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Differential Secretion of Interleukin-8 by Human Epithelial Cell Lines upon Entry of Virulent or Nonvirulent *Yersinia enterocolitica*

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Epithelial cells of the intestinal mucosa are among the first cells encountered by invasive pathogens. Bacterial invasion of the mucosa gives rise to an inflammatory response, characterized by the influx of polymorphonuclear leukocytes. The chemotactic stimulus responsible for this accumulation is unknown, but several in vitro studies have demonstrated that epithelial cells secrete the chemokine interleukin-8 (IL-8), a potent chemoattractant of polymorphonuclear leukocytes, upon bacterial entry. In this study we analyzed the secretion of IL-8 by human intestinal (T_{84}) and cervical (HeLa) epithelial cell lines in response to infection with the enteric pathogen *Yersinia enterocolitica*. IL-8 was secreted by T_{84} and HeLa cells in response to invasion by *Y. enterocolitica*. Virulent *Y. enterocolitica* induced a significantly lower level of IL-8 secretion than nonvirulent *Y. enterocolitica*. Subsequent analysis employing a mutant defective in Yop secretion and various *yop* mutants showed that the reduced secretion of IL-8 is due to the presence of Yop proteins. Our data suggest that YopB and YopD are required for the suppressive effect.

The chemokine interleukin-8 (IL-8) is secreted by enteric (T_{84}) and cervical (HeLa) epithelial cells in response to bacterial entry. Bacteria, including those that remain inside phagosomal vacuoles, e.g., *Salmonella* spp., and those that enter the cytoplasm, e.g., *Listeria monocytogenes*, stimulate this response (20). Neither bacterial lipopolysaccharide (LPS) nor noninvasive bacteria induce an IL-8 response in these cell lines. In a recent investigation, using monolayers of human colon epithelial cell lines (T_{84}, HT29, and Caco-2) as well as freshly isolated human intestinal epithelial cells, Jung et al. (35) demonstrated that infection with a variety of different invasive bacteria (*Salmonella dublin*, *Shigella dysenteriae*, *Yersinia enterocolitica*, *L. monocytogenes*, and enteroinvasive *Escherichia coli*) results in the coordinate expression and upregulation of a specific array of four proinflammatory cytokines, IL-8, monocyte chemotactic protein-1, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor alpha (TNF-α). These cytokines have well-established roles in acute inflammation. IL-8 is a potent chemoattractant for polymorphonuclear leukocytes (PMNs), basophils, and T cells, and it activates several PMN functions (2, 45).

The enterobacterium *Y. enterocolitica* causes a broad range of gastrointestinal syndromes, ranging from acute enteritis and enterocolitis to mesenteric lymphadenitis (8, 14). The virulence of *Y. enterocolitica* is controlled by chromosomal (*yst* and *inv*) (16, 34, 41, 46) and plasmid-encoded genes. The pYV (for *Yersinia* virulence) plasmid directs the production of the outer membrane protein YadA and the secretion of 11 antihost proteins called Yops (for recent reviews, see the work of G. R. Cornelis [12, 13]).

In vitro, *Y. enterocolitica* displays a number of properties relevant for its pathogenic character: *Y. enterocolitica* has the capacity to adhere to and to invade cultured mammalian epithelial cells (37, 43) and to adhere to extracellular matrix proteins. Adherence and internalization of *Y. enterocolitica* depend on a series of surface proteins, namely, Inv (33, 48, 61), Ail (41, 42), and YadA (6, 22, 32, 54, 60).

Further virulence-associated properties are conveyed by the Yop proteins in vitro: in *Yersinia pseudotuberculosis*, YopE and YopH, which are both highly conserved in *Y. enterocolitica* (39, 40), confer resistance to phagocytosis by macrophages (23, 25, 49, 50). YopH is a protein tyrosine phosphatase (28) related to eukaryotic phosphatases which acts on multiple target cell proteins (5, 7, 31). Furthermore, the tyrosine phosphatase activity of YopH inhibits the Fc-receptor-mediated oxidative burst in J774A.1 cells (4). YopE has a contact-dependent cytotoxic effect on HeLa cells and macrophages (50, 51), an effect which requires the presence of YopD (51). YopD was also shown to be associated with the suppression of the oxidative burst in exogenously stimulated macrophages by *Y. enterocolitica* (31). YopB may enhance virulence by suppressing the production of TNF-α by macrophages (3). YpkA of *Y. pseudotuberculosis*, the homolog of YopO in *Y. enterocolitica*, contains sequence motifs found in eukaryotic Ser/Thr kinases of the PSK family and has a kinase activity (26). The presumed action of the Yops, mainly YopH and YopE, implies that these bacterial virulence proteins function inside the target cells. The translocation of YopE and YopH to the intracellular compartments of target cells was shown to be dependent on intact *yopB* and *yopD* genes (51, 52, 56, 57).

When invasive enteric pathogens penetrate intestinal mucosae, they evoke an inflammatory response. Data presented in the studies mentioned above demonstrated that *Y. enterocolitica* induces a comparatively lower level of IL-8 secretion by epithelial cells than other bacteria tested (20, 24). We wondered whether this reduced level of secretion is due to the action of one of the Yop proteins. For this purpose we analyzed IL-8 secretion by HeLa and T_{84} cells upon infection with virulent (pYV^+) or nonvirulent (pYV^-) *Y. enterocolitica*. We

