by the pronase treatment. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique may not have been sensitive enough to detect a small amount of protein.

PMN were challenged with zymosan together with OMP isolated from whole cells after pronase treatment (Table 3). Inhibition of zymosan-induced CL by OMP derived from culture MCH700S-37°C decreased significantly ($P < 0.02$) after pronase treatment. However, as with whole cells, the CL response to the OMP of pronase-treated culture MCH700S-37°C was still not as high as the CL responses observed for the OMP derived from other cultures.

Effect of *Y. enterocolitica* on viability of PMN. Previous studies showed that plasmid-bearing strains of *Y. enterocolitica* were cytotoxic to tissue culture cells (37, 44). To test the possibility that inhibition of the CL response by

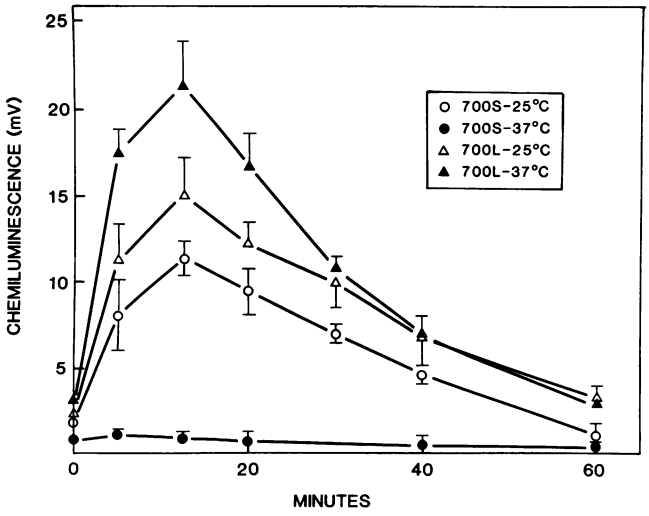

FIG. 5. CL responses of human PMN to OMP derived from *Y. enterocolitica* strains. OMP were prepared by sucrose density gradient centrifugation from *Y. enterocolitica* strains grown for 3 h at 25 or 37°C. The reaction mixtures contained 500 μg of OMP per ml. Each point represents the mean ± standard deviation from two separate experiments.

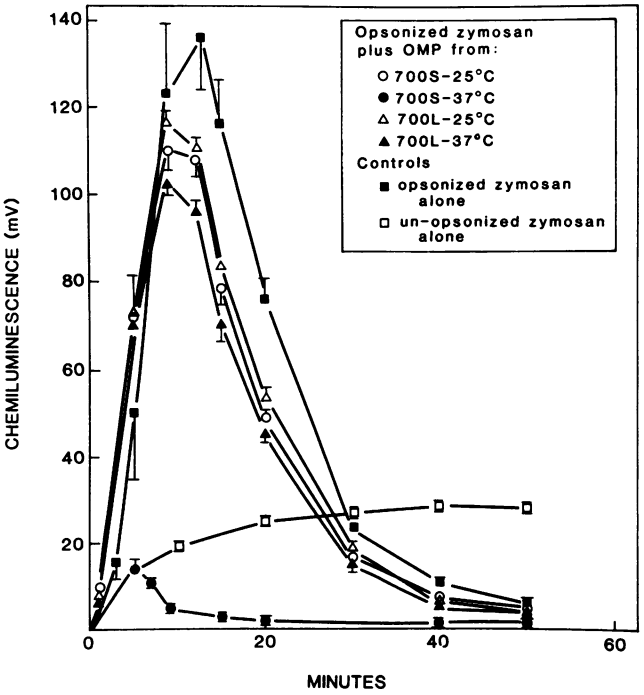

FIG. 6. Effects of OMP derived from *Y. enterocolitica* on zymosan-induced CL responses. OMP were prepared by sucrose density gradient centrifugation from cultures grown for 3 h at 25 or 37°C. The reaction mixtures contained OMP (1,000 μg/ml), PMN (8.3×10^5 cells per ml), pooled normal human serum (4.2%), luminol (8.3×10^{-7} M), and zymosan A (0.5 mg/ml). Each point represents the mean ± standard deviation from two separate experiments.

