Production of Proinflammatory Cytokines and Inflammatory Mediators in Human Intestinal Epithelial Cells after Invasion by *Trichinella spiralis*

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Epithelial cells are the first point of host contact for invasive intestinal pathogens and may initiate mucosal inflammatory responses via production of proinflammatory cytokines and mediators. The aim of the present study was to investigate in vitro the initial invasion of a parasitic nematode (*Trichinella spiralis*), to measure the early production of specific epithelial cytokines and inflammatory mediators after invasion, and to compare these responses with those to invasive bacteria. Monolayers of human colonic epithelial cell lines (HT29, T84, and Caco-2) were infected by *T. spiralis* or *Listeria monocytogenes*. Bile-activated infective larvae of *T. spiralis* invaded and migrated into the epithelial cell monolayers, leaving trails of dead cells. Transmission electron microscopy studies of damaged cells along the trail showed a progressive increase in size, disruption of cell membranes, loss or dilution of cytoplasmic proteins, and swelling of mitochondria and nuclei. However, no nuclear fragmentation was observed. With reverse transcription-PCR and an enzyme-linked oligonucleotide chemiluminescent assay, mRNA transcripts of interleukin-1β (IL-1β), IL-8, and epithelial neutrophil-activating peptide 78 were shown to increase in epithelial cells invaded by *T. spiralis* or *L. monocytogenes*, but only *L. monocytogenes* elicited increased inducible nitric oxide synthase (iNOS) mRNA. No increase in tumor necrosis factor alpha or transforming growth factor β mRNA was seen after *T. spiralis* invasion. Increased levels of IL-8 were also released from the basolateral surfaces of infected monolayers as detected by sandwich enzyme-linked immunosorbent assay. Induction and secretion of proinflammatory cytokines in epithelial cells after nematode or bacterial invasion may initiate the acute inflammatory response of the small intestine. The upregulation of iNOS in bacterial infections may contribute to mucosal defense and may also be associated with subsequent cell death, whereas different mechanisms appear to operate after nematode invasion.

The response of the intestine to invasion by pathogens represents a complex interaction between nonspecific inflammatory mechanisms and immunologically specific adaptive events. Epithelial cells are the first site of entry for invasive intestinal pathogens and may provide early signals for the acute mucosal inflammatory response via the release of proinflammatory cytokines and inflammatory mediators. A number of studies have examined the process in vitro with human colonic epithelial cell lines. These have been shown to produce a wide range of proinflammatory cytokines (IL-1α, IL-6, IL-8, GM-CSF, GRO-α, MCP-1, and TNF-α) in response to invasive microbial pathogens (*Entamoeba histolytica*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella dublin*, *Shigella dysenteriae*, and *Yersinia enterocolitica*) (8, 14). However, the patterns of epithelial cytokine response vary with the site of infection and type of pathogen (3, 14, 21).

Intestinal inflammation is mediated by proinflammatory cytokines and inflammatory mediators. NO is an important mediator and is also known to have antimicrobial activity (13). Excessive formation of NO by iNOS has been associated with cellular toxicity and tissue damage in experimental models of active intestinal inflammation (24). With immunoperoxidase staining, iNOS was localized in inflamed epithelium in ulcerative colitis, Crohn's disease, and diverticulitis (27). Therefore, epithelial iNOS may be important in acute mucosal inflammatory responses after invasion by pathogens.

Recently, an in vitro model of epithelial invasion by intestinal nematodes has been developed (20). Analysis of epithelial invasion by these parasites may broaden our understanding of epithelial cytokine responses to invasion by pathogens. *Trichinella spiralis* is a human intestinal pathogen that is becoming increasingly prevalent in Europe and North America because of changes in dietary habits and consumption of ethnic food. It has been used as an experimental model to study mucosal and inflammatory responses in animals. *T. spiralis* initiates infection by penetrating the columnar epithelium of the small intestine. During the early stages of infection, the inflammatory response is characterized by infiltration of neutrophils and macrophages/monocytes in the lamina propria (18). However, during the later stages of infection mast cells increase in number both in the lamina propria and between epithelial cells (26). In the murine model, the mucosal inflammatory response, which mediates expulsion of adult worms from the small intestine, is T-cell dependent (32). Moreover, data from many studies have shown that protective T-cell responses against intestinal nematodes are dependent on T helper 2 cytokines (10). However, the inductive and regulatory mechanisms of mucosal inflammatory responses to invasion by *T. spiralis* are still largely unknown.