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
Y. enterocolitica W22703	pYV^+ (serotype O:9)	9
Y. enterocolitica W22703c	pYV^- (serotype O:9)	This study
Y. enterocolitica KNG22703	pYV^+ *bla luxAB*$^+$	36
Plasmids		
pYVe227	Virulence plasmid from *Y. enterocolitica* W22703	10
pGC153	pGB63*yopB*–GC153::mini-Mu d1 *lac* (YopB and YopD deficient)	10
pGC559	pGB63*yopOP*–559::mini-Mu d1 *lac* (YopO and YopP deficient)	11
pGC1152	pGB63*yopH*–1152::mini-Mu d1 *lac* (YopH deficient)	11
pGC1256	pGB63*yopE*–1256::mini-Mu d1 *lac* (YopE deficient)	11
pBB2	pMS3 plus *yopD* (*yopD* under the control of the *yopE* promoter)	59
pAA210	pYVe227*yscK*::*aphA-3* (nonpolar mutation)	1
pPW72	pBluescriptII (KS−)*yopB* (PCR fragment)	This study
pPW74	pPW72Δ, 450-bp *Eco*RV fragment (in-frame deletion of codons 89–217 in *yopB*)	This study
pPW75	pKNG101-*yopB*$^{\Delta 89-217}$ (*Pvu*II fragment of pPW74, mutator for *yopB*)	This study
pPW2275	pYVe227*yopB*$^{\Delta 89-217}$ (in-frame deletion in *yopB*, defective in *YopB* secretion)	This study
pEH301	pYVe227 *yopD*::Tn*2507* (YopD deficient)	31
pEH500	pRK404-P*yopH*^{yopB}	This study
pEH503	pRK404-4P*yopH*^{yopD}	This study
pEH504	pRK404-P*yopH*^{yopBD}	This study

show herein that the amount of IL-8 secreted by epithelial cell lines upon infection with plasmidless *Y. enterocolitica* is greater than that induced by plasmid-bearing *Y. enterocolitica*. Our data suggest that YopB and YopD play important roles in the suppression of IL-8 secretion.

MATERIALS AND METHODS

Bacterial strains. *Y. enterocolitica* W22703 (nalidixic acid resistant) is a restriction mutant (Res$^-$ Mod$^+$) of the serotype O:9 W227 (9). A plasmid-cured isogenic derivative of strain W22703 was generated by plating the plasmid-carrying strain on MOX agar at 37°C (58). Strain KNG22703 is a *blaA* mutant of W22703 in which the gene encoding β-lactamase A was replaced by the *luxAB* genes from *Vibrio harveyi* (36). The *Salmonella dublin* strain was isolated by G. Wauters (Université Catholique de Louvain, Brussels, Belgium). *E. coli* SM10 lambda *pir*$^+$ constructed by Miller and Mekalanos (44) or *E. coli* S17.1 (55) was used to deliver mobilizable plasmids into *Y. enterocolitica*. Strains and plasmids used are listed in Table 1.

Bacterial growth conditions. Bacteria were routinely grown in tryptic soy broth (Oxoid, Basingstoke, England). Induction of the *yop* regulon and Yop protein analysis were done as described previously (10, 40). For infection experiments, overnight cultures of *Yersinia* spp. were diluted to an optical density at 600 nm of 0.2 in brain heart infusion (Difco, Detroit, Mich.) and incubated for 3 h at 25°C. In order to gain greater reproducibility of infection, we decided in the course of this study to use aliquots of frozen stocks of bacteria. For this purpose, bacteria grown for 3 h at 25°C were collected by centrifugation and washed twice with sterile phosphate-buffered saline (PBS) (pH 7.4). Aliquots of bacteria corresponding to 5×10^8 CFU were resuspended in PBS–10 mM $MgCl_2$–20% glycerol (final concentration) and immediately frozen in liquid nitrogen. The survival rate after freezing and thawing, for each strain used, was determined by plating for CFU before and after freezing and was higher than 90%. In control experiments, infections with freshly cultured bacteria or frozen bacterial stocks induced essentially the same level of IL-8. Antibiotics (Boehringer, Mannheim, Germany) were used at the following final concentrations: chloramphenicol, 20 μg ml^{-1}; gentamicin, 40 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; tetracycline, 20 μg ml^{-1}; and nalidixic acid, 35 μg ml^{-1}.

Cell lines. Human HeLa cervical epithelial cells (ATCC CCL 2) were obtained from the American Type Culture Collection, Rockville, Md. Human colon epithelial carcinoma T_{84} cells (ATCC CCL-248) were a kind gift from the Ludwig Institute for Cancer Research, Brussels, Belgium. HeLa cells were grown in RPMI 1640 (Seromed; Biochrome Université Catholique de Louvain, KG, Berlin, Germany)–10% fetal bovine serum (Gibco BRL, Paiseley, Scotland) supplemented with 2 mM L-glutamine (Seromed) and streptomycin (100 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). T_{84} cells were cultured in 50% Iscove's modified Dulbecco's medium–50% Ham's F12 (Gibco BRL)–5% fetal bovine serum (Gibco BRL) in the presence of streptomycin (100 μg/ml). Cells were grown in a humidified 8% CO_2 atmosphere at 37°C.

DNA methods and cloning techniques. Plasmid DNA extraction, restriction enzyme digests, DNA ligations, and transformation of *E. coli* were performed by standard techniques (53).

Construction of the *yopB* mutant and complementing plasmids (pEH500, pEH503, and pEH504). To mutagenize *yopB* we amplified the *yopB* gene by PCR using primers MIPA224 and MIPA124 (Fig. 1). The 5′ primer (MIPA224) is located upstream of *yopB* within *lcrH* (29). The amplified fragment was cloned into pBluescriptII (KS−) (Stratagene, La Jolla, Calif.) cut by *Bam*HI and *Sal*I, yielding pPW72. Codons 89 to 217 of the *yopB* gene were deleted by *Eco*RV digestion of pPW72 and religation, giving pPW74. This mutant *yopB* allele was subcloned as a *Pvu*II fragment into *Sma*I-digested pKNG101 (36), yielding pPW75, and exchanged with the wild-type allele of pYVe227, giving rise to pPW2275.

