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Salmonella typhi Stimulation of Human Intestinal Epithelial Cells Induces Secretion of Epithelial Cell-Derived Interleukin-6

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Interleukin 6 (IL-6) is a multifunctional cytokine that has been shown to be associated with both systemic and tissue-specific responses within the host. Moreover, IL-6 is produced by both lymphoid and nonlymphoid cells and has been identified as a growth-inducing, growth-inhibiting, and differentiation-inducing factor for these cells. Recent studies of uropathogenic and upper respiratory pathogens have suggested that epithelial cell-derived IL-6 plays a role in mucosal host-parasite interactions. Since many mucosal enteric pathogens enter the host through the epithelial cells of the distal small intestine, a role for intestinal epithelial cell-derived IL-6 in the initial interaction between bacteria and host might also be predicted. However, no studies to date have determined whether the interaction of any bacteria with the epithelial cells that line the small intestine of the host can induce IL-6. To address this issue, we have established an in vitro model to evaluate the capacity of the gram-negative bacterium *Salmonella typhi* to induce IL-6 in the small intestine epithelial cell line Int407 and in other intestinal epithelial cell lines. The results demonstrate that both wild-type and live, attenuated *S. typhi* vaccine strains induce small and large intestine epithelial cells to secrete IL-6, and kinetic analysis suggests that IL-6 may be one of the earliest responses following adherence and invasion of enteric organisms. Thus, these studies suggest a physiologic role for epithelial cell-derived IL-6 in the initial interactions between host and bacterium in the small intestine.

Interleukin 6 (IL-6) is a multifunctional cytokine that is involved in diverse biological responses of the host (reviewed in references 4, 26, 42, 43, 80). In addition to playing roles in hematopoiesis (30, 36) and in acute-phase reactions (9, 20, 26, 60), this cytokine has been shown to be a growth factor for both normal and neoplastic cells (6, 8, 29, 56). IL-6 also plays a role in normal immune responses to foreign antigens as illustrated by studies that show that IL-6 is involved in mucosal B-cell differentiation (10, 11, 31, 54), as well as in proliferation and differentiation of T cells (13, 32, 34, 46, 65). Moreover, IL-6 is elevated in the sera of patients who have urinary tract infections (24) and septic shock (28, 79) and of children and adults with shigellosis (63, 64) and typhoid fever (40, 66). Peripheral blood mononuclear cells isolated from volunteers immunized orally with attenuated vaccine strains of *Salmonella typhi* can also be stimulated in vitro by *S. typhi* flagella to secrete levels of IL-6 that are significantly greater than the controls (71). Finally, IL-6 has been shown to be associated with host responses in several experimental infection models (14, 44, 48). Taken together, these studies suggest that IL-6 may play an important role in host-parasite interactions.

Recent investigations have proposed that the early inflammatory stage of enteric diseases could, in part, be regulated by cytokines derived from the intestinal epithelial mucosa (12, 39, 50, 64, 70). DeMan and colleagues have demonstrated that IL-6 can be detected in the mucosa of mice infected with uropathogenic *Escherichia coli* (15). In addition, these investigators showed that IL-6, IL-8, and IL-1α are secreted by bladder epithelial cell lines in the in vitro response to uropathogenic *E. coli* (2, 3, 25). Arnold et al. (5) also showed that the human laryngeal epithelial cell line, HEp-2, secreted significant levels of IL-6 after invasion of both *Listeria monocytogenes* and *Yersinia enterocolitica*. Moreover, McGee et al. (51–53) demonstrated that the proinflammatory cytokine IL-1β and transforming growth factor β1 could act synergistically to induce IL-6 secretion in a rat small intestinal epithelial cell line (IEC-6) and that cholera toxin also stimulated IL-6 in these cells. Collectively, these studies suggest that epithelial cell-derived IL-6 may be involved in the early inflammatory response which occurs after exposure to members of the family *Enterobacteriaceae* and may contribute to the induction of host immune responses.

Since relatively little is known about the host factors that regulate either the early innate immune response or the late, adaptive, systemic stages of typhoid fever, we have developed an in vitro model to evaluate the early innate immune responses after exposure to the mucosal pathogen *S. typhi*. In this report, we analyzed the capacity of *S. typhi* to induce IL-6 in human small intestine epithelial cells. Our results demonstrate that significant quantities of IL-6 are induced in intestinal epithelial cells following exposure to virulent *S. typhi* and suggest that intestinal epithelial cell-derived IL-6 may be one of the earliest host responses following adherence and invasion by this enteric pathogen. These studies begin to elucidate the role(s) of intestinal epithelial cell-derived IL-6 in the initial stages of typhoid fever and suggest that IL-6 may also play a role in the early response to other enteric pathogens. These studies also expand the notion that host intestinal epithelial cells play an active role in the mucosal innate response to infectious agents (25, 50).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The following bacteria were used in this study: *S. typhi* ISP1820 (clinical isolate; gift of David Hone, Center for Vaccine Development, Baltimore, Md.), *S. typhi* Ty2 (clinical isolate; gift of Eric Elsinghorst, University of Kansas, Lawrence, Kans.), *S. typhi* Quailes strain (clinical isolate; gift of Alison D. O'Brien, Uniformed Services University of the

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Health Sciences, Bethesda, Md.), *S. typhi* CVD906 (33), and *S. typhi* CVD 908 (72). Luria-Bertani (LB) broth or agar (67) with 0.3 M NaCl was used for routine growth of bacteria, unless otherwise specified (75).

Cell lines and culture conditions. The culture system was established by utilizing the human embryonic intestinal epithelial cell line, Intestine 407 (27) (Int407; ATCC CCL6; American Type Culture Collection [ATCC], Rockville, Md.). The human colonic adenocarcinoma cell line Caco-2 (22) (ATCC HTB-37; ATCC), the human colonic adenocarcinoma cell line HT-29 (21) (ATCC XRB; ATCC), and the normal rat small intestine cell line IEC-6 (61) (ATCC CRL 1592; ATCC) were also used. The Int407, Caco-2, and HT-29 cell lines were grown and maintained in minimal essential medium (MEM; GibcoBRL Life Technologies, Inc., Gaithersburg, Md.) to which 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, Utah) and 2 mM glutamine (GibcoBRL) were added. The IEC-6 cell line was grown and maintained in Dulbecco's modified Eagle's medium with 4.5 g of glucose (Bio Whittaker, Walkersville, Md.) per liter to which 5% heat-inactivated FBS (Hyclone), 2 mM glutamine, and 0.1 U of bovine insulin (Sigma Chemical Co., St. Louis, Mo.) per ml were added. Stock epithelial cell cultures were maintained in 75-cm^2 culture flasks (Becton Dickinson Labware, Lincoln Park, N.J.) at 37°C in a 6% CO_2 atmosphere and split weekly. Monolayers for adherence and invasion assays were prepared by seeding either 2.5×10^5 cells in 1 ml of growth medium into each well of a 24-well tissue culture plate (Costar Corp., Cambridge, Mass.) or 1.3×10^6 cells in 5 ml of growth media in each well of a 6-well tissue culture plate (Costar Corp.). Confluent monolayers were obtained after 24 h. Immediately before adherence and invasion assays, the medium was removed from each well of the 24-well or 6-well tissue culture plates and replaced with 0.3 ml or 1 ml of fresh growth medium, respectively.

