Cryptosporidium parvum Infection of Human Intestinal Epithelial Cells Induces the Polarized Secretion of C-X-C Chemokines

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Cryptosporidium parvum infects intestinal epithelial cells and does not invade deeper layers of the intestinal mucosa. Nonetheless, an inflammatory cell infiltrate that consists of neutrophils and mononuclear cells is often present in the lamina propria, which underlies the epithelium. This study investigated the host epithelial cell response to C. parvum by assessing in vitro and in vivo the expression and production of proinflammatory cytokines by intestinal epithelial cells after infection. The human colon epithelial cell lines HCT-8 and Caco-2 and human intestinal xenografts in SCID mice were infected with C. parvum. The expression and secretion of the C-X-C chemokines interleukin-8 (IL-8) and GROα were determined by reverse transcription-PCR analysis and enzyme-linked immunosorbent assay. Our results demonstrate that upregulated expression and secretion of IL-8 and GROα after C. parvum infection of intestinal epithelial cells first occurred 16 to 24 h after infection and increased over the ensuing 1 to 2 days. The kinetics of C-X-C chemokine production by C. parvum-infected epithelial cells contrast markedly with the rapid but transient expression of C-X-C chemokines by epithelial cells infected with invasive enteric bacteria. C-X-C chemokine secretion in C. parvum-infected epithelial cells occurred predominantly from the basolateral surface in polarized monolayers of Caco-2 cells grown in Transwell cultures, whereas cell lysis occurred at the apical surface. The basolateral secretion of IL-8 and GROα from C. parvum-infected epithelial cells suggests that C-X-C chemokines produced by those cells contribute to the mucosal inflammatory cell infiltrate in the underlying intestinal mucosa.

Cryptosporidium parvum is a common cause of diarrhea in humans and animals (5). Infection most commonly involves the small intestine and is usually self-limited, and the diarrhea which accompanies infection is transient in immunocompetent hosts (6). However, in immunocompromised individuals (e.g., patients with AIDS), diarrhea persists and frequently the infection and pathological changes are more widespread, possibly involving the biliary tract, pancreas, stomach, esophagus, and respiratory tract as well as the small intestine (3, 12, 19, 35).

In the intestine, C. parvum resides in epithelial cells (31). The life cycle of C. parvum in the intestinal mucosa includes several stages (14). When oocysts are ingested, sporozoites excyst and rapidly infect host epithelial cells. C. parvum establishes itself intracellularly but extracytoplasmically in a parasinusoidal vacuole beneath the apical membrane of the epithelial cell. After asexual multiplication in epithelial cells over the ensuing 16 to 48 h, type I and II merozoites are released and infect new epithelial cells. Type II merozoites differentiate into microgamonts and macrogamonts and initiate sexual reproduction between 3 and 5 days postinfection (p.i.). After macrogametes are fertilized by microgametes, they develop into oocysts, which sporulate in situ. By approximately 4 to 7 days p.i., oocysts either are shed in the feces or release sporozoites, which can infect other host epithelial cells and initiate a new cycle of parasite development (5).

C. parvum infection results in a spectrum of pathologic changes in the intestine. The severity of mucosal injury correlates with the numbers of infecting organisms and is characterized by minimal-to-marked villus atrophy (22, 31). Moderate-to-severe infections can be accompanied by a patchy or prominent neutrophil and mononuclear infiltrate in the lamina propria and numerous intraepithelial neutrophils (11, 22). Nonetheless, the mechanism by which this epithelial infection can cause mucosal inflammation and injury is not known.

Chemokines are low-molecular-weight proteins with pleiotropic effects on the recruitment and activation of leukocytes at sites of inflammation (2). The C-X-C chemokines, such as interleukin-8 (IL-8) and GROα, play an important role in the chemotaxis of neutrophils to sites of inflammation and in the activation of those cells (2). We and others have previously reported rapid upregulated expression of members of the C-X-C chemokine family in human intestinal epithelial cells after invasion of those cells with gram-negative or gram-positive bacteria (9, 16, 20, 30). Those studies have suggested that epithelial cells which line the human intestinal mucosa can act as sensors for bacterial invasion and provide early signals for initiation of the mucosal inflammatory response (17, 20, 30). In contrast to enteroinvasive bacteria, C. parvum resides within intestinal epithelial cells and does not invade deeper layers of the intestinal mucosa (5). Nonetheless, infection with C. parvum is associated with the recruitment of leukocytes to the lamina propria of the mucosa and with marked diarrhea.

To investigate the host epithelial cell response to C. parvum...
infection, we assessed the expression and production of proinflammatory cytokines in intestinal epithelial cells after infection of human epithelial cell lines and human intestinal xenografts in SCID mice. The data reported here demonstrate that *C. parvum* infection of human intestinal epithelial cells results in upregulated expression and basal secretion of the C-X-C chemokines IL-8 and GROα.