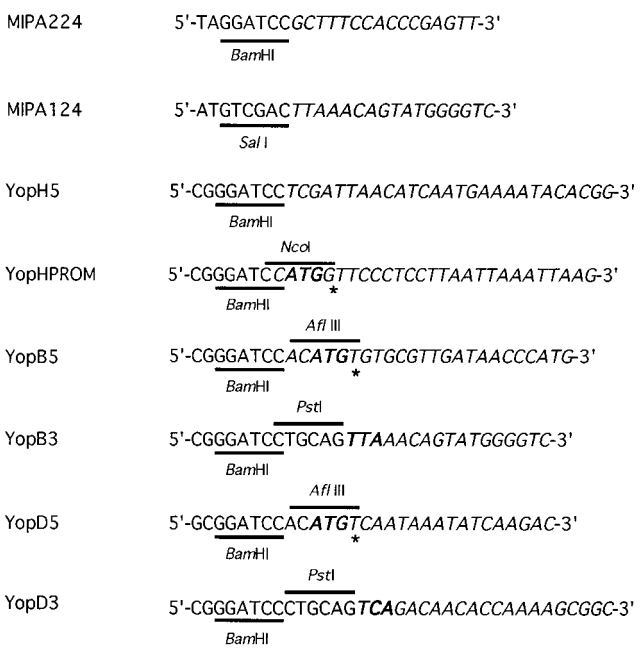

FIG. 1. Sequences of the deoxyribonucleoside primers used to amplify the *yopH* promoter or the *yopB*, *yopD*, and *yopBD* genes. The sequences of the *yop* genes from *Y. enterocolitica* and the *lcrH* gene from *Y. pseudotuberculosis* (29, 39) are shown in italics. In primers MIPA124, YopHPROM, YopB3, and YopD3 the sequences are reversed and complemented. The translational start and stop codons are shown in boldface type. Recognition sites of *Afl*III, *Bam*HI, *Nco*I, and *Pst*I are underlined. Nucleotide changes introduced in order to create the *Afl*III or *Nco*I site in the amplification product are indicated by asteriks. RBS, the putative ribosome binding site of the *yopH* gene (39).

The *yopH* promoter was amplified by PCR with primer YopH5 and YopHPROM (Fig. 1). The 148-bp product was cloned into the *Bam*HI site of pBluescriptII (KS−), giving rise to plasmid pPROM20. *yopB*, *yopD*, and *yopBD* were amplified with primers YopB5/YopB3, YopD5/YopD3, and YopB5/YopD3, respectively (Fig. 1), and cloned as *Bam*HI fragments into pBluescriptII (KS−). DNA fragments containing the *yopB*, *yopD*, and *yopBD* genes were cut out with *Afl*III-*Pst*I and cloned downstream of the *yopH* promoter into pPROM20 digested with *Nco*I-*Pst*I. The *Bam*HI-*Pst*I fragments containing the *yopH* promoter and the respective *yop* gene(s) were ligated into pRK404 (18) digested with *Bam*-*Pst*I, resulting in plasmids pEH500, pEH503, and pEH504.

Infection protocol. Epithelial cells were seeded into 24-well culture plates (Falcon) at 1×10^5 to 2×10^5 cells per well in 1 ml of tissue culture medium containing 100 μg of streptomycin per ml. Cells were allowed to adhere for 20 to 24 h. Viability of the cells was routinely controlled by trypan blue exclusion. One to two hours before the infection, cells were washed three times with sterile PBS (pH 7.4) and incubated in medium containing heat-inactivated fetal bovine serum without streptomycin.

Bacteria grown for 3 h in brain heart infusion at 25°C (see bacterial growth conditions) were collected by centrifugation and washed twice in sterile PBS. After determination of the optical density, appropriate dilutions of the bacteria in PBS were performed before infection. Cell cultures were infected with 50 to 100 μl of bacterial suspension per well, at a bacterium/cell ratio of 50:1 or as indicated in the text. The actual number of bacteria administered was determined by plating 0.1 ml of 1:10 serial dilutions on tryptic soy agar plates and counting CFU after 36 h of incubation at 28°C.

Cells and bacteria were incubated for 2 h to allow bacterial adherence and entry. After removal of the medium, cultures were washed three times to remove extracellular bacteria and further incubated for 4 h in the presence of 40 μg of gentamicin per ml to kill remaining extracellular bacteria. Culture supernatants were then removed and centrifuged for 20 min to pellet residual bacteria and cells before IL-8 determination. Cells of the monolayers were lysed in 24-well plates with 0.1% Triton X-100 in physiological saline. The number of released viable bacteria was determined by plating serial 10-fold dilutions on tryptic soy agar containing the appropriate antibiotics.

As an alternative to the use of bacterial cultures, the experiments reflected in Fig. 4 and 5 and Table 3 were done using frozen stocks of bacteria (see bacterial growth conditions) for infections. For this purpose frozen stocks were thawed on ice and centrifuged, and bacterial pellets were resuspended in PBS. Dilutions of bacteria, infections of cells, and platings for the actual inoculum were performed as described above.

TNF-α, a gift of the Ludwig Institute for Cancer Research, and bacterial LPS from *E. coli* (Sigma) were used in concentrations of 5 and 100 ng/ml, respectively.

Interleukin determination by ELISA. The amount of IL-8 secreted into the supernatant was determined by an enzyme-linked immunosorbent assay (ELISA) using a human IL-8 quantikine kit from R&D Systems (Oxon, United Kingdom). The sensitivity of the ELISA was 3 pg/ml.