Quantitation of cell-associated bacteria. The assays used in the present studies were a modification of the procedures developed by Elsinghorst et al. (19) and Tartera and Metcalf (75). Briefly, bacteria were grown overnight at 37°C in a rotary shaking water bath (200 rpm) and then subcultured by 1:100 dilution in 10 ml of fresh LB medium containing 0.3 M NaCl, unless otherwise stated. The bacteria were grown to an A_{600} of 0.5 (mid- to late logarithmic phase), and 1 ml of each culture was centrifuged at 5,000 × *g* for 10 min. The pellets were resuspended in an equal volume of MEM supplemented with 10% FBS and 2 mM L-glutamine. For the 24-well plate assay, 25 μl of this suspension was added to each of 2 to 4 wells of intestinal epithelial cell monolayers, representing an initial inoculum of 2.5×10^6 to 6×10^6 CFU per well. In the six-well plate assay, 127.5 μl of the suspension was added to each of four wells of epithelial cell monolayers, representing an initial inoculum of 1.3×10^7 to 2.6×10^7 CFU per well. The plates were centrifuged at 2,000 × *g* for 10 min to permit bacteria to interact optimally with the cell monolayers.

For quantitation of cell-associated bacteria, bacteria were incubated with the monolayers of intestinal epithelial cells for 60 or 90 min at 37°C in 6% CO_2, unless otherwise stated, and then each well was rinsed six times with Earle's balanced salt solution (EBSS; GibcoBRL). The cell-associated bacteria were released with 0.2 ml of 1% Triton X-100 (Sigma) in phosphate-buffered saline (PBS) and mixed for 10 min on a platform rocker at room temperature. PBS (0.8 ml) was then added to each well, and the suspension was diluted in sterile saline. The numbers of CFU were quantified by plating the appropriate dilutions on LB agar.

For the invasion assays, bacteria were incubated with monolayers of intestinal epithelial cells for 90 min or 2 h at 37°C in a CO_2 incubator and then washed three times with EBSS. One milliliter (for 24-well plates) or 2.5 ml (for 6-well plates) of prewarmed MEM containing 100 μg of gentamicin per ml was added per well, and incubation was continued for an additional 90 min or 2 h to kill extracellular bacteria (75). The supernatant from each well was collected into 1.5-ml microcentrifuge tubes, centrifuged (8,000 × *g* for 10 min), and/or filter sterilized through a 0.22-μm-pore-size low-protein-binding filter (Millipore, Bedford, Mass.) to remove bacteria and cell debris. The supernatants were frozen at −20°C until assayed for IL-6. Subsequently, the wells of the 24-well tissue culture plates were washed three times with EBSS, and intracellular bacteria were released by lysis of the monolayer with 0.2 ml of 1% Triton X-100 in PBS and mixed for 10 min. An additional 0.8 ml of PBS was then added per well to resuspend the cell lysate, which was then diluted in saline and plated on LB agar for determination of viable bacterial counts. The wells of six-well plates were washed two times with EBSS and then one time with 0.25% trypsin (GibcoBRL). The cells were removed from the plate with 0.5 ml of 0.25% trypsin and gentle agitation for 5 min, after which the cells were transferred to 15-ml conical tubes containing MEM and 10% FBS. The cells were pelleted by centrifugation (1,000 × *g* for 5 min) and resuspended in 1 ml of PBS. To determine the number of intracellular bacteria, 100 μl of the cell suspension was added to 100 μl of 2% Triton X-100 (resulting in a final volume of 200 μl in 1% Triton X-100) and mixed for 10 min to release the bacteria. PBS (0.8 ml) was then added to each tube, and the suspension was diluted in sterile saline and plated on LB agar to determine viable bacteria counts. The percent cell-associated bacteria or invasion was calculated as follows: (CFU released by 1% Triton X-100/CFU in the initial inoculum of bacteria added to each culture well) × 100 = percent adherence or invasion.

The remaining cells from the six-well plate assay were pelleted by centrifugation (3,000 × *g* for 10 min) and resuspended in 1 ml of RNA-STAT60 (Tel-Test "B," Friendswood, Tex.). These suspensions were immediately frozen at −70°C until the mRNA could be extracted.

To determine the effect of cytochalasin D on the adherence/invasion of *S. typhi* and IL-6 induction by Int407 cells, a modification of the procedures described by Elsinghorst et al. (19) and Tang et al. (74) was used. Briefly, 30 min prior to infection, epithelial cell monolayers were overlaid with fresh MEM supplemented with various concentrations of cytochalasin D (stock containing 1 mg/ml in dimethyl sulfoxide; Sigma). The cells were incubated in MEM containing each different concentration of cytochalasin D throughout the adherence and invasion steps.

Cytokine assay. The cell line B13.29, which is dependent on IL-6 for its growth, has been described previously (15). For IL-6 determinations, the more-sensitive subclone, B9, was used and was kindly provided by Philip Morrissey (Immunex, Seattle, Wash.). The B9 cells were maintained in Dulbecco's MEM (BioWhittaker) supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin, and 50 ng of recombinant human IL-6 (rhIL-6; Genzyme Diagnostics, Cambridge, Mass.) per ml. To quantify levels of IL-6 in the supernatants of *S. typhi*-stimulated epithelial cell cultures, a modification of a standard IL-6 assay was employed (58). Briefly, the IL-6-dependent B9 cells were washed three times in rhIL-6-free media. Six thousand cells per well were seeded into microtiter plates (Costar) containing twofold dilutions of supernatant or the rhIL-6 standard in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 100 μg of gentamicin per ml in a total volume of 0.2 ml. After 48 h of culture, 1 μCi of [^{3}H]thymidine (1 mCi/ml; ICN Radiochemicals, Costa Mesa, Calif.) in a 10-μl volume was added to each well, and the cells were harvested onto paper filters 5 h later with a Brandel (Gaithersburg, Md.) cell harvester. The incorporated radioactivity was determined and compared with a standard curve generated with rhIL-6. The number of counts per minute for each concentration of rhIL-6 in the standard curve was plotted, and the line was subjected to linear regression analysis. The linear equation from each standard curve was used to extrapolate the amount of IL-6 (in picograms per milliliter) in the highest dilution of the sample supernatant that gave at least 50% maximal stimulation of proliferation. The significance of the data was evaluated by the Student *t* test.