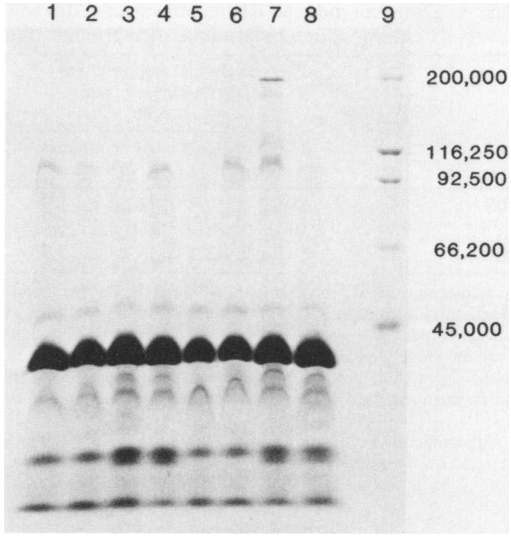

FIG. 7. Effect of pronase treatment on the OMP of *Y. enterocolitica* strains. Strains MCH700S and MCH700L grown at 25 or 37°C for 3 h were washed and incubated with pronase (1 mg/ml) for 30 min at 37°C. OMP were prepared from pronase-treated and nontreated cell suspensions by sucrose density centrifugation. Lane 1, Culture MCH700L-25°C control; lane 2, culture MCH700L-25°C, pronase treated; lane 3, culture MCH700L-37°C control; lane 4, culture MCH700L-37°C, pronase treated; lane 5, culture MCH700S-25°C control; lane 6, culture MCH700S-25°C, pronase treated; lane 7, culture MCH700S-37°C control; lane 8, culture MCH700S-37°C, pronase treated; lane 9, molecular weight standards.

culture MCH700S-37°C might have been due to its cytotoxicity to PMN, we examined the viability of PMN incubated with culture MCH700S-37°C or MCH700L-37°C by using the trypan blue exclusion technique. There was no significant change in the viability of PMN up to 1 h of incubation; the levels of viable PMN after 1 h of incubation were 94 and 95% with cultures MCH700S-37°C and MCH700L-37°C, respectively.

DISCUSSION

In this study, we found that the luminol-enhanced CL response of human PMN is inhibited by the plasmid-mediated OMP of *Y. enterocolitica*. This conclusion is based on the following observations: (i) the CL response to *Y. enterocolitica* expressing the plasmid-mediated OMP was much lower than the CL response observed with cells lacking the plasmid-mediated OMP; (ii) the CL response of zymosan-stimulated PMN was inhibited in a dose-related fashion by OMP derived from *Y. enterocolitica* expressing plasmid-mediated properties; and (iii) pronase treatment of whole cells, which was shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to remove the plasmid-mediated OMP, resulted in partial but significant elimination of CL inhibition by whole cells and by the OMP derived from them. The inhibition of the CL response was demonstrated under conditions which did not affect PMN viability.

The incomplete elimination of CL inhibition after pronase treatment (Tables 2 and 3) may have been due to the presence of residual OMP. The plasmid-mediated OMP of *Y. pseudotuberculosis* (4) and *Y. enterocolitica* (C. H. Pai, G. Meyers, and D. E. Woods, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, abstr. no. 783, 1983) have been shown by sodium dodecyl sulfate-polyacrylamide

TABLE 1. Dose-related inhibition by OMP of the PMN CL response to opsonized zymosan[a]

OMP concn (μg/ml)	Peak-height CL response (mV)	Inhibition
0	106.7 ± 21.4[b]	
50	106.3 ± 23.4	0.4
100	84.1 ± 4.5	21.2
500	39.9 ± 6.6	62.6
1,000	24.8 ± 9.6	76.8

[a] PMN were challenged with increasing concentrations of OMP derived from culture MCH700S-37°C and opsonized zymosan.
[b] Data are means ± standard deviations of two to four experiments.

TABLE 2. Effect of pronase treatment on the CL response to whole cells of *Y. enterocolitica*[a]

Strain	Growth temp (°C)	Peak-height CL response (mV)[b]		*P* value[c]
		Before pronase treatment	After pronase treatment	
MCH700S	37	29.4 ± 20.0	59.8 ± 19.9	<0.001
	25	126.6 ± 61.8	123.4 ± 61.5	NS[d]
MCH700L	37	117.9 ± 61.6	117.4 ± 54.9	NS

[a] For reaction mixtures, see the legend to Fig. 2.
[b] Data are means ± standard deviations of eight experiments in which each strain (with or without pronase treatment) was tested in pairs.
[c] As determined by Student's *t* test with paired values and a two-tailed hypothesis.
[d] NS, Not significant.