RESULTS

Secretion of IL-8 by epithelial cells infected with virulent or nonvirulent *Y. enterocolitica*. To study the release of IL-8 by epithelial cells after infection with *Y. enterocolitica*, we employed two cell lines: T_{84} colonic epithelial cells and HeLa cervical epithelial cells. Monolayers of T_{84} and HeLa cells were infected with bacteria as described in Materials and Methods. After the initial infection period of 2 h and a further cultivation for 4 h in the presence of gentamicin, the amounts of IL-8 secreted into the supernatant were measured. As a control we included a strain of *Salmonella dublin* in our experiments, an invasive bacterium shown to induce IL-8 secretion by human epithelial cell lines (20). As shown in Table 2, *Salmonella dublin* and pYV$^-$ *Y. enterocolitica* W22703 induced IL-8 secretion in both cell lines. In contrast, virulent (pYV$^+$) *Y. enterocolitica* W22703 induced a significantly lower level of secretion. For HeLa cells this amount was barely higher than the background level of noninduced cells, whereas for T_{84} cells the level was almost the same as that of the noninduced background (Table 2). After correction for background levels, the IL-8 secretion level induced by pYV$^-$ *Y. enterocolitica* was 27-fold (T_{84}) or 24-fold (HeLa) higher than the secretion level induced by pYV$^+$ *Y. enterocolitica*. As expected, while the cells respond normally to stimulation with the physiological agonist TNF-α (Table 2), bacterial LPS did not stimulate IL-8 secretion by these cell lines.

TABLE 2. *Yersinia*-induced IL-8 secretion by human epithelial cells[a]

Bacterium or other stimulus[b]	IL-8 secreted (pg/ml)[c] by:	
	T_{84} cells	HeLa cells
Salmonella dublin	492 ± 49	173 ± 5
Y. enterocolitica pYV$^+$	115 ± 20	17 ± 9
Y. enterocolitica pYV$^-$	322 ± 40	338 ± 41
LPS	119 ± 14	1 ± 1
TNF-α	696 ± 60	75 ± 9
None	107 ± 7	3 ± 2

[a] Semiconfluent monolayers of epithelial cells in 24-well plates were infected at a bacteria/cell ratio of 50:1 as described in Materials and Methods. Bacterial entry was allowed for 2 h before cells were washed and medium containing gentamicin (40 μg/ml) was added. After 4 h in culture, the supernatants were taken off and centrifuged for 2 min and the concentrations of IL-8 in the supernatants were determined. Intracellular bacterial counts were determined after lysis of the cells by plating serial dilutions.

[b] LPS and TNF-α were used at 100 and 5 ng/ml, respectively.

[c] Results are means ± standard errors of the means of values obtained from three separate experiments with triplicate samples.

Influence of bacterial invasion on IL-8 secretion. Eckmann et al. (20) have demonstrated that bacterial entry is required to induce IL-8 secretion by colonic and cervical epithelial cells. To determine if the differences observed between the amount of IL-8 released upon infection with pYV$^-$ or pYV$^+$ *Y. enterocolitica* simply reflect a difference in rates of bacterial entry, we infected HeLa cell monolayers with various doses of pYV$^-$ or pYV$^+$ bacteria and determined the number of viable intracellular *Yersinia* organisms. We found that at the end of the culture period the numbers of intracellular pYV$^-$ bacteria were much higher than those for pYV$^+$ bacteria (Fig. 2).

To analyze whether a lower rate of entry or intracellular multiplication of pYV$^+$ *Yersinia* organisms was responsible for the phenomenon, we monitored the number of viable intracellular bacteria for 4 h after the initial infection phase. The results showed that the pYV$^-$ and the pYV$^+$ strains multiplied only once or twice during this period (data not shown) and that

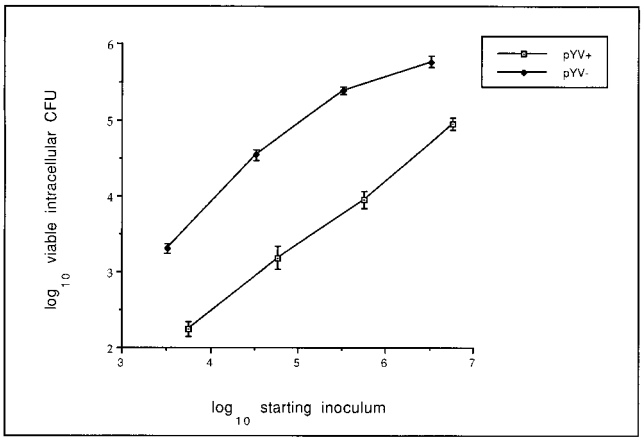

FIG. 2. Comparison of the numbers of viable intracellular pYV$^-$ and pYV$^+$ yersiniae with respect to those of the starting inoculum. Semiconfluent monolayers of HeLa cells in 24-well plates (1 ml) were infected with various doses of pYV$^-$ or pYV$^+$ *Y. enterocolitica* for 2 h to allow bacterial entry. After removal of extracellular bacteria by washing the plates three times with PBS, cultures were incubated for 4 h in the presence of gentamicin. At the end of the culture period, the numbers of intracellular bacteria were determined after HeLa cells were lysed. Values are means ± standard deviations of triplicate samples. Data are from a representative experiment. Comparable results were obtained in additional experiments.