In this paper, we describe the epithelial responses to invasion by the nematode *T. spiralis* with a recently described in vitro model (20) and compare these to responses elicited by invasive bacteria. The invasion and migration into epithelial
monolayers by infective larvae produced significant damage to cell membranes and organelles. Invasion by infective larvae or bacteria resulted in increases in mRNA transcripts of IL-1β, IL-8, and ENA-78, but only bacteria elicited increased iNOS mRNA levels. Expression and secretion of proinflammatory cytokines in epithelial cells after microbial invasion may initiate the acute inflammatory response of the small intestine. The upregulation of iNOS mRNA in bacterial infections may be important in mucosal defense and subsequent cell death.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: DIG, digoxigenin; DMEM, Dulbecco’s minimal essential medium; ELISA, enzyme-linked immunosorbent assay; ELOCA, enzyme-linked oligonucleotide chemiluminescent assay; ENA-78; IL-8, and ENA-78, but only bacteria elicited increased iNOS mRNA levels. Expression and secretion of proinflammatory cytokines in epithelial cells after microbial invasion may initiate the acute inflammatory response of the small intestine. The upregulation of iNOS mRNA in bacterial infections may be important in mucosal defense and subsequent cell death.

### Table 1. Primer and probe sequences for PCR and ELOCA

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequence(s)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’TCCCGGAGCTCAUCAGAAA3’</td>
<td>327</td>
</tr>
<tr>
<td></td>
<td>5’GGGGAATCTGCAAGCACTC3’</td>
<td>80</td>
</tr>
<tr>
<td>IL-8</td>
<td>5’TGGGCAAGCTTCTGTATTCT3’</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>5’TTTCTGGCCAGGCTTCT3’</td>
<td>80</td>
</tr>
<tr>
<td>ENA-78</td>
<td>5’TGTGGTGTCGCCGGCTG3’</td>
<td>212</td>
</tr>
<tr>
<td></td>
<td>5’CAAATCTCTCCCGGTCT3’</td>
<td>80</td>
</tr>
<tr>
<td>iNOS</td>
<td>5’ATGGAAACTCCAAAACAA3’</td>
<td>453</td>
</tr>
<tr>
<td></td>
<td>5’CAGCATCTCTGTGGAAAC3’</td>
<td>80</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’GTTGAACGGTGGAGGTAGG3’</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>5’GGAGAATCGCTTCGGAA3’</td>
<td>80</td>
</tr>
</tbody>
</table>

For each primer pair, the sense primer is given above the antisense primer. Labelled directly with DIG via a six-carbon spacer.

**RT-PCR.** RT-PCR was carried out as described previously (23). Briefly, total RNA was obtained by lysing the cells in RNAzolB (Biogenesis Ltd., Poole, United Kingdom), extraction with chloroform, and precipitation with isopropyl alcohol. After being washed and dried, the RNA pellet was resuspended in 50 μl of diethyl pyrocarbonate-treated H2O. Random hexamer primer RT was carried out with 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco). Control tubes without reverse transcriptase were set up with half of the RNA from each sample. Primers and probes for ENA-78, GAPDH, IL-1β, IL-8, and iNOS were kindly designed by R. Seth and R. A. Robins (Immunology, QMC Lewes, United Kingdom), sequencing was performed at the QMC, and the primers were synthesized in-house (Table 1). GAPDH was used as reference marker to correct for any variation in procedure. Specific oligonucleotide probes to detect PCR products were synthesized and labelled with DIG by R&D Systems Europe Ltd. Reaction conditions are given in Table 1. PCR products were quenched by ELOCA with the labelled probes to identify PCR products after blotting and hybridization with rabbit anti-DIG antibody labelled with alkaline phosphatase (Boehringer Mannheim, Lewes, United Kingdom), membranes were incubated with chemiluminescent substrate (CL-HPR-Star; Boehringer Mannheim) and the photons emitted were measured by a microplate scintillation spectrophotometer. The signal for the cytokine PCR product was recorded as counts per second and expressed as a ratio of the counts per second for the GAPDH product. Expression of mRNA of IL-1β, IL-8, ENA-78, or iNOS by the corresponding density of GAPDH expression. The result obtained was then used to calculate the relative ratio of expression of cytokines or inflammatory mediators in infected cells to the medium control. This final ratio was used to construct the graphs presented in Fig. 3. The data were calculated as means ± standard errors of the means, and the medium control value was normalized as 1.