TABLE 3. Zymosan-induced CL responses of PMN to OMP derived from *Y. enterocolitica* before and after pronase treatment[a]

Strain	Growth temp (°C)	Peak-height CL response (mV) to OMP prepared from whole cells[b]		*P* value[c]
		Before pronase treatment	After pronase treatment	
MCH700S	37	80.7 ± 19.4	122.0 ± 23.2	<0.02
	25	160.6 ± 26.9	160.0 ± 21.9	NS[d]
MCH700L	37	183.4 ± 42.3	152.8 ± 37.7	NS

[a] Outer membranes were prepared from whole cells that had been incubated with pronase (1 mg/ml) at 37°C for 30 min. Reaction mixtures contained 1,000 μg of OMP per ml.

[b] Data are means ± standard deviations of five experiments in which OMP from whole cells (with or without pronase treatment) were tested in pairs.

[c] As determined Student's *t* test with paired values and a two-tailed hypothesis.

[d] NS, Not significant.

gel electrophoresis followed by autoradiography to be expressed within minutes of a temperature shift. However, in the present study the plasmid-mediated OMP were not detected by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles until 60 min after the temperature shift, suggesting that sodium dodecyl sulfate-polyacrylamide gel electrophoresis was not sensitive enough to detect a small amount of protein. The insensitivity of sodium dodecyl sulfate-polyacrylamide gel electrophoresis may also account for a slight delay in the expression of the plasmid-mediated OMP after the temperature shift (Fig. 4) compared with the expression of CL-depressing factor(s) (Fig. 3).

To our knowledge, this study showed for the first time that interactions of *Y. enterocolitica* with phagocytic cells are altered by the presence of the plasmid-mediated OMP. The CL response is a sensitive, indirect measure of the degree of phagocytosis in human neutrophils (1, 20) and has been used to demonstrate effects of bacterial cell surface structures, such as capsule, protein A, lipopolysaccharide "O" chain, Vi antigens, and unidentified surface components, on the phagocytic activities of PMN (24, 25, 31). Inhibition of CL responses, as demonstrated in the present study, may be interpreted as due to the anti-phagocytic role of the plasmid-mediated OMP. Since normal human serum is required for a PMN CL response to occur, we may speculate that the OMP interfere with C3b receptor recognition or with signal transmission after the C3b-receptor complex has been formed.

Recently, Lachica et al. (26) described a hydrophobic fibrillar surface structure of *Y. enterocolitica* that is associated with the virulence plasmid. These authors suggested that the fibrillar structure may promote the attachment of the bacteria to the intestinal epithelial cells and to phagocytes. In describing nonflagellar surface appendages in *Y. enterocolitica* and *Y. pseudotuberculosis*, Kapperud et al. (23) presented evidence suggesting that the plasmid-mediated OMP which has a molecular weight of ca. 180×10^3 may be related to the surface fibrillae. It is possible that the plasmid-mediated OMP described in this study and the fibrillar structure of Lachica et al. (26) and Kapperud et al. (23) are the same outer membrane component. Certain types of fimbriae and fibril proteins have been shown to promote attachment to epithelial cells or resistance to phagocytosis or both. Some examples are fimbriae of gonococci (14), the M protein of streptococci (17), and the CFA/I fimbriae of *Escherichia coli* (3). Purification of the fibrillar structure of *Y. enterocolitica* may allow identification of the factor associated with CL inhibition.

The presence of virulence plasmids in *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* and the high degree of homology demonstrated among these plasmids suggest that similar mechanisms of pathogenicity may exist in all three species of *Yersinia* (2, 5, 16, 19, 38–40). However, the observation that plasmid-mediated OMP are expressed in *Y. enterocolitica* and *Y. pseudotuberculosis* (4, 30, 37, 38) but not in *Y. pestis* (38, 41) indicates that *Y. pestis* possesses virulence-associated surface properties different from those of the other two species. This is further supported by the finding that *Y. pestis* is constitutively resistant to the bactericidal action of normal human serum, whereas *Y. enterocolitica* is extremely sensitive unless the plasmid-mediated OMP are expressed (30, 32, 34). Thus, the inhibition of the CL response, or possibly of phagocytosis, by plasmid-mediated OMP may not be common to all *Yersinia* species. However, the plasmid DNA from *Y. pestis* has been shown by *E. coli* minicell analysis to contain the coding capacity for production of OMP which are immunologically related to the OMP produced by *Y. enterocolitica* (38). Furthermore, *Y. pestis* cultivated in vivo has been shown to express plasmid-mediated polypeptides that are recognized by absorbed antisera to *Y. enterocolitica* (39), although it is not known whether the polypeptides are located on the cell surface. Thus, the possibility that inhibition of the CL response by OMP or another plasmid-mediated surface structure that is common to all pathogenic species of *Yersinia* cannot be ruled out completely.