**MATERIALS AND METHODS**

**Cell lines and cell culture.** The human ileocecal adenocarcinoma cell line HCT-8 (ATCC CCL244) was obtained from the American Type Culture Collection, Rockville, Md., and the human ileocecal adenocarcinoma cell line Caco-2 was kindly provided by S. Tzipori, Tufts University. Cells were maintained in growth medium (RPMI 1640 with heat-inactivated fetal bovine serum [FBS] [10% FBS for HCT-8 cells and 20% FBS for Caco-2 cells], 2 mM l-glutamine, 50 U of penicillin G per ml, and 50 μg of streptomycin per ml). Polarized monolayers of Caco-2 cells were prepared by seeding 5 × 10⁶ Caco-2 cells on the tops of collagen-coated microporous supports (0.4-μm pore size; 4.7-cm² growth area) in Transwell chambers (Costar, Cambridge, Mass.). The medium was replaced every 2 days, and cells were allowed to differentiate for 15 days before use. The medium volumes in the apical and basal compartments were 1.5 and 2.6 ml, respectively. The differentiation of polarized Caco-2 monolayers was assessed by transmission electron microscopy, demonstrating the formation of microvilli and tight junctions, and by the increased expression over time of alkaline phosphatase, as determined by enzymatic assay (15) (data not shown). In addition, the formation of tight junctions was functionally assessed by measuring trans-epithelial electrical resistance across the monolayers by using a Millicell electrical resistance system (Millipore, Bedford, Mass.). The electrical resistance of uninfected monolayers in the experiments reported here ranged from 430 to 493 Ω cm² after subtraction of resistance across a cell-free filter.

**Purification and excretion of *C. parvum* oocysts.** *C. parvum*, initially isolated from an infected child (1), was maintained in calves at the INRA, Nouzilly, France, from an infected child (1), was maintained in calves at the INRA, Nouzilly, France. *C. parvum* oocysts, isolated from feces by filtration and diethyl ether sedimentation, were treated with 1.25% sodium hypochlorite, washed, and then stored at 4°C in phosphate-buffered saline (PBS; pH 7.4) containing 50 U of penicillin G per ml, 50 μg of streptomycin per ml, and 0.25 μg of amphotericin B per ml. With this treatment, subsequent excretion rates in growth medium were 40 to 60%.

Oocysts in medium equilibrated with 5% CO₂-95% air were gently agitated for 2 h at 37°C to enhance excretion. Various numbers of oocysts that had been excreted in growth medium were used to infect Caco-2 cells, whereas various numbers of oocysts that had been excreted in supplemented growth medium (34) (the supplement consisted of 50 mM glucose, 35 μg of ascorbic acid per ml, 1.0 μg of folic acid per ml, 4 μg of 4-aminobenzoic acid per ml, 2.0 μg of calcium pantothenate per ml, 0.1 U of insulin per ml, and 0.25 μg of amphotericin B per ml) were used to infect HCT-8 cells. For heat inactivation, oocysts were incubated at 57°C in growth medium for 2 h prior to use.

**Infection protocols for cell lines.** For the infection of HCT-8 cells, 2 × 10⁶ cells were seeded into six-well Costar tissue culture plates and cultured for 24 h to form monolayers. Oocysts, previously reported to be implementable growth stages of *C. parvum* organisms, as heat-killed *C. parvum* oocysts were resuspended in 100 μl of PBS. Xenografts were removed 5 days after infection. For histology, portions were fixed in 10% formalin in PBS and embedded in paraffin, and sections were prepared and stained with hematoxylin and eosin. For molecular studies, tissues were prepared and immediately snap-frozen in liquid nitrogen. Xenografts were studied by transmission electron microscopy with full approval from the Cambridge Local Ethics Committee and in accordance with the Home Office guidelines specified in the Polyclar TARTE Report (29).

**Transmission electron microscopy.** Tissue pieces were immersion fixed in 4% glutaraldehyde–2% sucrose–0.1 M sodium phosphate (pH 7.2) for 1 to 2 h at room temperature. Postfixation was performed for 1 h in 1% osmium tetroxide–0.1 M calcium cacodylate (pH 7.2), followed by 1 h of staining in 1% aqueous uranyl acetate. Samples were dehydrated in ethanol and propylene oxide and embedded in Araldite epoxy resin (Sigma Chemical Co.). All processing was carried out at room temperature. Sections were stained with uranyl acetate and lead citrate.

**RESULTS**

**Increased IL-8 and GROα release in response to *C. parvum* infection of HCT-8 and Caco-2 cells.** IL-8 and GROα are potent chemoattractants and activators of neutrophils. We first assessed IL-8 and GROα production in response to *C. parvum* infection of HCT-8 cells, a human intestinal epithelial cell line known to support the development of *C. parvum* in vitro (33). The levels of IL-8 and GROα release were determined for up to 96 h after infection by ELISA. As shown in Fig. 1, HCT-8 cells constitutively secreted low levels of IL-8 and GROα and the release of these chemokines increased two- to threefold after *C. parvum* infection. Increases in IL-8 and GROα release were first noted at 16 to 24 h p.i. and continued for up to 72 to 96 h p.i.

