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Translocation of *Yersinia enterocolitica* through an Endothelial Monolayer by Polymorphonuclear Leukocytes

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An endothelial cell monolayer grown on a microporous membrane coated with basement membrane protein matrix was used to study translocation of yersinia-infected human polymorphonuclear leukocytes (PMNs). PMNs infected with one to eight bacteria were able to translocate living yersiniae from the upper chamber to the chemoattractant-containing lower chamber. This process may contribute to extravasation and dissemination of yersiniae in the infected host.

Yersinia enterocolitica is enteropathogenic for humans, causing a variety of clinical syndromes such as enterocolitis, mesenteric adenitis, terminal ileitis, reactive arthritis, and erythema nodosum (4, 8, 10). Ingestion of the pathogen leads to the formation of primary lesions in the mucosa of the lower small intestine. *Y. enterocolitica* probably transits the intestinal mucosa through M-cells and gains access to the underlying lymphoid tissue (e.g., Peyer's patches [PPs]), where the bacteria multiply and from which they may be disseminated throughout the body (9).

There is evidence that the invasion gene *inv* expressed by enteropathogenic *Yersinia* species is required for the initial entry process via M-cells (14). Surprisingly, an isogenic *inv* mutant was unable to invade PPs efficiently but was able to infect the liver and spleen, and finally the mice challenged with it succumbed. From these results, it was concluded that different invasion pathways can be used by yersiniae, one of which is probably the M-cell of the PPs. Another proposed mechanism for the translocation of intestinal bacteria is that microorganisms are phagocytosed by interepithelial leukocytes and subsequently carried to extraintestinal sites, where the phagocytes liberate viable bacteria (20). Bacterial translocation by transmigrating phagocytes as a vehicle may also be relevant for extravasation of phagocytosed bacteria in the blood circulation and in particular for yersiniae to gain access to the synovial tissue (13).

Enteropathogenic *Yersinia* species have developed a strategy to survive and multiply essentially extracellularly. Several plasmid-encoded polypeptides of yersiniae have been identified as antiphagocytic factors: (i) YopH, the protein tyrosine phosphatase; (ii) YopE, the cytotoxin; and (iii) YadA, the complement inhibitory factor (1, 3, 16–18). In spite of these factors, a small portion of infecting yersiniae can be found internalized by professional and nonprofessional phagocytes (2, 5, 7, 19). The contribution of intracellularly surviving yersiniae to the infection process is unclear. It is conceivable that internalized bacteria can escape the immune defense of the host and use phagocytes for transmigration (e.g., extravasation) and further dissemination of the pathogen.

In this study, we investigated the ability of human polymorphonuclear leukocytes (PMNs) to transport internalized *Y. enterocolitica* through an artificial endothelial monolayer on a microporous membrane (Biocoat Matrigel invasion chambers; Becton Dickinson, Bedford, Mass.). The upper and lower reservoirs of these chambers are separated by an 8-μm-pore-size polyethylene membrane coated with Matrigel basement membrane matrix (solubilized basement membrane preparation extracted from EHS mouse sarcoma, consisting mainly of laminin, collagen type IV, and heparan sulfate proteoglycan) to seal the pores, thus mimicking the situation in connective tissue in vivo.

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as previously described (15) and seeded onto the membrane in the upper reservoir. Briefly, after collagenase treatment of the cords and centrifugation of the resultant cell suspension, the pellet was resuspended in 5 ml of M199 medium with 20% normal human serum plus penicillin and streptomycin (50 U/ml). This cell suspension was transferred to a gelatin-coated tissue culture flask. After overnight incubation at 37°C in 5% CO_2, the flask was rinsed several times with M199 medium to remove contaminating cell types. HUVEC were passaged every 3 to 4 days. In this study, only cells from passage 3 or 4 were used.

To prepare monolayers in the invasion chamber for PMN transmigration, 5×10^4 HUVEC were seeded onto the membrane. Total confluency and integrity of monolayers were assessed by microscopic control of the chambers and by measuring the electrical resistance with an Evom epithelial voltohmmeter (World Precision Instruments). We used 2-day-old monolayers that had developed an electrical resistances of 900 to 1,000 Ω/cm^2. The Matrigel-coated membranes alone showed electrical resistances of 500 to 600 Ω/cm^2. Three hours before starting the PMN transmigration assay, the monolayers were washed several times with antibiotic-free medium.