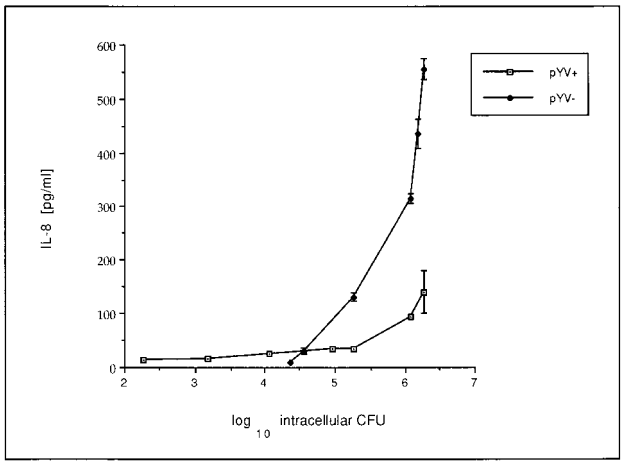

FIG. 3. Relationship between the number of intracellular virulent or nonvirulent *Y. enterocolitica* and IL-8 secretion by HeLa epithelial cells. Cultures of HeLa cells in 24-well plates were infected with various doses of pYV$^+$ or pYV$^-$ *Y. enterocolitica* and incubated for 2 h to allow bacterial invasion. After removal of extracellular bacteria, cultures were further incubated for 4 h in the presence of gentamicin. After the culture period, the concentrations of IL-8 in the supernatants were measured and the numbers of viable intracellular bacteria were determined after lysis of the cells. Values represent means ± standard deviations from three independent experiments with triplicate samples.

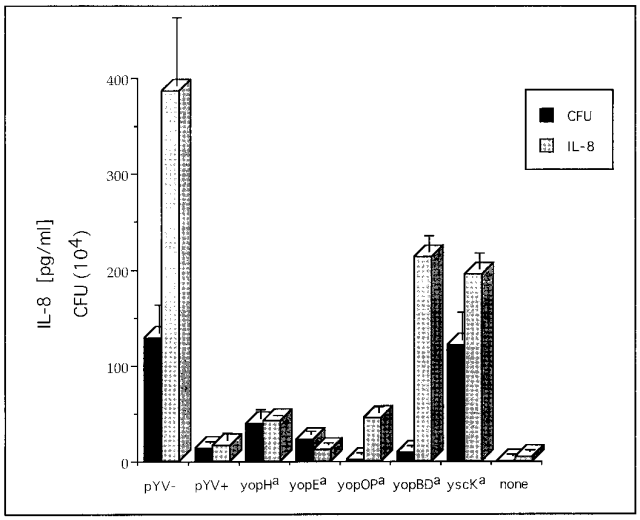

FIG. 4. IL-8 secretion by HeLa cells after infection with various *Y. enterocolitica yop* mutants and a secretion mutant. Semiconfluent monolayers of HeLa cells in 24-well plates were infected with pYV$^-$ or pYV$^+$ wild-type *Y. enterocolitica*, different *yop* mutants, and a secretion mutant at a bacterium-to-cell ratio of 50:1. Bacteria were allowed to enter cells for 2 h before they were removed and gentamicin was added. After 4 h, the concentrations of IL-8 in the supernatants were measured and the amounts of viable intracellular bacteria were estimated by plating serial dilutions of HeLa cell lysates. Values represent means ± standard deviations from three independent experiments with triplicate samples. a, mutant strains.

no difference in levels of intracellular multiplication was detectable (data not shown). With respect to the pYV$^+$ strain, our finding is in agreement with results of Devenish and Schiemann (17), who reported the lack of intracellular multiplication of virulent *Y. enterocolitica* in HeLa cells.

These data seemed to indicate that differences in the abilities to invade HeLa cells may be the reason for the observed difference in amounts of IL-8 released. To achieve similar numbers of intracellular pYV$^+$ and pYV$^-$ *Yersinia* organisms, we infected HeLa cells with various numbers of bacteria at multiplicities of infection of between 50 and 1,000. As depicted in Fig. 3, at very high multiplicities of infection intracellular counts for pYV$^+$ *Yersinia* organisms reached levels comparable to those of the pYV$^-$ strain. Surprisingly, however, there was still a significantly smaller amount of IL-8 secreted into the culture supernatant (Fig. 3). Thus, approximately 2×10^6 viable intracellular pYV$^+$ *Yersinia* organisms induced a level of IL-8 secretion of 131 pg/ml while about the same number of pYV$^-$ *Yersinia* organisms gave rise to an amount of 546 pg/ml (Fig. 3). Although we tried to obtain a higher number of intracellular bacteria, in particular pYV$^+$ bacteria, by using a multiplicity of infection higher than 1,000, we could never obtain higher counts of intracellular *Y. enterocolitica*. This could indicate that under our experimental conditions, with a statistical mean of 10 to 20 bacteria per cell, the maximal possible level of infection was reached. The data presented in Fig. 3 suggested that, beside the difference in the level of invasion, a second effect, exerted by a pYV-encoded factor, is responsible for the low-level IL-8 secretion by epithelial cells infected with virulent *Y. enterocolitica.*

Rosqvist et al. (50) have reported that YopE is cytotoxic for cultured HeLa cells. To exclude the possibility that the reduced level of IL-8 response was due to this cytotoxic effect, we analyzed the viability of HeLa cells at the end of the experiment by trypan blue exclusion. The results showed no significant difference for cells infected with pYV$^+$ or pYV$^-$ *Y. enterocolitica* under these conditions.

Effect of YopB and YopD on IL-8 release. To investigate if Yop proteins are responsible for the suppression of IL-8 secretion, we monitored the IL-8 response to infection with a Yop secretion mutant. HeLa monolayers were infected with the *yscK* mutant KNG22703(pAA210) which is defective in the secretion of all Yop proteins (1). Infection with this strain gave rise to intracellular bacterial counts comparable to those of the pYV$^-$ strain. In addition, the amount of IL-8 secreted by HeLa cells was strongly increased above levels induced by the pYV$^+$ strain: 195 pg/ml compared with 17 pg/ml (Fig. 4).