The specificity of the assay for IL-6 was tested by determination of the capacity of a murine anti-human IL-6 monoclonal antibody, MAB206, (R&D Systems, Minneapolis, Minn.) to neutralize the IL-6 activity in the supernatants. An isotype-matched, murine anti-human tumor necrosis factor alpha (TNF-α) monoclonal antibody, MAB210 (R&D Systems), was used as a negative control.

RNA extraction and RT-PCR detection of cytokine mRNA. RNA was extracted from intestinal epithelial cell cultures prepared as described above, with an RNA-STAT 60 kit (Tel-Test "B", Inc.) according to the directions supplied by the manufacturer. Reverse transcriptase-PCR (RT-PCR) was performed by using a modification of the procedure previously described (49) to determine the quantities of mRNA for hypoxanthine guanine phosphoribosyltransferase (HPRT) and IL-6. Briefly, cDNA was prepared in a 25-μl volume containing 0.25 mM (each) deoxynucleotide triphosphate, 1× reverse transcription buffer (GibcoBRL), 8 mM dithiothreitol, 0.5 U of random hexamer oligonucleotides (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 1 μg of total RNA, and 0.5 U of RNasin (Promega, Madison, Wis.). The reaction mixture was heated to 70°C for 5 min and then cooled on ice for 5 min. Following this cooling step, 200 U of Superscript II RT (GibcoBRL) was added, and the reaction mixture was incubated first at 37°C for 60 min and then at 90°C for 5 min to inactivate the Superscript II RT, cooled on ice for 3 min, and stored at −20°C. The final reaction mixture was diluted 1:8 by the addition of 175 μl of distilled H_2O prior to freezing.

The primers and probes for human HPRT and IL-6 were prepared on a DNA synthesizer (Applied Biosystems, Foster City, Calif.) and have been previously described (57). Specific cytokine gene cDNA transcripts were PCR amplified with 10 μl of cDNA in a 50-μl 1× reaction mixture (0.25 mM mix of each deoxynucleoside triphosphate, PCR buffer [Promega], 0.2 μM sense and antisense primers, 1 U of *Taq* polymerase [Promega]). The samples were heated at 95°C for 5 min, and then temperature cycling was started (1 min of denaturation at 95°C, 2 min of annealing at 50°C, and 3 min of extension at 72°C). The PCR conditions for each primer set were adjusted to ensure that a linear relationship between the added cDNA and amplified product was obtained; in each experiment, the housekeeping gene, HPRT, was also amplified. The number of PCR cycles selected for each cytokine was 26 for HPRT and 30 for IL-6. After the appropriate number of PCR cycles, 15 μl of each amplified DNA was separated by electrophoresis in a 1% agarose gel. The gel was subjected to denaturation and neutralization, and the DNA was transferred by capillary action to a Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, Calif.) and cross-linked to the membrane both by UV light with a UV Stratalinker 1800 (Stratagene, La Jolla, Calif.) and by being baked in a vacuum hybridization oven at 80°C for 2 h (67). The membranes were hybridized at 42°C for 2 h with a non-radioactive probe (57), as described in the ECL gene detection kit (Amersham Corporation, Arlington Heights, Ill.). The blots were washed two times for 5 min each at room temperature in 5× SSC (0.075 M sodium citrate, 0.75 M NaCl) with 0.1% sodium dodecyl sulfate (SDS) and then two times at 42°C for 15 min each in 1× SSC (0.015 M sodium citrate, 0.15 M NaCl) with 0.1% SDS. The hybridized probes were detected as described in the ECL detection system (Amersham Corp.) by the manufacturer. The membranes were exposed at room temperature for 2 to 10 min to X-Omat AR film (Kodak, Rochester, N.Y.). The chemiluminescent sig-

nals were scanned with a Datacopy GS Plus scanner (Xerox), and the area of each band was calculated by using the National Institutes of Health Image 1.57 program, recently described by Manthey et al. (49). Briefly, the magnitude of change in gene expression was determined by the development of a standard curve for every PCR amplification. This standard curve was established by making twofold dilutions of the PCR-amplified product from cDNA of a sample known to be positive for each transcript and then detecting the diluted PCR products as described above. The area of each band of the standard curve was plotted, and the line generated was subjected to linear regression analysis. The equation for each line was used to calculate the relative levels of gene expression in experimental samples. Expression of human HPRT mRNA was also evaluated as a housekeeping gene in every experimental sample to ensure uniformity of a constitutively expressed gene. The calculated HPRT mRNA concentration did not differ more than two- to threefold. The relative level of expression of HPRT in each experimental sample was compared to that of HPRT for uninfected samples to normalize for RNA in the RT reaction. Expression of a given RNA by uninfected epithelial cell cultures was arbitrarily assigned a value of 1, and the expression of IL-6 RNA by intestinal epithelial cell cultures in other experimental groups was expressed relative to this baseline.

RESULTS

Intestinal epithelial cells are stimulated by *S. typhi* strains to secrete IL-6. Since previous studies had shown that production of cytokines played a role in inflammatory responses induced by enteric bacteria (39, 50, 64), the current study was undertaken to assess the capacity of the host intestinal epithelial cells to secrete IL-6 in response to exposure to *S. typhi*. IL-6 was chosen for these initial studies because this cytokine has been shown to play a central role in numerous diverse biological systemic responses and has been implicated as a physiologic molecule in epithelium-parasite interactions in other systems (14, 44, 48). Although results from this laboratory (75) and others (14, 44, 48) have shown that *S. typhi* can adhere to (75) and invade (23, 55, 59, 75) human intestinal epithelial cell lines, few studies, to date, have focused on the effect(s) of such adherence and invasion on the eukaryotic host. Since *S. typhi* is a host-restricted pathogen and initially penetrates the small intestine epithelium, a human cell line of small intestine origin, Int407, was selected for our initial studies. Confluent monolayers of Int407 cells were exposed to fresh medium or medium containing one of three wild-type strains of *S. typhi*. After the monolayers were washed, medium containing gentamicin was added to kill extracellular organisms. Subsequently, the supernatants were collected and the monolayers were lysed to determine the percent invasion. IL-6 induction was assessed by a bioassay that utilizes the IL-6-dependent B9 cell line. The results of our initial experiments are shown in Table 1 and demonstrate that all three *S. typhi* strains induce Int407 cells to secrete significant quantities of IL-6 compared to the control, uninfected Int407 cell monolayers. Our observation is the first demonstration that IL-6 is induced by small intestine epithelial cells after specific stimulation with pathogenic enteric bacteria. In contrast, the noninvasive *E. coli* strain DH5α induced secretion of a maximum of 180 pg of IL-6 per ml over a range of bacteria (1 to 1×10^3) (data not shown).

The ability of *S. typhi* to associate with and invade this cell line is also indicated by the results in Table 1. The results indicate that all three strains of *S. typhi* significantly adhered to and invaded this human intestinal epithelial cell line. The percent invasion for two of these *S. typhi* strains was greater than 100%, which reflects bacterial multiplication in the culture well during the first 90 to 120 min of the assay, since previous studies from this laboratory have shown that *S. typhi* does not replicate intracellularly in this cell line (75).