**Sandwich ELISA for IL-8.** To confirm whether increased mRNA expression correlated with protein secretion, levels of one representative cytokine (IL-8, a marker for neutrophil inflammation) were measured in the culture supernatants of infected cells. The epithelial cell lines were grown to confluency in 24-mm diameter Transwell plates (Costar). Seven hours after infection with *T. spiralis* or *L. monocytogenes* from each sample, 50 μl of supernatant was collected and assayed for IL-8 by sandwich ELISA using Mab618-biotinylated BAF218 (R&D Systems) as the cytokine-specific antibodies. Cytokine levels were quantified against recombinant human IL-8 standard (208-IL-010) (R&D Systems). The lower sensitivity of the assay was 10 pg/ml calculated as the mean optical density of [20 replicated medium controls plus two times the standard deviations]. Any test wells with optical density values above this sensitivity were considered positive for IL-8.

**Statistical analysis.** The results were analyzed by one-way analysis of variance and Student’s t test. A P value of <0.05 was considered statistically significant.
RESULTS

Behavior of *T. spiralis* in human intestinal epithelial monolayers. Light microscopy studies showed that bile-activated infective larvae invaded and migrated within the epithelial monolayers shortly after the agar overlay was applied (Fig. 1A). The presence of the agar was critical in providing initial mechanical support for the larvae. Without the agar, infective larvae could move only over the epithelial monolayers and did not penetrate the epithelial cells. However, once the infective larvae were embedded in the epithelial monolayers, they continued to invade and migrate even when the agar was removed.

The invasive behavior of *T. spiralis* varied with the state of the larvae and the nature of the epithelial cell lines. Bile-treated infective larvae invaded epithelial cells more rapidly than freshly pepsin-digested infective larvae. Moreover, the degree of invasion of infective larvae in epithelial cells was in the order of Caco-2, T84, and HT29 cells, as indicated by the speed of invasion of the larvae and the extent of damage to epithelial cells (data not shown).

The morphology of the damaged epithelial cells after *Trichinella* invasion was studied by trypan blue and toluidine blue staining. With trypan blue staining, it was observed that the infective larvae left behind trails of dead cells (Fig. 1B). These cells were larger in size than adjacent cells. With toluidine blue staining, these dead cells were lightly stained whereas unaffected cells were stained deeply blue. The zigzag pattern of the trails reflected the serpentine movement of the larvae in the monolayers.

Ultrastructural changes in epithelial cells after *Trichinella* invasion. TEM studies showed that the epithelial cells were seriously damaged after invasion by *T. spiralis*. The infective larvae injured the cells sequentially as they moved through the monolayers, and only the cells in direct contact with the larvae were damaged (Fig. 2A). The infected cells were larger in size, and their cytoplasm and nuclei were less electron dense. The cell membranes of infected cells were severely disrupted (Fig. 2B). However, only the membrane in direct contact with the cuticular surface of the larva was disrupted (Fig. 2C), the apical and lateral membranes of the same cell remaining intact. Moreover, the infected cells showed either loss or dilution of cytoplasmic proteins. Other ultrastructural changes in the infected cells included swelling of nuclei and mitochondria (Fig. 2D). Disruption of cell membranes also extended to the nuclear membrane. When this was in direct contact with the larval surface, the nuclear membrane ruptured and the nucleus disintegrated (Fig. 2E). However, no nuclear fragmentation was observed. Therefore, it appeared that the larvae damaged the cells by disrupting the cell membranes. In consequence, the cells lost osmotic control, their organelles became swollen, and the infected cells eventually burst.

Production of cytokines and iNOS in epithelial cells in response to invasion by pathogens. Three different intestinal epithelial cell lines were used to study the production of proinflammatory cytokines and inflammatory mediators in response to invasion. The response to *T. spiralis* was compared to that elicited by bacterial invasion, with *L. monocytogenes* used as a positive control.

By RT-PCR and ELOCA, it was shown that invasion by *T. spiralis* caused an elevation of mRNA of IL-1β, IL-8, and ENA-78 in the epithelial cells after 5 h of infection (Fig. 3). The observed increase was dose dependent. Levels of IL-1β, IL-8, and ENA-78 were increased even when 1,000 larvae were used, but much greater increases were seen with 5,000 larvae. However, no increase in iNOS mRNA was seen after *Trichinella* invasion at any dose level. No changes in mRNA for TNF-α or TGF-β were seen (data not shown). Infection with *L. monocytogenes* also caused an increase of IL-1β, IL-8, and ENA-78 in all cell lines, but the iNOS mRNA transcript was also upregulated. Similar results were obtained with other enteroinvasive bacteria (*Salmonella enteritidis* and *E. coli*[ data not shown]).