In conclusion, the CL response of human PMN is inhibited by a virulent strain of *Y. enterocolitica*, and this inhibition appears to be mediated by the plasmid-encoded OMP. These observations suggest a possible antiphagocytic role of the OMP in the pathogenesis of *Y. enterocolitica*. Further studies will be required for resolution of the basis for inhibition of CL responses.

ACKNOWLEDGMENTS

We thank Gwyneth Meyers for providing an outer membrane preparation, Brenda Croucher for collecting blood samples, Allan Godfrey for technical assistance in the use of the densitomer scanner, and Don Woods for a critical review of the manuscript. Secretarial assistance was provided by Shirley Eikerman.

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

LITERATURE CITED

1. **Allen, R. C., R. L. Stjernholm, and R. L. Steel.** 1972. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. **47**:679–684.
2. **Ben-Gurion, R., and A. Shafferman.** 1981. Essential virulence determinants of different *Yersinia* species are carried on a common plasmid. Plasmid **5**:183–187.
3. **Björkstein, B., and T. Wadström.** 1982. Interaction of *Escherichia coli* with different fimbriae and polymorphonuclear leukocytes. Infect. Immun. **38**:298–305.
4. **Bölin, I., L. Norlander, and H. Wolf-Watz.** 1982. Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. Infect. Immun. **37**:506–512.
5. **Bölin, I., and H. Wolf-Watz.** 1984. Molecular cloning of temperature-inducible outer membrane protein 1 of *Yersinia pseudotuberculosis*. Infect. Immun. **43**:72–78.
6. **Boyum, A.** 1968. Separation of leukocytes from blood and bone