Although the IL-8 response induced by the *yscK* mutant did not reach the same levels as those for the nonvirulent strain, we took this result as an indication that the suppression of IL-8 is caused by a secreted Yop(s). Subsequent analysis of *yopH*, *yopE*, and *yopOP* mutants (Table 1) revealed some variation with respect to the numbers of intracellular bacteria as well as the amounts of IL-8 secreted but did not show a strong effect on the IL-8 response (Fig. 4). However, the *yopBD* mutant W22703(pGC153) induced a high level of IL-8 secretion (213 pg/ml), although the number of viable intracellular bacteria was quite low (Fig. 4). This mutant contains a polar insertion in *yopB* and does not produce YopB and YopD, but it secretes all the other Yops (10). When this mutant was used to infect T_{84} cells, we observed the same effect, i.e., high levels of IL-8 secretion (data not shown).

The significance of these findings for the *yopBD* mutant was confirmed by dose-response experiments, the results of which are shown in Fig. 5. HeLa cells were infected with increasing doses of pYV$^-$ or pGC153 *Y. enterocolitica* W22703. After the culture period the numbers of viable intracellular bacteria and the IL-8 concentrations in the supernatants were determined. As depicted in Fig. 5A, an increase in the amount of starting inoculum resulted in an increase in the number of intracellular bacteria for the pYV$^-$ and the *yopBD* mutant strain (pGC153). However, the counts for intracellular *yopBD Y. enterocolitica* organisms were 5- to 10-fold lower than for the plasmidless

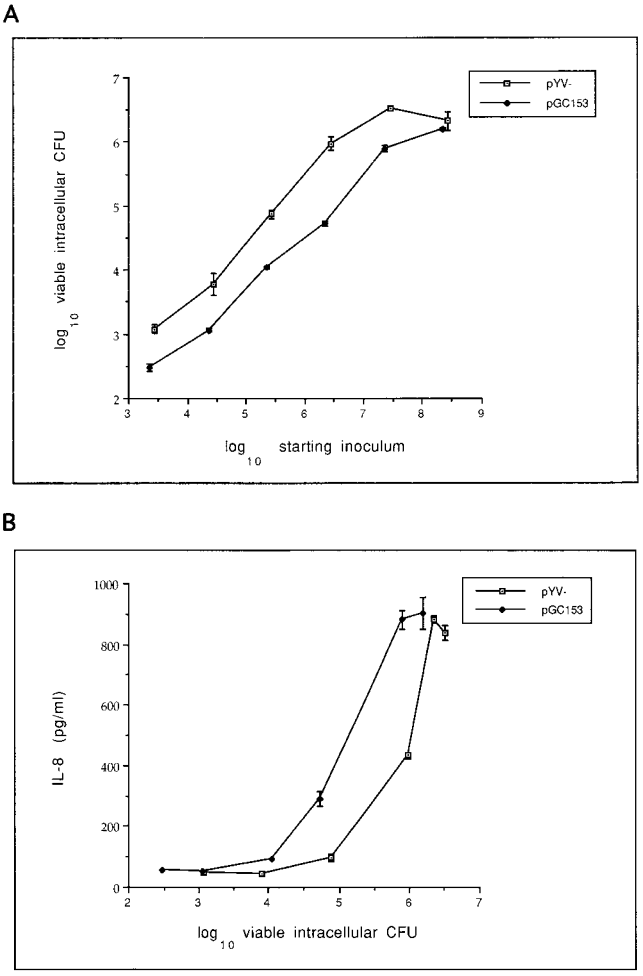

FIG. 5. Comparison of the numbers of viable intracellular pYV$^-$ and *yopBD* mutant *Y. enterocolitica* organisms with respect to those of the starting inoculum (A) and dose-response curve for IL-8 secretion (B). Semiconfluent monolayers of HeLa cells in 24-well plates (1 ml) were infected with various doses of pYV$^-$ or pGC153 (*yopBD*) *Y. enterocolitica* for 2 h to allow bacterial entry. After removal of extracellular bacteria by washing the plates three times, cultures were incubated for 4 h in the presence of gentamicin. At the end of the culture period, IL-8 in the supernatants was measured and the amounts of viable intracellular bacteria were determined by plating serial dilutions of cell lysates. Values are means ± standard deviations of triplicate samples. Data are from a representative experiment. Comparable results were obtained in additional experiments.

strain. Nevertheless, infection with the *yopBD* mutant *Y. enterocolitica* stimulated high levels of IL-8 secretion (Fig. 5B). IL-8 levels were even slightly higher than those found for comparable numbers of intracellular pYV$^-$ *Y. enterocolitica* (Fig. 5B).

To exclude the possibility that the pYV plasmid derivative pGC153 of the *yopBD* mutant strain is less stable and that IL-8 release resulted from bacteria that had lost the plasmid during the course of the experiment, we determined the number of plasmidless bacteria at the end of the experiment. For this purpose, we took advantage of the kanamycin resistance of the mini-Mu element inserted in *yopB* of pGC153 (10). Selective plating of intracellular *yopBD* mutant *Y. enterocolitica* W22703 (pGC153) on nalidixic acid or nalidixic acid and kanamycin did not reveal any significant differences in bacterial counts, indicating that pGC153 was retained during the course of the experiment.