PMNs were obtained from the venous blood of healthy adult donors as previously described (7). Fresh cells were resuspended in RPMI 1640, counted, and adjusted to the desired concentration. Cell viability was >98%, as tested by dye exclusion.

In this study, two different *Y. enterocolitica* strains were used to infect PMNs. Strain Y-108-P (serotype O3) harbors the 70-kb virulence plasmid, whereas the isogenic strain Y-108-C is plasmid cured (12). Bacteria were cultivated in Luria-Bertani (LB) broth overnight at 26°C. The following day, bacterial cultures were diluted 1:20 in fresh LB broth and grown for 4 h at 26°C in a rotary shaker to repress expression of plasmid-encoded antiphagocytic factors and to enable expression of

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TABLE 1. Results of PMN transmigration through a confluent HUVEC monolayer Matrigel-coated filter[a]

Chemoattractant in lower reservoir	Condition of PMNs in upper reservoir	Total no. of transmigrated PMNs	% Yersinia-associated PMNs	Total no. of yersiniae associated with PMNs	CFU/well
None	Noninfected	0			
	Infected with Y-108-P	0			
	Infected with Y-108-C	0			
FMLP	Noninfected	284 ± 12			
	Infected with Y-108-P	214 ± 13	89 ± 2	564 ± 28	460 ± 28
	Infected with Y-108-C	132 ± 13	87 ± 1	242 ± 11	121 ± 19
Y-108-P	Noninfected	102 ± 12	7 ± 2.5		
Y-108-C	Noninfected	75 ± 11	5 ± 3.9		

[a] Either 10^{-7} M FMLP or 10^7 *Y. enterocolitica* were used for stimulation, and 10^5 noninfected or infected PMNs were allowed to cross the membrane for 30 min. Both the total number of transmigrated PMNs and the number of yersinia-associated PMNs on the lower surface of the membrane and in the lower reservoir were determined after cytocentrifugation. Translocated viable bacteria are indicated as CFU. All values are means ± standard deviation for five separate assays.

invasin. To enhance the interaction of yersiniae with PMNs, *Y. enterocolitica* strains were opsonized by incubation in RPMI 1640 medium containing 5% pooled human serum from healthy adult volunteers for 10 min at 37°C (16a). A total of 10^5 PMNs were incubated with 10^7 opsonized bacteria in a total volume of 200 μl in microtiter plates (Nunc, Roskilde, Denmark) with constant mixing for 30 min at 37°C in a 5% CO_2 atmosphere. Afterwards, 30 μg of gentamicin per ml was added to the wells for 30 min to kill extracellular bacteria. The wells were washed twice with medium to remove loosely attached bacteria. This protocol turned out to be suitable for rapid internalization of plasmid-positive as well as plasmid-negative yersiniae by PMNs. Using the double immunofluorescence method for differentiation between intracellularly and extracellularly located bacteria, we found that about 95% (strain Y-108-C) and 70% (strain Y-108-P) of PMN-associated bacteria were localized intracellularly after 60 min (7, 12). This high phagocytosis rate for strain Y-108-P is not surprising because of the 26°C precultivation temperature. However, as the environmental conditions of internalized yersiniae (low Ca concentration and 37°C) are believed to favor expression of plasmid genes, we thought it worthwhile to compare an isogenic pair of yersiniae with respect to translocation by PMNs. To determine the percentage of infected PMNs before starting each transmigration assay, aliquots of cell suspensions were cytocentrifuged onto glass slides at 1,200 × *g* for 10 min. Slides were the stained either with Giemsa or, in some controls, by the double immunofluorescence method (7, 12). For transmigration assays, only those cell suspensions in which 97 to 99% of the PMNs were infected with bacteria were used.

To start the transmigration assay, 10^5 infected PMNs in RPMI 1640 (plus 30 μg of gentamicin per ml to prevent extracellular growth of yersiniae) were added to the upper reservoir when the endothelial cells had formed a confluent monolayer (electrical resistance of at least 900 Ω/cm^2). The lower reservoir contained 500 μl of RPMI 1640 medium (without gentamicin) either with or without a chemoattractant, such as 10^{-7} M FMLP, or 10^7 *Y. enterocolitica* (grown at 26°C as described above).