Elevated mRNA expression for IL-8 correlated with increased secretion of IL-8 by infected epithelial cells. By sandwich ELISA, secreted IL-8 was found to be increased in all cell lines after infection with *T. spiralis* or *L. monocytogenes* (Table 2). Compared with medium and agar controls, epithelial cells produced significantly higher levels of IL-8 in the lower compartments of the cluster plate after 7 h of infection with *T. spiralis* or *L. monocytogenes*. The levels of IL-8 released by epithelial cells were similar whether they were infected with *T. spiralis* or *L. monocytogenes*.

DISCUSSION

The outcome of infection of the gastrointestinal tract is determined by the host’s ability to mount innate and adaptive immune responses. The inflammatory changes which accompany these responses may play an important role in host defense but can also have pathological consequences. Studies with gastrointestinal nematodes have contributed to a greater understanding of the capacity of the intestine to mount immune and inflammatory responses to infection, especially those mediated by T cells (33). The majority of studies, however, have concentrated on the development and expression of these responses rather than on their induction. Recent studies of host defense against other pathogens have shown that early expression of innate, nonspecific immunity may determine the outcome of later adaptive, specific immune responses (9). For many invasive intestinal nematodes, epithelial cells are the first point of host contact. Data from many studies have shown that epithelial cells play an integral role in mucosal immune responses (1, 15). Epithelial responses to invasion by nematodes may therefore provide data relevant to the induction of mucosal immune and inflammatory responses.

Invasion by *T. spiralis* produced significant damage in epithelial cells at the ultrastructural level and resulted in increased mRNA for IL-1β, IL-8, and ENA-78. Cell damage was associated with disruption of the cell membrane. With the loss of osmotic control, the damaged cells showed increased size, dilution of cytoplasmic proteins, and swelling of organelles, eventually becoming necrotic and bursting. This is very different from bacterial infections in which epithelial cells die by apoptosis. For example, in *Clostridium difficile* infection, bacterial toxin A induced cell rounding, detachment, and apoptosis in epithelial cell lines and organ cultures of human colonic biopsy specimens (19). Moreover, apoptosis in HT29 epithelial cell lines induced by invasive *S. dublin* and *E. coli* was related to NO production (16).

Epithelial cell invasion is necessary for most intestinal pathogens to establish infection. Although various molecules which mediate adherence and invasion have been identified in some intracellular pathogens, in many cases the mechanism of invasion is still unclear. The in vitro model used here suggests that *T. spiralis* may injure epithelial cells by mechanical and biochemical means. Based on the speed of invasion, mechanical movement is likely to be the major method. However, the loss of membrane integrity of epithelial cells in direct contact with the larvae suggests that there may be molecules either on the surface or released in secretions which facilitate invasion. Excretory-secretory (ES) products of *T. spiralis* are known to contain serine proteases, cysteine proteases, and metallopro-
FIG. 1. Light microscopy of human colonic epithelial monolayers infected by *T. spiralis*. (A) With trypan blue staining, the infective larva (arrow) can be seen to have left a trail of dead cells after invasion and migration in Caco-2 epithelial monolayers. (B) Trails of damaged T84 epithelial cells consist of many dead cells (arrow), which are larger than adjacent cells and have enlarged nuclei.
teinases (2, 30) and may contain pore-forming proteins similar to those of Trichuris nematodes, which also develop exclusively within the intestinal epithelium (6).

Data from many sources, with a variety of cell lines and pathogens, suggest that cell line models of pathogen invasion provide data relevant to in vivo events (8, 14, 25). Although these cell lines are all transformed, they are well established in studies of infection with bacteria, viruses, and protozoa. Primary cell lines, although closer to in vivo characteristics, are not suitable for experiments involving invasion of worm parasites, as they are more difficult to establish and maintain for long periods. The damage to epithelial cells by T. spiralis de-
Monolayers of HT29 (A), T84 (B), and Caco-2 (C) cells were infected with 1 \times 10^3, 3 \times 10^3, and 5 \times 10^3 infective larvae of T. spiralis or 10^7 CFU of L. monocytogenes per ml. After 5 h of infection, total RNA extracted from each sample was used for semiquantitative RT-PCR and ELOCA to examine the levels of expression of mRNA of IL-1β, IL-8, ENA-78, and iNOS. The experiments were repeated twice, and results are presented as ratios of infected to control cell lines, as described in Materials and Methods. *, statistically significant compared with uninfected medium control (P < 0.05).