To confirm that the biologically active IL-6 detected in the Int407-derived culture supernatants was specific IL-6 and not a cross-reactive factor capable of stimulating the IL-6-dependent B9 cell line, five 50% effective doses of supernatant from *S. typhi* ISP1820-stimulated Int407 cells were incubated with various concentrations of the monoclonal murine anti-human IL-6 antibody, MAB206. The results in Fig. 1 demonstrate that 2 μg of the anti-human IL-6 antibody per ml neutralized the B9-stimulating activity to the level of the background control. In contrast, the isotype-matched murine anti-human TNF-α antibody did not inhibit any of the *S. typhi*-induced IL-6 activity derived from the Int407 cells at any concentration of antibody tested. Taken together, these data support the notion that *S. typhi* stimulates specific, biologically active IL-6 secretion in Int407 cells.

TABLE 1. Stimulation of human intestinal epithelial cells by *S. typhi* induces secretion of biologically active IL-6[a]

Culture	% of cell-associated bacteria[b]	% Invasion[c]	IL-6 secreted (pg/ml) (fold increase)[d]
Control (uninfected)			690 ± 255[e]
S. typhi ISP1820	62	92	7,157 ± 1,389 (10.4)
S. typhi Ty2	25	304	15,570 ± 5,472 (22.6)
S. typhi Quailes	23	245	16,653 ± 5,379 (24.1)

[a] Monolayers (in quadruplicate) of Int407 cells were infected with 3×10^6 to 5×10^6 *S. typhi* organisms of each strain. After 2 h of incubation, the cultures were washed and gentamicin was added for an additional 2 h. The supernatants were collected for IL-6 determination, the monolayers were lysed, and the number of CFU was determined. Monolayers of Int407 cells infected with *E. coli* HB101 did not secrete levels of IL-6 significantly above those for uninfected cells (data not shown).

[b] Percentage of bacteria associated with the monolayers after 1 h of incubation with respect to the initial inoculum.

[c] Percentage of bacteria resistant to gentamicin treatment after 2 h with respect to the initial inoculum.

[d] Values are the mean IL-6 concentrations in the supernatants from quadruplicate cultures ± standard deviations as determined by the B9 bioassay; the data are representative of five separate experiments. The results in parentheses are the fold increases above the uninfected control.

[e] Statistically significantly different from values obtained for *S. typhi*-stimulated cultures ($P < 0.01$).

***S. typhi* induces IL-6 specific mRNA in Int407 cells.** To assess specific induction of IL-6, steady-state levels of IL-6 mRNA in *S. typhi*-infected Int407 cells were also analyzed. RT-PCR was performed with the RNA isolated from both infected and uninfected Int407 cells, and the results in Fig. 2 demonstrate that IL-6-specific mRNA from *S. typhi*-infected cells increased 40- to 60-fold compared to that of the uninfected control cells. These data are consistent with the B9 bioassay data showing protein secretion of IL-6 (Table 1).

Epithelial cell lines derived from both the small and large intestine are able to secrete IL-6 after stimulation by *S. typhi*. To substantiate further our observations that intestinal epithelial cells are able to secrete IL-6 after exposure to *S. typhi*, and to determine if IL-6 production was a general consequence of epithelial cell infection by *S. typhi*, three other intestinal epithelial cell lines were stimulated with *S. typhi* ISP1820 and their supernatants were tested for secretion of IL-6 by the B9 bioassay. Caco-2 and HT-29, both of colonic origin, can be invaded by *S. typhi* and other *Salmonella* spp. (75, 76). Previously, McGee et al. (53) showed that IEC-6, a normal, rat, small intestine, epithelial cell line, could be stimulated by purified cholera toxin to secrete IL-6, but cytokine induction by whole bacteria was not assessed. We have demonstrated that *S. typhi* can significantly adhere to and invade these IEC-6 cells (data not shown). More importantly, the results in Fig. 3 demonstrate that *S. typhi* infection induced both Caco-2 and IEC-6 cells to secrete significant quantities of biologically active IL-6 compared to those secreted by uninfected control monolayers. Moreover, increased quantities of IL-6-specific mRNA pre-

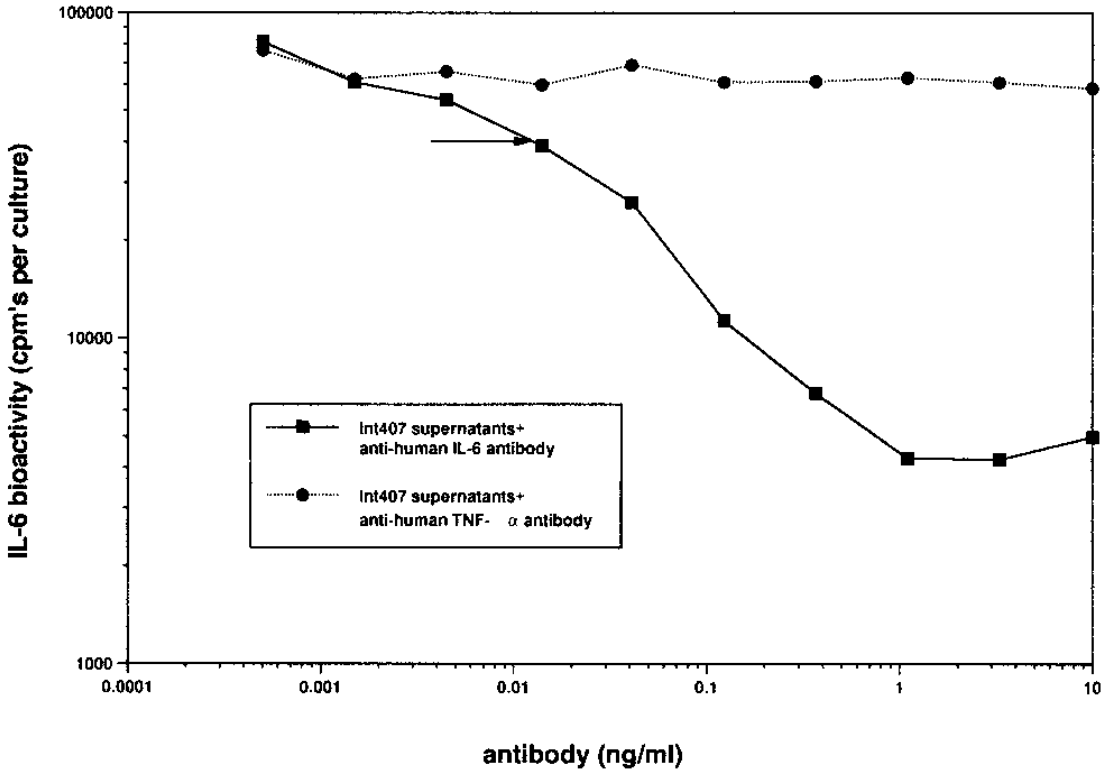

FIG. 1. Monoclonal murine anti-human IL-6 antibody neutralizes Int407-derived IL-6. Int407 supernatant, from cells stimulated with *S. typhi* ISP1820 in a standard invasion assay, was incubated 1 h with dilutions of monoclonal murine anti-human IL-6 antibody MAB206 (■) or isotype matched murine anti-human TNF-α monoclonal antibody MAB210 (●). The proliferation of the B9 cells after 48 h is shown as counts per minute of [^{3}H]thymidine incorporated by proliferating B9 cells. The arrow indicates the concentration of antibody required to inhibit half of the maximal IL-6 activity when the concentration of IL-6 in that supernatant is present at five times its normal 50% effective dose. These results are representative of two separate experiments.