After the chambers were incubated at 37°C for 30 min, membranes were fixed in pure methanol at −15°C for 5 min, removed with a scalpel, and prepared for immunostaining and for subsequent confocal laser scanning microscopy (CLSM) analysis. Membranes were overlaid with 50 μl of fluorescein isothiocyanate (FITC)-labeled anti-O3 mouse monoclonal antibody (Progen Biotechnik, Heidelberg, Germany) diluted in phosphate-buffered saline (PBS) and incubated for 45 min at 37°C. After the coverslips were dipped three times in PBS, they were mounted under a glass coverslip.

Specimens were examined with a Leica TCS 4D confocal laser scanning microscope (Leica, Bensheim, Germany). The number of intracellular bacteria was counted by using the FITC filter system, whereas the autofluorescence of the cell bodies was visualized by using the rhodamine filter system. Confocal images were recorded at variable pixel densities, with the highest resolution at a setting of 1,024 by 1,024 pixels. Membranes were analyzed by performing *xy* and *xz* sections.

In a variation, PMNs were removed from the lower surface of the membrane with a cotton swab and resuspended in the medium of the lower reservoir. Aliquots of this suspension were cytocentrifuged onto glass slides as described above to determine the number of transmigrated PMNs attached to the lower side of the membrane and trapped in the lower reservoir of the chamber, or PMNs were lysed by resuspension in PBS containing 0.5% bovine serum albumin and 0.5% Tergitol. After serial dilutions of the resultant suspension, 100 μl was plated on Mueller-Hinton agar. CFU were counted after 36 h at 26°C to determine the number of viable cell-associated bacteria as previously described (7).

Neither noninfected PMNs nor PMNs infected with *Y. enterocolitica* Y-108-P or Y-108-C were able to transmigrate through the confluent HUVEC monolayer into the lower chamber in the absence of chemoattractant (Table 1). From these results, we concluded that the endothelial monolayer in combination with the Matrigel-coated membrane is a sufficient barrier to random transmigration of PMNs for at least 30 min. Moreover, the high electrical resistance, from 900 to 1,000 Ω/cm^2, of the tissue model did not change after PMN incubation. Examination of the HUVEC monolayer by CLSM showed no alteration of endothelial cell integrity or adherence of infected or noninfected PMNs (results not shown). In an additional experiment, we added 10^7 yersiniae (cultivated at 26°C) suspended in RPMI 1640 medium to the upper chamber in the absence of PMNs. After incubation for 2 h at 37°C in 5% CO_2, we were unable to detect any bacteria in the medium of the lower reservoir or attached to the lower side of the membrane (results not shown).

In the next series of experiments, we added FMLP (10^{-7}M) as a chemoattractant to the lower reservoir containing RPMI 1640 medium without serum (Table 1). PMNs were allowed to incubate in the upper part of the invasion chamber for 30 min, and the transmigration rate of PMNs was then determined.

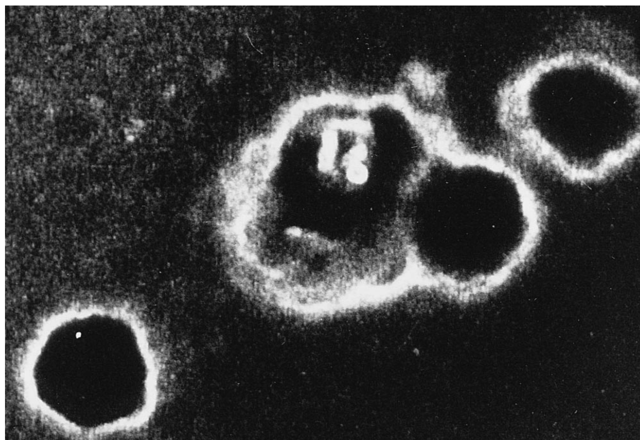

FIG. 1. CLSM photograph showing a yersinia-infected (four bacteria) PMN migrating through a pore of a membrane coated with Matrigel and an endothelial cell monolayer. The bacteria were stained with FITC-conjugated anti-O3 mouse monoclonal antibody.