TABLE 3. Effects of YopB and -D on *Yersinia*-induced IL-8 secretion by HeLa epithelial cells[a]

Y. enterocolitica W22730 plasmid(s)	Relevant genotype	Intracellular CFU (10^4)[b]	IL-8 secreted (pg/ml)[b]
pYV$^-$	NA[c]	164 ± 69	477 ± 86
pYV$^+$	Wild type	11 ± 6	33 ± 6
pGC153	*yopBD*	16 ± 2	355 ± 60
pEH301	*yopD*	12 ± 2	482 ± 5
pPW2275	*yopB*	16 ± 3	347 ± 7
pGC153/pEH500	*yopBD/yopD*$^+$	14 ± 2	321 ± 37
pGC153/pEH503	*yopBD/yopB*$^+$	3 ± 0	154 ± 14
pGC153/pEH504	*yopBD/yopBD*$^+$	3 ± 1	125 ± 14
None	NA	NA	5 ± 2

[a] Semiconfluent monolayers of epithelial cells in 24-well plates were infected at a bacteria/cell ratio of 50:1 as described in Materials and Methods. Bacterial entry was allowed for 2 h before cells were washed and medium containing gentamicin (40 μg/ml) was added. After 4 h in culture, the supernatants were taken off and centrifuged for 20 min and the concentrations of IL-8 in the supernatants were determined. Intracellular bacterial counts were determined after lysis of the cells by plating serial dilutions.

[b] Results are means ± standard deviations of values obtained from three separate experiments with triplicate samples.

[c] NA, not applicable.

The results described above demonstrated that YopB and/or YopD are required for the suppression of IL-8 secretion by HeLa and T_{84} cells upon infection with virulent *Y. enterocolitica*. To determine which of these Yops is necessary to exert the suppressive effect, we tested a mutant defective in the production of YopD. HeLa cells infected with the *yopD* mutant strain W22703(pEH301) (31) secreted levels of IL-8 comparable to those induced by the *yopBD* mutant (Table 3). However, when we introduced pBB2, a plasmid which directs the expression of YopD from the strong *yopE* promoter (59), into the *yopBD* mutant W22703(pGC153), we could not restore the suppression of IL-8 release (data not shown), suggesting an important role for YopB in IL-8 suppression. Introduction of pBB2 into the pYV$^+$ wild-type strain did not alter IL-8 suppression (data not shown).

To elucidate the contribution of YopB to IL-8 suppression, we mutagenized *yopB* by introducing an internal in-frame deletion into the *yopB* gene on the pYV plasmid by allelic exchange. The mutant strain carrying the *yopB*Δ allele was deficient in the secretion of YopB, while the secretion of other Yops was not affected. After infection of HeLa cell monolayers with this strain, we detected high IL-8 concentrations in the supernatants, confirming that both YopD and YopB are required for IL-8 suppression. In a further attempt to analyze the role of YopB and -D, we cloned the *yopB*, *yopD*, and *yopBD* genes under the control of the *yopH* promoter on a medium-copy-number plasmid. The plasmids were introduced into the W22703(pGC153) strain, and the transconjugant strains were examined for their Yop secretion profiles. Infection of HeLa cells with the *yopBD* mutant complemented for YopBD (pGC153/pEH504) gave rise to an intermediate level of IL-8 secretion into the supernatant (Table 3). Levels of IL-8 were reduced but were significantly higher than for the pYV$^+$ wild-type strain. This result indicates that the cloned genes under the control of the *yopH* promoter were not able to complement the mutation in W2270(pGC153) entirely. Surprisingly, introduction of the *yopB* (pEH503) gene alone into the *yopBD* mutant (pGC153) also effected the induction of IL-8. The IL-8 concentrations in the supernatants were higher than for the wild-type strain but were reduced in comparison with that of the *yopBD* mutant (Table 3). Complementation of the *yopBD*

mutant by YopD alone (pEH500) had no significant effect (Table 3).

DISCUSSION

In this study we demonstrated that the amounts of IL-8 secreted by human intestinal (T_{84}) and cervical (HeLa) epithelial cells in response to infection with virulent or nonvirulent *Y. enterocolitica* differed. The amount of IL-8 released upon infection by virulent pYV^+ *Y. enterocolitica* was reduced in comparison with that released upon infection by nonvirulent pYV^- *Y. enterocolitica* and also by *Salmonella dublin*, an observation previously reported by Eckmann et al. (20). Bacterial LPS did not stimulate this IL-8 response. This is in agreement with the concept suggested by Eckmann et al. (20, 21) that bacterial entry into intestinal epithelial cells but not LPS, which is quite abundant in human colons, serves as a stimulus for the IL-8 response. Noninvasive bacteria are unable to induce the release of IL-8 by T_{84} and HeLa cells. Furthermore, a direct relationship between the number of intracellular bacteria and the amount of IL-8 secreted into the supernatant has been demonstrated (20). A correlation between the number of intracellular *Y. enterocolitica* organisms and the amount of IL-8 secreted was also observed in this study (Fig. 3). Analysis of the number of intracellular bacteria in HeLa cells revealed that the pYV^+ strain invaded to a lesser extent than the pYV^- strain. These diverse rates of entry into epithelial cells could explain the distinct IL-8 responses. However, IL-8 measurements performed on epithelial cells that contained similar numbers of intracellular bacteria nevertheless showed different responses to infection with virulent and nonvirulent *Y. enterocolitica*, suggesting that a reason other than a reduced rate of invasion may be responsible for the differential levels of IL-8 secretion. Analysis of a *Y. enterocolitica* mutant compromised in the secretion of all Yop proteins revealed that Yop secretion is essential to suppress IL-8 release. Subsequent examination of various *yop* mutants demonstrated that both YopB and YopD are required for IL-8 suppression while the loss of YopE, YopH, and YopOP did not have a significant effect.

It is noteworthy that the *Y. enterocolitica yopE* and *yopH* mutants did not induce a stronger IL-8 release than the wild-type strain, indicating that the cytotoxic effects of these Yops were not the reason for the detected suppression. Viabilities of HeLa cells infected with pYV^+ or pYV^- bacteria did not differ significantly, as it was determined microscopically by trypan blue exclusion, although we occasionally observed some rounding up of HeLa cells (50) in response to infection with the pYV^+ strain.