pared from *S. typhi*-infected Caco-2 cells were also observed (Fig. 2). To confirm that the biologically active IL-6 detected in the *S. typhi*-infected Caco-2 cells was specific IL-6, supernatants were neutralized by a monoclonal anti-human IL-6 antibody but not a monoclonal anti-human TNF-α antibody. In contrast, HT-29 cells secreted small quantities of IL-6 constitutively, and these levels of cytokine secretion were not further augmented after exposure to *S. typhi* (data not shown). Taken together, these results support the notion that epithelial cells derived from both the small and large intestines can be stimulated by *S. typhi* to secrete IL-6.

The parameters of IL-6 induction in intestinal epithelial cells are consistent with a physiologic role for epithelial cell-derived IL-6. Previous studies in this laboratory (75) demonstrated that environmental conditions can significantly affect the capacity of *S. typhi* to adhere to and invade intestinal epithelial cells. Therefore, the possibility that such variables could also affect the level of cytokine induction in the host cells was next explored. The optimal osmolarity of the bacterial growth media for IL-6 inducibility was determined, and the B9 assay results in Table 2 demonstrate that the *S. typhi* wild-type strain Ty2 was more adherent and invasive and better able to induce Int407 cells to secrete IL-6 when the bacteria were cultured in high-osmolarity medium, LB supplemented with 0.3 M NaCl. Similar results were shown for *S. typhi* ISP1820 (data not shown). These data extend and confirm the previous findings of Tartera and Metcalf, who reported that attachment of *S. typhi* Ty2 to intestinal epithelial cells was osmoregulated (75).

Since an earlier study with *Salmonella dublin* had shown that the levels of the intestinal epithelial cell-derived chemokine, IL-8, correlated with the multiplicity of infection (MOI) (18), the optimal MOI for induction of IL-6 was also investigated in our experimental model. Int407 monolayers were exposed to increasing concentrations of *S. typhi*, and the quantity of IL-6 cytokine secreted in the supernatants was assessed. Figure 4 illustrates that the optimal MOI for IL-6 secretion by Int407 cells was approximately 20 bacteria per epithelial cell. Lower MOIs induced smaller quantities of IL-6 and MOIs of less than 0.3 bacterium per cell did not induce secretion of IL-6 levels above the level in the uninfected control. Since 90 *S. typhi* organisms per epithelial cell were observed microscopically to lead to cell detachment, the slight decrease in IL-6 induction observed at the higher MOI is most likely a consequence of eukaryotic cell death and disruption of the cell monolayers. The amount of IL-6 induced by each MOI directly correlated with the relationship between the MOI and the number of bacteria surviving gentamicin treatment in each well.

To determine if the production of IL-6 by epithelial cells was an early response to *S. typhi* exposure, the kinetics of IL-6 production were examined. In a preliminary study, Int407 cells were incubated with various concentrations of *S. typhi* ISP1820, and the percentage of cell-associated bacteria was determined every 30 min for 4 h. These data established that a 90-min incubation step prior to the addition of gentamicin was optimal for the induction of biologically active IL-6. This study was expanded, and as shown in Table 3, IL-6-specific mRNA was detected by 60 min postinfection, peaked at 150 min, and then gradually declined. Under these culture conditions, cells in the monolayer begin to slowly detach from the culture wells at

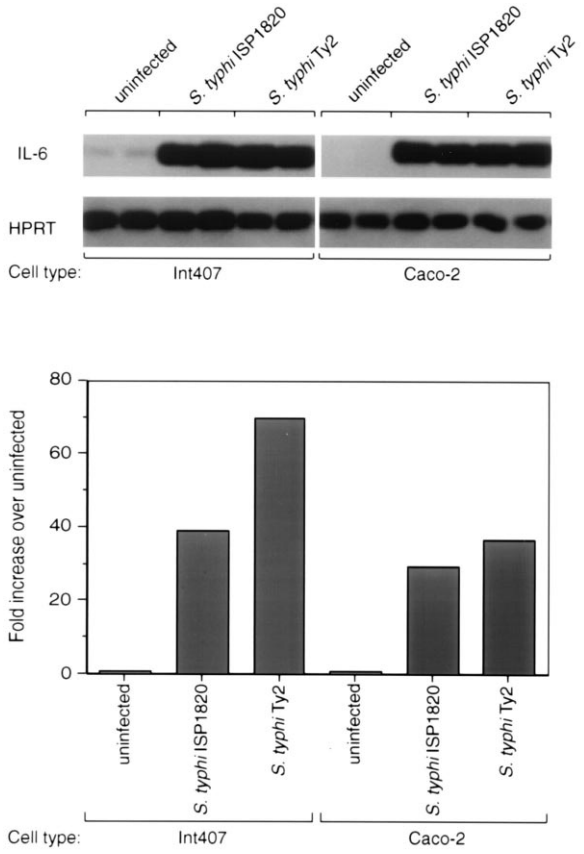

FIG. 2. Cytokine gene expression by Int407 and Caco-2 cells stimulated with *S. typhi* ISP1820 and Ty2. RNA was prepared from 2.6×10^6 uninfected Int407 or Caco-2 cells stimulated with *S. typhi* ISP1820 or Ty2 at MOIs of 20 to 30 bacteria per cell in a standard invasion assay. cDNA was prepared from 1 μg of each mRNA sample, and RT-PCR was performed with primers specific for human IL-6 and the housekeeping gene HPRT. Blots were hybridized with ECL-labelled oligonucleotide probes specific for the corresponding genes. Autoradiographs were scanned, and the fold increase of the IL-6-specific cytokine gene was calculated by comparing the signals elicited by bacteria to those elicited by medium alone. These results are representative of two separate experiments.