Table 2. Secretion of IL-8 by human colonic epithelial monolayers after infection with T. spiralis and L. monocytogenes

<table>
<thead>
<tr>
<th>Infecting organism</th>
<th>HT29 (IL-8 pg/ml)</th>
<th>T84 (IL-8 pg/ml)</th>
<th>Caco-2 (IL-8 pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (medium control)</td>
<td>24.31 ± 0.1*</td>
<td>885.2 ± 64*</td>
<td>24.34 ± 0.1*</td>
</tr>
<tr>
<td>None (agar control)</td>
<td>31.42 ± 0.2*</td>
<td>1,057 ± 120*</td>
<td>25.64 ± 0.2*</td>
</tr>
<tr>
<td>T. spiralis</td>
<td>45.56 ± 0.1*</td>
<td>1,467 ± 106*</td>
<td>35.97 ± 0.5*</td>
</tr>
<tr>
<td>L. monocytogenes</td>
<td>41.89 ± 0.3*</td>
<td>1,557 ± 112*</td>
<td>33.75 ± 0.3*</td>
</tr>
</tbody>
</table>

* Monolayers of HT29, T84, and Caco-2 cells were grown on microporous membranes (Transwell; Costar) and infected with 10^3 infective larvae of T. spiralis or 10^7 CFU of L. monocytogenes per ml. After 7 h of infection, culture supernatants in the lower compartments were collected and levels of secreted IL-8 were measured by sandwich ELISA. The experiments were repeated twice.

T. spiralis (28). IL-8 and ENA-78 are C-X-C chemokines that are potent chemoattractants and activators of neutrophils (31). Epithelial cell-derived IL-8 released as a result of bacterial invasion was shown to attract neutrophils by transforming the matrix components of epithelial cells (22). Moreover, IL-8 was recently shown to attract T cells and monocytes via degranulation of neutrophils (29). Both IL-1β and IL-8 were shown to stimulate intestinal restitution in IEC-6 cell lines (4, 5). ENA-78 is a newly discovered neutrophil chemotactic receptor initially isolated from the conditioned media of the IL-1- or TNF-activated A549 human lung type II alveolar epithelial cell line (35). The amino acid sequence of ENA-78 shows 22% similarity to that of IL-8 and 48 to 51% similarity to GRO-α/β/γ Based on cross-desensitization experiments, it has been suggested that ENA-78 activity is mediated through the IL-8 receptor type (17). Increases in the level of ENA-78 have been detected in epithelial cells in patients with inflammatory bowel diseases (38). Recent studies of activated human colonic epithelial cells suggested that the patterns of production of IL-8 and ENA-78 were different (37). IL-8 production was very short-lived, whereas ENA-78 was long lasting but delayed in onset, occurring only when IL-8 secretion was downregulated. This suggested that IL-8 and ENA-78 may be important in both early and later stages of mucosal inflammation and could therefore contribute to the pattern of inflammatory responses seen in T. spiralis infection.

The upregulation of mRNA transcripts of iNOS in epithelial cells after invasion by bacteria suggests that NO may be important in mucosal defense. NO from activated macrophages is known to control and inhibit intracellular pathogens, and NO from epithelial cells has recently been shown to inhibit the intraepithelial growth of Chlamydia species (12). The differences in the expression of iNOS in epithelial cells injured by bacteria and T. spiralis may be due to the expression of iNOS in epithelial cells that undergo apoptosis. NO has been shown to induce apoptosis in intestinal epithelial cells after bacterial invasion (16). Epithelial NO may not be important in innate...
defense mechanisms against multicellular parasites, and this may explain the lack of iNOS induction in T. spiralis-injured epithelial cells. However, other animal studies showed that when rats were given L-NAME, a nonselective inhibitor of nitric oxide synthase, orally for 6 days, there was increased NO activity in the mucosa and a reduced number of adult worms of T. spiralis in the small intestine (11). Therefore, it appears that iNOS from other cellular sources may be involved in the acquired effector mechanism against intestinal nematodes.

In conclusion, the data presented here show that transcriptional activation of proinflammatory cytokines follows invasion of intestinal epithelial cells by both prokaryotic and eukaryotic pathogens. In the case of T. spiralis, these cytokines may initiate the protective acute inflammatory response by recruiting and activating cells in the lamina propria. The patterns of production of proinflammatory cytokines and inflammatory mediators are different between different groups of invasive pathogens.

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REFERENCES