marrow. Scand. J. Clin. Lab. Invest. **21**(Suppl. 97):77–89.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
8. **Bradford, W., P. S. Noce, and L. T. Gutman.** 1974. Pathologic features of enteric infection with *Yersinia enterocolitica*. Arch. Pathol. **98**:17–22.
9. **Brubaker, R. R.** 1972. The genus *Yersinia*: biochemistry and genetics of virulence. Curr. Top. Microbiol. Immunol. **57**: 111–158.
10. **Brubaker, R. R.** 1983. The Vwa^+ virulence factor of yersiniae: the molecular basis of the attendant nutritional requirement for Ca^{++}. Rev. Infect. Dis. **5**(Suppl. 4):748–758.
11. **Carter, P. B.** 1975. Pathogenicity of *Yersinia enterocolitica* for mice. Infect. Immun. **11**:164–170.
12. **Carter, P. B., R. J. Zahorchak, and R. R. Brubaker.** 1980. Plague virulence antigens from *Yersinia enterocolitica*. Infect. Immun. **28**:638–640.
13. **Chang, M. T., and M. P. Doyle.** 1984. Identification of specific outer membrane polypeptides associated with virulent *Yersinia enterocolitica*. Infect. Immun. **43**:472–476.
14. **Dilworth, J. A., J. O. Hendley, and G. L. Mandell.** 1975. Attachment and ingestion of gonococci by human neutrophils. Infect. Immun. **11**:512–516.
15. **Easmon, C. S. F., P. J. Cole, A. J. Williams, and M. Hastings.** 1980. The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence. Immunology **41**:67–74.
16. **Ferber, D. M., and R. R. Brubaker.** 1981. Plasmids in *Yersinia pestis*. Infect. Immun. **31**:839–841.
17. **Foley, M. J., and W. B. Wood, Jr.** 1959. Studies on the pathogenicity of group A streptococci. II. The antiphagocytic effects of the M protein and the capsular gel. J. Exp. Med. **110**:617–628.
18. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid-associated pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27**:682–685.
19. **Gemski, P., J. R. Lazere, T. Casey, and J. A. Wohlhieter.** 1980. Presence of a virulence-associated plasmid in *Yersinia pseudotuberculosis*. Infect. Immun. **28**:1044–1047.
20. **Grebner, J. V., E. L. Mills, B. H. Gray, and P. G. Quie.** 1977. Comparison of phagocytic and chemiluminescence response of human polymorphonuclear neutrophils. J. Lab. Clin. Med. **89**:153–159.
21. **Hancock, R. E. W., and H. Nikaido.** 1978. Outer membranes of gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and use in reconstitution and definition of the permeability barrier. J. Bacteriol. **136**:381–390.
22. **Heesemann, J., B. Algermissen, and R. Laufs.** 1984. Genetically manipulated virulence of *Yersinia enterocolitica*. Infect. Immun. **46**:105–110.
23. **Kapperud, G., E. Nawork, and H. Skarpeid.** 1985. Temperature-inducible surface fibrillae associated with the virulence plasmid of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. Infect. Immun. **47**:561–566.
24. **Kossack, R. E., R. L. Guerrant, P. Densen, J. Schadelin, and G. L. Mandell.** 1981. Diminished neutrophil oxidative metabolism after phagocytosis of virulent *Salmonella typhi*. Infect. Immun. **31**:674–678.
25. **Kreger, A., L. DeChatelet, and P. Shirley.** 1981. Interaction of *Vibrio vulnificus* with human polymorphonuclear leukocytes: association of virulence with resistance to phagocytosis. J. Infect. Dis. **144**:244–248.
26. **Lachica, R. V., D. L. Zink, and W. R. Ferris.** 1984. Association of fibril structure formation with cell surface properties of *Yersinia enterocolitica*. Infect. Immun. **46**:272–275.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
28. **Laird, W. J., and D. C. Cavanaugh.** 1980. Correlation of autoagglutination and virulence in yersiniae. J. Clin. Microbiol. **11**:430–432.
29. **Magnusson, K. E., J. Davies, T. Grundstrom, E. Kihlström, and S. Normark.** 1980. Surface charge and hydrophobicity of *Salmonella*, *E. coli* and gonococci in relation to their tendency to associate with animal cells. Scand. J. Infect. Dis. **24**:135–140.
30. **Martinez, R. J.** 1983. Plasmid-mediated and temperature-regulated surface properties of *Yersinia enterocolitica*. Infect. Immun. **41**:921–930.
31. **Musher, D. M., H. A. Verbrugh, and J. Verhoef.** 1981. Suppression of phagocytosis and chemotaxis by cell wall components of *Staphylococcus aureus*. J. Immunol. **127**:84–88.
32. **Pai, C. H., and L. deStephano.** 1982. Serum resistance associated with virulence in *Yersinia enterocolitica*. Infect. Immun. **35**:605–611.
33. **Pai, C. H., V. Mors, and T. A. Seemayer.** 1980. Experimental *Yersinia enterocolitica* enteritis in rabbits. Infect. Immun. **28**:238–244.
34. **Perry, R. D., and R. R. Brubaker.** 1983. Vwa^+ phenotype of *Yersinia enterocolitica*. Infect. Immun. **40**:166–171.
35. **Portnoy, D. A., H. F. Blank, D. T. Kingsbury, and S. Falkow.** 1983. Genetic analysis of essential plasmid determinants of pathogenicity in *Yersinia pestis*. J. Infect. Dis. **148**:297–304.
36. **Portnoy, D. A., and S. Falkow.** 1981. Virulence-associated plasmids from *Yersinia enterocolitica* and *Yersinia pestis*. J. Bacteriol. **148**:877–883.
37. **Portnoy, D. A., S. L. Moseley, and S. Falkow.** 1981. Characterization of plasmids and plasmid-associated determinants of *Yersinia enterocolitica* pathogenesis. Infect. Immun. **31**: 775–782.
38. **Portnoy, D. A., H. Wolf-Watz, I. Bölin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43**:108–114.
39. **Skurnik, M.** 1985. Expression of antigens encoded by virulence plasmid of *Yersinia enterocolitica* under different growth conditions. Infect. Immun. **47**:183–190.
40. **Skurnik, M., I. Bölin, H. Heikkinen, S. Piha, and H. Wolf-Watz.** 1984. Virulence plasmid-associated autoagglutination in *Yersinia* spp. J. Bacteriol. **158**:1033–1036.
41. **Straley, S. C., and R. R. Brubaker.** 1981. Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. Proc. Natl. Acad. Sci. U.S.A. **78**:1224–1228.
42. **Une, T.** 1977. Studies on the pathogenicity of *Yersinia enterocolitica*. I. Experimental infections in rabbits. Microbiol. Immunol. **21**:349–363.
43. **Van Oss, C. J.** 1978. Phagocytosis as a surface phenomenon. Annu. Rev. Microbiol. **32**:19–39.
44. **Vesikari, T., C. Sundqvist, and M. Mäki.** 1983. Adherence and toxicity of *Yersinia enterocolitica* 0:3 and 0:9 containing virulence-associated plasmids for various cultured cells. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **91**:121–127.
45. **Zink, D. L., J. C. Feeley, T. G. Wells, C. Vanderzant, J. C. Vickery, W. C. Roof, and G. A. O'Donovan.** 1980. Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*. Nature (London) **283**:224–226.