Published work suggests that the principal function of YopB and YopD is to facilitate the translocation of effector Yops across the cytoplasmic membrane of mammalian cells. This suggestion is supported by analysis of the translated protein sequences of the *yopB* and *yopD* genes, which predict the existence of transmembrane domains (29). Translocation of YopE into HeLa cells is reduced in a *yopBD* mutant of *Y. enterocolitica* (57) as well as in a *yopD* mutant of *Y. pseudotuberculosis* (52). In addition, the translocation of YopH-adenylate cyclase hybrid proteins is reduced in a *yopBD* mutant (56). *Yersinia*-mediated antiphagocytosis associated with YopH is impaired in YopB and YopD mutant strains (23). We previously reported that *yopD* mutants showed impaired ability to suppress the oxidative burst in stimulated bone marrow-derived macrophages in vitro (31). Although our data did not rule out the possibility that YopD acts as a virulence determinant on its own, it is more likely that YopD acts by allowing other Yops, including YopE and YopH, to cross the host cell membrane. With respect to the suppression of IL-8, we also favor the idea that YopD and -B act by facilitating the translocation of a so-far-unidentified Yop which abrogates the secretion of IL-8 by epithelial cells. Another possibility could be that two or more Yops, translocated by a YopBD-dependent pathway, act in a synergistic fashion to block the IL-8 response.

The level of invasion of HeLa cells by the *Y. enterocolitica yopBD* mutant (pGC153) was 5- to 10-fold lower than for the pYV^- strain. This was somehow surprising, since the role of YopB and -D in the translocation of the antiphagocytic effectors YopE and YopH has been established (52, 56, 57). This observation points to the possibility that the internalization of *Y. enterocolitica* by epithelial cells is not sensitive or is less sensitive to YopE and YopH than phagocytosis by PMNs or macrophages and/or that another factor is involved in blocking the uptake. Such a blocking effect on the internalization of *Y. enterocolitica* by human epithelial cells has already been reported before and was ascribed to YadA (32).

Recently, Beuscher et al. (3) reported that YopB inhibits the production of TNF-α by murine macrophages in vitro. YopB has sequence similarity to contact hemolysins (29), such as IpaB of *Shigella* spp. Pore formation by YopB in the target cell membrane may cause suppression of TNF-α (3). YopB may act in a similar manner to reduce secretion of IL-8. According to this model, YopD could act in an indirect way on IL-8 secretion by translocating or inserting YopB in the host cell membrane. An intriguing question that arises from this possibility is whether there is a common pathway for the induction of IL-8 and TNF-α release that could be blocked by YopB.

Eckmann et al. (19) have demonstrated that IL-8 expression in human intestinal epithelial cell lines is also stimulated by the physiological agonist IL-1β or TNF-α. We are currently investigating the possibility that the reduced IL-8 response level is caused by a block of the production of the physiological agonist TNF-α.

It is not clear by what means YopB, YopD, or the putative effector Yop is delivered to the epithelial cells to suppress IL-8 secretion: by extracellularly adhering *Y. enterocolitica* organisms during the initial infection phase (before they are destroyed by gentamicin) or by intracellularly located *Y. enterocolitica* organisms in the phagosome. We also do not know whether the suppression of IL-8 secretion depends on effects on transcription and translation or an effect on its secretion. The mechanism by which the entry of bacteria leads to the release of IL-8 is presently unknown, making it difficult to speculate about the possible effects of YopB and -D on the process. The study of the functions of YopB and YopD or of the unknown effector Yop on the release of IL-8 may help to elucidate this mechanism. Further investigations should also analyze whether the suppression of IL-8 secretion is specific for mucosal epithelial cells or whether it also works on other cell types known to secrete IL-8, e.g., macrophages and T cells.

An important issue that arises from our finding is the effect on natural infections of animals or humans. It is generally accepted that *Y. enterocolitica* penetrates ileal epithelia via M cells overlying Peyer's patches (27), but there are only a few reports describing invasion of *Y. enterocolitica* into epithelial cells of ilea. Lian et al. (38) reported early lesions, consisting of epithelial cell degeneration, at 1 to 12 h after infection of rabbits with pYV^+ and pYV^- *Y. enterocolitica*. This epithelial degeneration was associated with an increase in levels of PMNs in laminae propriae. Investigations of orally infected mice showed colonization of ileal mucosae by *Y. enterocolitica*, although to a lesser extent than that of Peyer's patches (30). Histopathological changes like those observed in rabbits have not been described for mice, but the studies of infected mice

were done at relatively late time points postinfection. Another explanation for the lack of observed epithelial cell invasion by *Y. enterocolitica* in mice could be that the mouse model does not accurately represent invasion of intestinal epithelia by this species. In contrast, rabbits which develop diarrhea in response to infection with *Y. enterocolitica* (15, 47) may serve as more relevant models in this respect and may exhibit a form of infection closer to that of humans.

Eckmann et al. (20, 21) have suggested that IL-8 secreted by epithelial cells is the initial signal for the acute inflammatory response that follows bacterial invasion of mucosal surfaces. IL-8 secretion in response to bacterial entry is predominantly basolateral, indicating that secreted IL-8 accumulates in mucosae underlying the epithelial cell layers where IL-8-responsive effector cells reside. The data presented here suggest that virulent *Y. enterocolitica* organisms have the capacity to suppress the release of IL-8, which they would induce by invading intestinal epithelia. We speculate that this ability favors *Y. enterocolitica* especially during the early phase of infection. Further histopathological and histoimmunological studies with experimentally infected animals are required to determine whether reduced secretion of IL-8 by infected ileal epithelial cells and subsequent reduced rates of infiltration by PMNs are of importance in the early steps of the pathology of *Y. enterocolitica.*

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