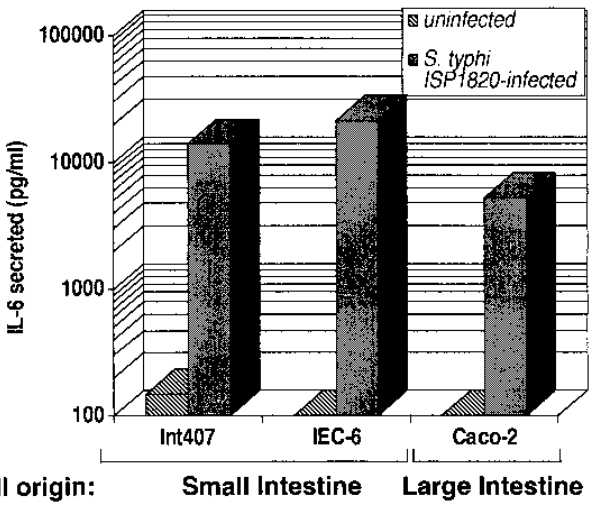

FIG. 3. Induction of IL-6 secretion by *S. typhi* ISP1820-stimulated epithelial cells from both small and large intestines. Monolayers of Int407, IEC-6, and Caco-2 (2.5×10^5 cells per well of a 24-well tissue culture plate) were overlaid with medium (uninfected control) or infected at a bacterium-to-cell ratio of 20:1 with *S. typhi* ISP1820 and incubated for 2 h to allow the bacteria to adhere and invade. Cultures were washed and incubated in the presence of gentamicin for an additional 2 h to eliminate extracellular bacteria. At the end of the culture period, the supernatants were removed, and the concentration of IL-6 in each supernatant was determined by B9 bioassay. The intestinal epithelial cells were lysed, and the number of viable intracellular bacteria was determined. These results are representative of two independent experiments.

approximately 3.5 to 4 h. However, since RNA values are based on micrograms of RNA per milliliter, detachment of monolayer cells would not influence the levels of cytokine-specific mRNA. Nonetheless, this detachment could account for the decrease observed in adherence during the later times.

Biologically active IL-6 was detected by 90 min postinfection and peaked at 210 min. The seemingly larger quantities of IL-6 observed at later times in this experiment are a result of using 2×-concentrated supernatants from the six-well culture dishes. Collectively, these data indicate that optimal secretion of IL-6 is influenced by the environment of the small intestine and occurs early after infection with relatively low concentrations of bacteria.

Cytochalasin D inhibits bacterial invasion but does not abolish IL-6 induction. The results of the time course experiment (Table 3) suggested that only adherence of *S. typhi* to Int407 cells was required for stimulation of IL-6. To confirm that bacterial cell adherence was sufficient, Int407 cells were preincubated with various concentrations of cytochalasin D. Since previous studies have shown that cytochalasin D inhibits bacterial invasion of epithelial cells by disruption of actin rearrangement and membrane ruffling (41, 74), it is possible to assess adherence independently of invasion in the presence of cytochalasin D. Thus, a standard assay for quantitation of cell-associated bacteria and invasion was performed, and supernatants were assessed for the presence of bioactive IL-6. The results in Table 4 show that preincubation of the cultures with 0.75 and 0.50 μg of cytochalasin D inhibited invasion of the *S. typhi* by greater than 90% but did not affect the percentage of cell-associated (adherent) bacteria, compared to media alone. Cytochalasin D did not affect the viability of either the Int407 cells or *S. typhi* (data not shown). Most importantly, the quantity of IL-6 secreted by the *S. typhi*-infected Int407 cells treated with cytochalasin D was essentially the same as that secreted by Int407 cells produced in the absence of cytochalasin D treatment, at all concentrations tested. These data suggest that bacterial invasion is not required for IL-6 induction in Int407 intestinal epithelial cells.

***S. typhi* vaccine strains are able to induce IL-6 secretion in Int407 cells.** Since there is no small-animal model for *S. typhi*,

TABLE 2. Increasing the osmolarity of the growth medium for *S. typhi* Ty2 increases the percent invasion and the concentration of IL-6 induced in Int407 cells[a]

NaCl (g/liter of LB)	Osmolarity[b]	% Invasion	IL-6 secreted (pg/ml)
0 (uninfected control)			490
0.06	283	33	8,100
0.17	446	112	21,980
0.30	676	184	31,505

[a] *S. typhi* Ty2 was grown in LB supplemented with increasing concentrations of NaCl. Monolayers (in duplicate) of Int407 cells were infected with 3×10^6 to 5×10^6 bacteria. After 2 h of incubation, the cultures were washed and gentamicin was added for an additional 2 h. The supernatants were collected for IL-6 determination, the monolayers were lysed, and the number of CFU was determined. These results are representative of three similar experiments.

[b] Osmolarity (in milliosmoles per kilogram of water) was calculated as described by Tartera and Metcalf (75).