The transmigration rate of noninfected neutrophils in response to FMLP chemoattractant was higher than that for infected PMNs (Table 1). During the interaction of PMNs with yersiniae and subsequent ingestion of the bacteria, neutrophils may release oxygen radicals and various toxic granule components, which could result in impaired transmigration capability for a certain portion of PMNs. This might explain the slight decrease in the percentage of infected PMNs from 97 to 98% in the upper reservoir to approximately 86 to 91% of infected PMNs on the lower side of the membrane and the lower part of the invasion chamber. Interestingly, 70 to 80% of the transmigrated PMNs contained only one to three yersiniae (maximum, eight) (Fig. 1), whereas about 80% of the neutrophils in the upper chamber harbored 10 to 15 (maximum, 22) internalized bacteria after gentamicin treatment (data not shown). From these data, we conclude that an overload of PMNs with yersiniae reduces the transmigration capability.

During transmigration assays with chemoattractants, we measured the electrical resistances in the invasion chambers. Initial values (900 to 1,000 Ω/cm^2) dropped to 600 to 700 Ω/cm^2, indicating that the endothelial barrier was partially compromised in our in vitro model. In parallel experiments, transmigrated PMNs collected from the lower surface of the membrane and the lower reservoir of the chamber were lysed with Tergitol, and aliquots of the suspension obtained were plated on Mueller-Hinton agar to determine the number of transported viable yersiniae by FMLP-stimulated PMNs.

Approximately 80% of *Y. enterocolitica* Y-108-P cells detected as PMN associated were viable, in contrast to only 50% of strain Y-108-C cells (see Table 1). Evidently, the 70-kb virulence plasmid in yersiniae appeared to play a role in the survival of yersiniae after interaction with PMNs. Similar results have been obtained by us with infected PMNs in suspension (7).

Finally, we examined whether transmigrated PMNs are able to interact with yersiniae and whether yersiniae are able to attract PMNs.

Noninfected PMNs were loaded in the upper reservoir, and nonopsonized yersiniae (10^7 in 500 μl of RPMI 1640) were used as a target and a chemoattractant in the lower reservoir. About 7% $\pm$ 2.5% (in case of Y-108-P) or 5% $\pm$ 3.9% (in case of Y-108-C) of the transmigrated PMNs were found to be associated with yersiniae in the lower compartment (Table 1). By Giemsa staining, we determined that the majority of bacteria were ingested. Extension of the transmigration time of noninfected PMNs to 90 min resulted in an increase in yersinia-associated neutrophils in the lower chamber of 50 to 60% (data not shown), indicating phagocytic activity of PMNs after transmigration.

Interestingly, only 20 to 25% of the transmigrated PMNs were attached to the lower surface of the membrane, whereas the remaining transmigrated neutrophils were found in the medium of the lower part of the invasion chamber. This was determined by examination of the membranes by CLSM in five additional transmigration assays.

The present study demonstrates that PMNs infected with viable *Y. enterocolitica* are able to migrate through an artificial barrier composed of an endothelial monolayer and a basement membrane matrix protein layer in response to a chemoattractant. PMNs infected with Y-108-P showed a transmigration rate similar to that of neutrophils infected with Y-108-C. The presence of the virulence plasmid had no significant influence on the motility of PMNs when viable yersiniae were located intracellularly. After translocation by PMNs, the plasmid-positive strain was clearly better able to survive within PMNs. Our laboratory has previously shown that *Y. enterocolitica* can survive within PMNs for at least 24 h (7). The microbial mechanisms that protect yersiniae from oxidative and nonoxidative killing by PMNs are not clearly identified (1, 5, 7, 19). It remains to be determined whether viable plasmid-positive yersiniae attached to PMNs (precultivation of yersiniae at 37°C, no gentamicin treatment) are able to inhibit the transmigration process.

Taken together, the results of this study support the concept that the survival of *Y. enterocolitica* within PMNs can contribute to the pathogenicity of the organism, as has also been assumed for *Salmonella typhimurium* (6). The bulk of extracellularly localized yersiniae in PPs during the early stage of infection may activate phagocytes infected with bacteria to transport yersiniae to other sites of the body, such as mesenteric lymph nodes, liver, spleen, and joints. Further studies with the experimental mouse or rat model (11) are required to evaluate whether PMN-mediated translocation of yersiniae contributes to pathogenicity in vivo.

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