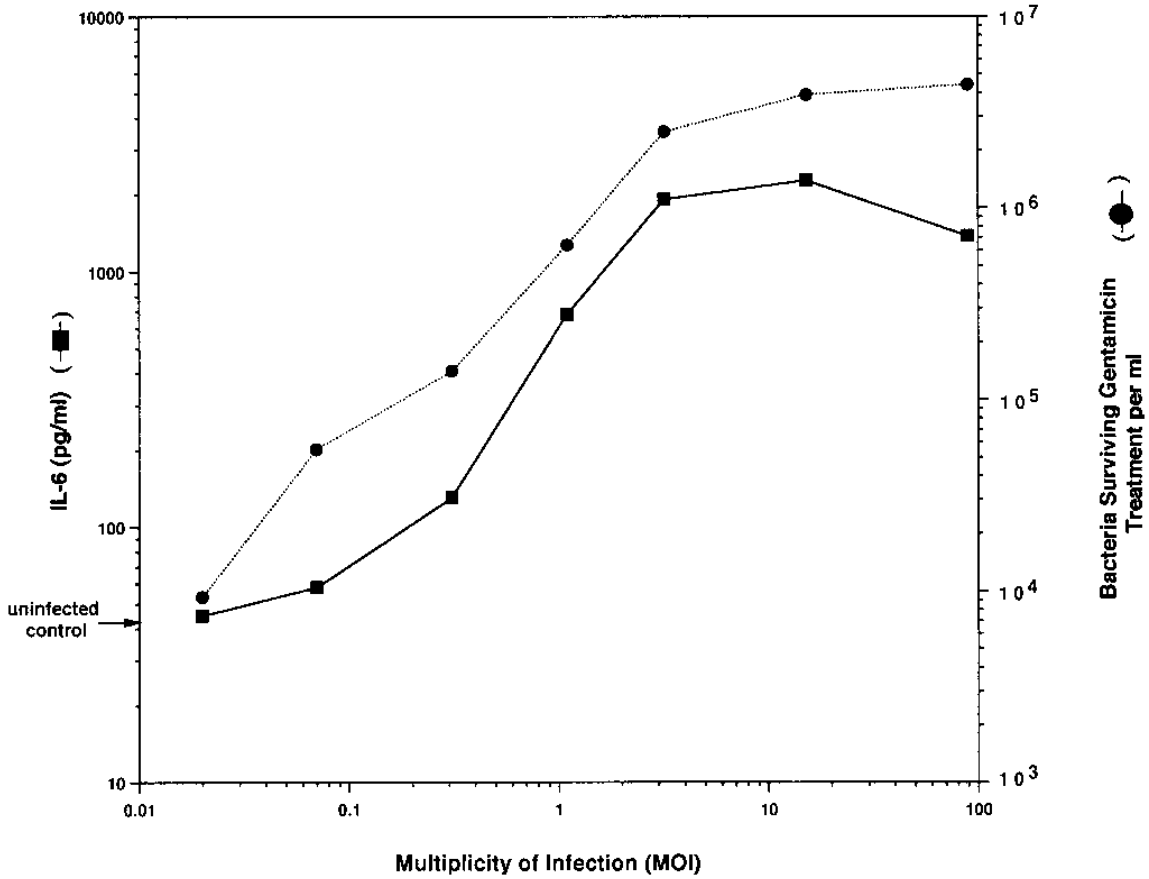

FIG. 4. Relationship of MOI of *S. typhi* ISP1820-induced IL-6 secretion by intestinal epithelial cells. Confluent monolayers of Int407 cells (2.5×10^5 cells per well of a 24-well tissue culture plate) were infected with various doses of *S. typhi* ISP1820 and incubated for 90 min to allow the bacteria to adhere and invade. After removal of the extracellular bacteria by washing, the cultures were further incubated in the presence of gentamicin for an additional 90 min. At the end of the culture period, the supernatants were removed, and the concentration of IL-6 in each supernatant was determined by bioassay. The Int407 cells were lysed, and the number of bacteria surviving gentamicin treatment was determined. These results are representative of two independent experiments.

one possible alternative approach for the in vitro assessment of vaccine strains might be to determine if such strains induced cytokine responses in the host cells. To this end, the live, attenuated *S. typhi* vaccine strains CVD906 (33) and CVD908 (72) were analyzed for their capacities to induce IL-6 in Int407 cells. These vaccine strains are Δ*aroC* Δ*aroD* mutants which have been shown to be relatively nonpathogenic in human volunteer studies (33, 72, 73). The results in Table 5 show that both CVD906 and CVD908 were able to invade Int407 cells and to induce significant secretion of IL-6 by the Int407 cells. Taken together, these results suggest that IL-6 secretion may be a physiologically relevant parameter to measure in this in vitro experimental model of host-parasite interactions and may provide an additional approach to analyzing the efficacy of potential vaccine strains prior to human volunteer trials.

DISCUSSION

These studies were undertaken to determine whether intestinal epithelial cell-derived IL-6 could be one of the initial host responses that follow the interaction of *S. typhi* with the intestinal mucosa. Our studies demonstrate that exposure of both small and large intestine epithelial cells to *S. typhi* induces secretion of significant quantities of biologically active IL-6. In addition, IL-6 production occurs early after infection, does not require invasion of the epithelial cells by the bacterium, is influenced by the microenvironment of the small intestine, and requires only small numbers of bacteria. Taken together, these data imply a physiological role for intestinal epithelial cell-derived IL-6 in the initial responses between host and parasite in typhoid fever.

Several studies in other experimental systems have contributed to the notion that intestinal epithelial cell-derived IL-6 may play a physiologic role in the initial responses of the host to *S. typhi* and perhaps other enteric pathogens. Shirota et al. (70) and Jones et al. (37) showed that IL-6 was expressed in human small intestine epithelial cells by immunohistochemical staining of normal tissue specimens. More recently, McGee and his colleagues (51–53) demonstrated that IL-6 was secreted by the rat intestinal epithelial cell line, IEC-6, after exposure to transforming growth factor β, cholera toxin, and IL-1β. Until the present study, the induction and secretion of IL-6 by small intestine epithelial cells in response to a mucosal, enteric, pathogenic bacterium had not yet been evaluated. Thus, the data presented herein are the first to demonstrate that exposure of human small intestine epithelial cells to *S. typhi* induces biologically active IL-6 secretion, which may play an early role in the response of the host to *S. typhi.*

In the present study, significant increases of IL-6-specific mRNA and biologically active IL-6 protein could be detected within 90 min after exposure of intestinal epithelial cells to *S. typhi* compared to that in uninfected controls. This early ex-

TABLE 3. *S. typhi* ISP1820 induces IL-6-specific mRNA by 60 min postinfection and secretion of IL-6 in Int407 cells by 90 min postinfection[a]

Treatment and time postinfection (min)	% of cell-associated bacteria	Fold increase in IL-6-specific mRNA[b]	IL-6 secreted (pg/ml)
None (uninfected control)		1.0	460
30	36	3.3	330
60	55	5.6	370
90	88	17.0	3,410
Gentamicin			
120	94	24.2	2,790
150	98	48.0	11,900
210	73	6.0	37,960
270	68	13.1	45,090
330	78	14.8	55,870

[a] Monolayers of Int407 cells were infected as described in Table 1, footnote *a*. Samples were taken every 30 min, and mRNA was prepared, the total number of CFUs was determined, or IL-6 activity in supernatants was assessed, as described in Table 1, footnote *a*. The results of this experiment are representative of those for two similar experiments.

[b] Fold increase over level in uninfected cells.

pression of IL-6 after exposure to bacteria is consistent with the results of studies by Hedges et al. (25), who demonstrated secretion of IL-6 in a bladder epithelial cell line as early as 2 h after stimulation with uropathogenic *E. coli*. Indirect support for an early role for IL-6 after infection has also been shown in in vivo studies. Klimpel et al. (44) demonstrated that IL-6 was secreted into murine ligated intestinal loops in response to *Salmonella typhimurium* exposure, although the cellular source of this cytokine was not determined. Liu and Cheers (47) and Liu et al. (48) showed that IL-6 was secreted by nonlymphoid cells early after infection by the gram-positive organism *L. monocytogenes*. Moreover, Dalrymple et al. (14), using IL-6-deficient mice, showed that IL-6 also was critical early after infection with *L. monocytogenes*. In contrast, secretion of IL-8 and other chemokines (17, 39) appears to be a later event in the interaction between bacteria and intestinal epithelial cells. In some cases, secretion did not occur until 8 to 10 h after exposure of the epithelial cells to the bacteria (35, 69). Taken together, these data suggest that IL-6 induction may represent one of the earliest host responses to live, virulent, enteric bacteria.

TABLE 4. Cytochalasin D inhibits invasion of *S. typhi* ISP1820 into Int407 cells but does not abolish IL-6 induction[a]

Sample and amt of cytochalasin D (μg/ml) added	% of cell-associated bacteria (fold inhibition)	% Invasion (fold inhibition)	IL-6 secreted (pg/ml)
Uninfected control (0)			220
Infected cultures			
0	100	114	15,420
0.75	95 (1.1)	2 (57)	10,180
0.50	104 (1.0)	7 (16)	14,450
0.10	93 (1.1)	35 (3)	17,580
0.02	80 (1.2)	57 (2)	11,730
0.004	72 (1.4)	111 (1)	17,330

[a] Cytochalasin D was freshly prepared on the day of each experiment and added to the incubation mixtures at the indicated concentrations for the duration of the experiment. Quantitation of cell-associated bacteria, invasion, and IL-6 was as described in Table 1, footnote *a*, and Materials and Methods. These results are representative of five similar experiments.

A second feature of these results which suggests that IL-6 secretion by intestinal epithelial cells is a physiologically relevant event in host-parasite interactions is that the optimal parameters for IL-6 stimulation paralleled the optimal conditions for *S. typhi* adherence to and invasion of intestinal epithelial cells. Previous findings from this laboratory showed that high osmolarity and growth phase regulate *S. typhi* adherence and invasion (75). The present study demonstrates that optimal IL-6 production occurred after growth of *S. typhi* in high-osmolarity medium. In addition, the observation that an optimal MOI of approximately 20 bacteria/epithelial cell indicates that relatively few pathogenic *S. typhi* organisms need to interact with the intestinal epithelium in order to initiate the induction of IL-6. In contrast, the nonpathogenic *E. coli* strain DH5α stimulated only minimal levels of IL-6 even at a high MOI. The capacity of small numbers of pathogenic bacteria to induce this host response would be consistent with a physiologic role for IL-6, since a host response would presumably be more effective if it were sensitive to low doses of infecting pathogenic bacteria.

The kinetics of induction of IL-6 suggested that adherence of *S. typhi* to intestinal epithelial cells was sufficient to induce IL-6 production. To extend and confirm this notion, epithelial cells were treated with cytochalasin D prior to exposure to *S. typhi*, and IL-6 secretion was subsequently assessed. Since cytochalasin D inhibited *S. typhi* invasion but not adherence, the observation that IL-6 secretion was not abolished by this pretreatment supports the contention that adherence of *S. typhi* is sufficient for IL-6 induction by small intestine epithelial cells. In contrast, Eckmann and colleagues (17, 18) have suggested that bacterial entry is required for induction of the chemokine IL-8 by colonic epithelial cells after stimulation with *S. dublin*. Nevertheless, our studies are in accord with other reports that demonstrate that only adherence to epithelial cells is required for the secretion of IL-8 in response to *E. coli* (3), *Pseudomonas aeruginosa* (16), *Helicobacter pylori* (35, 69), and *L. monocytogenes* (5). More importantly, Hedges et al. (25) showed that only adherence of *E. coli* to bladder epithelial cells was required for IL-6 induction. This issue could be resolved by construction of an *S. typhi* strain that is as adherent as the wild type but noninvasive in epithelial cells. It would be predicted that such a mutant could induce significant levels of epithelium-derived IL-6. These studies are currently under way.

Involvement of IL-6 in mucosal infections has been implicated by several investigators. First, Beagley et al. (10, 11) have shown that IL-6 can act as terminal differentiation factor for B cells activated to secrete immunoglobulin A in the lamina

TABLE 5. *S. typhi* live, attenuated vaccine strains CVD 906 and CVD 908 induce IL-6 in human intestinal epithelial cells[a]

Bacterial strain genotype	Relevant	% Invasion	IL-6 secreted (pg/ml)
None (uninfected control)			410
S. typhi ISP1820	Wild type	180	5,040
S. typhi CVD 906	Δ*aroC* Δ*aroD* ISP1820	163	5,310
S. typhi Ty2	Wild type	261	8,000
S. typhi CVD 908	Δ*aroC* Δ*aroD* Ty2	103	5,910

[a] Int407 monolayers were not infected or infected with 3×10^6 to 6×10^6 *S. typhi* organisms of either the wild type or vaccine strain, and standard invasion and IL-6 assays were conducted. Values are the means for duplicate cultures from a representative experiment repeated three times.

propria. Since Ramsey et al. (62) and Kopf et al. (45) have also observed that mice with targeted disruptions in their IL-6 gene express a significant reduction in mucosal immunoglobulin A antibody responses to *L. monocytogenes*, it is possible that locally produced, intestinal epithelial cell-derived IL-6 may provide the terminal differentiation step for these mucosal B cells. Second, de Man et al. (15) have shown that uropathogenic *E. coli* can activate IL-6 secretion at the mucosal surface of the urinary tract. Finally, McGee et al. (53) have suggested that a localized infusion of IL-6 to the lamina propria of the small intestine may enhance an anti-cholera toxin-specific antibody response. In a physiologic system, epithelial cell-derived IL-6 could provide the equivalent exposure. Taken together, these data are consistent with our findings and imply that small intestine epithelial cells infected with *S. typhi* may secrete IL-6 locally and, as a consequence, enhance mucosal immune responses.

A second role for intestinal epithelial cell-derived IL-6 is suggested from other studies that show an anti-inflammatory role for IL-6. Our results imply that epithelial cell-derived IL-6 could serve as an anti-inflammatory cytokine to down-regulate the local inflammatory response in the gastrointestinal tract that occurs after infection with *S. typhi*. Support for this contention comes from the study of Aderka et al. (1), who demonstrated that IL-6 could reduce LPS-induced TNF-α secreted by peripheral blood monocytes and U937 macrophage-like cells. Schindler et al. (68) also reported that IL-6 had a suppressive effect on the production of LPS-induced TNF-α and IL-1β by peripheral blood monocytes. Ulich et al. (78) observed that injected IL-6 will inhibit acute inflammation, while Barton and Jackson (7), studying the role of IL-6 in a galactosamine-sensitized, LPS-induced model of sepsis, concluded that IL-6 played a regulatory role by down-regulating production of TNF-α and IL-1. Recently, Tilg et al. (77) showed that cancer patients receiving rIL-6 therapy had enhanced levels of tissue macrophage-derived IL-1 receptor antagonist (IL-1Ra) in their sera that has been shown to mitigate the activity of IL-1 in an inflammatory response. Finally, and perhaps most intriguing, is the study by Jordan and colleagues (38) which demonstrated that IL-6 induced after oral infection with *Y. enterocolitica* subsequently stimulated IL-1Ra production and secretion by circulating granulocytes. Thus, it is feasible that IL-6 secreted by intestinal epithelial cells after infection by *S. typhi* could subsequently induce IL-1Ra in the lamina propria by infiltrating granulocytes and/or macrophages, which, in turn, could counteract the IL-1 produced as a result of the localized inflammatory response.

In conclusion, this study is the first demonstration of IL-6 production by small intestine epithelial cells in response to a human mucosal pathogen. The expression and secretion of epithelium-derived IL-6 after infection by *S. typhi* strongly suggest that this cytokine plays a relevant physiologic role in the initial interaction between the host and parasite in typhoid fever and perhaps in other infections with enteric bacteria.

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