Increased chemokine release required infection with viable *C. parvum* organisms, as heat-killed *C. parvum* organisms did not induce IL-8 or GROα release (data not shown). To relate increased chemokine release to cell lysis, which is a consequence of *C. parvum* infection, lysis of HCT-8 cells after infection was assessed by LDH release. As shown in Fig. 1, cell lysis increased markedly between 16 and 48 h p.i. and the LDH levels in supernatants were maximal at between 48 and 72 h p.i., indicating that the time course of cell lysis after infection overlapped that of the increases in IL-8 and GROα release. The maximum levels of LDH release from monolayers infected with *C. parvum* were ~50% of the levels obtained by lysing parallel monolayers by sonication.

The magnitude of the chemokine response was dependent on the inoculum of infecting oocysts per epithelial cell. Thus, infection of HCT-8 cells with increasing ratios of *C. parvum* oocysts per HCT-8 cell was paralleled by increased IL-8 release. At ratios of 0.3, 1, and 3 oocysts/HCT-8 cell, IL-8 release was...
increased (1.3 ± 0.3)-, (3.4 ± 0.4)-, and (5.1 ± 0.7)-fold, respectively, relative to that of uninfected controls (data are means ± standard errors of the means of six separate experiments).

Caco-2 is another human intestinal epithelial cell line known to support C. parvum infection (4). In addition, Caco-2 cells differentiate in culture and can be grown as polarized monolayers on microporous supports. As shown in Fig. 2, C. parvum infection of Caco-2 cells increased IL-8 and GROα release in a dose- and time-dependent manner, with kinetics similar to those in the HCT-8 line. Whereas HCT-8 cells infected with C. parvum released higher levels of IL-8 than of GROα, the opposite was true for Caco-2 cells. Neither cell line produced detectable levels of the C-X-C chemokine ENA-78 before or after C. parvum infection (<100 pg/ml). Lysis of Caco-2 cells, like that of HCT-8 cells, increased markedly between 24 and 48 h p.i. and the LDH levels in supernatants continued to increase over the 72-h culture period (Fig. 2).

C. parvum infection upregulates IL-8 mRNA levels in HCT-8 cells. To assess whether increased chemokine secretion was paralleled by increased mRNA levels, the IL-8 mRNA levels in control and C. parvum-infected HCT-8 cells were assessed. As shown in Table 1, the levels of IL-8 mRNA transcripts increased by 5-fold at 24 h p.i., peaked at 12-fold by 48 h p.i., and

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>IL-8 mRNA levels (Control/ + C. parvum)</th>
<th>Infected/control ratio</th>
<th>β-Actin mRNA levels (Control/ + C. parvum)</th>
<th>Infected/control ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>3.8 × 10^6</td>
<td>1.9 × 10^6</td>
<td>5.0</td>
<td>1.3 × 10^7</td>
</tr>
<tr>
<td>48</td>
<td>5.0 × 10^6</td>
<td>6.0 × 10^6</td>
<td>12.0</td>
<td>2.0 × 10^7</td>
</tr>
<tr>
<td>72</td>
<td>8.3 × 10^6</td>
<td>2.0 × 10^6</td>
<td>2.6</td>
<td>2.4 × 10^7</td>
</tr>
<tr>
<td>96</td>
<td>1.1 × 10^6</td>
<td>1.0 × 10^6</td>
<td>0.9</td>
<td>1.5 × 10^7</td>
</tr>
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</table>

*Confluent monolayers of HCT-8 cells in six-well plates were infected (+ C. parvum) at an oocyst/cell ratio of 3. Total cellular RNA was extracted at the indicated times p.i., and mRNA levels for IL-8 and the housekeeping gene β-actin were determined by quantitative RT-PCR with internal standards. Data are the numbers of mRNA transcripts per microgram of cellular RNA.
was changed daily and the IL-8 and GRO in the top and bottom compartments of Transwell chambers. V3 monolayers decreased only slightly after infection (430 to 495 compartment (Fig. 3) and the electrical resistance across ers, since LDH was released predominantly into the apical not related to a reduced barrier function of infected monolay-

determined. As shown in Fig. 3, 80% or more of IL-8 and LDH release, in the apical and basolateral compartments were with electron microscopy. Infection showed a patchy distribution, toxylin- and eosin-stained paraffin sections and by transmission and tissues were removed 5 days later and analyzed on hema-
xenografts were injected intraluminally with C. parvum human intestinal xenografts could be infected with C. parvum origin over a 10- to 20-week period (29). To establish that transplanted subcutaneously into SCID mice. Such xenografts which human fetal intestine (gestational age, 10 to 14 weeks) is expression in intestinal epithelial cells induces IL-8 and GRO a-induced by mediators released from infected or lysed cells. Polarized basolateral secretion of IL-8 and GRO α after C. parvum infection. Intestinal epithelial cells are functionally polarized. If IL-8 and GRO α produced by C. parvum-infected epithelial cells play a physiologic role in the influx of neutrophils into the mucosa, they predictably would be released from the basolateral surfaces of infected epithelial cells. To determine the polarity of chemokine secretion by C. parvum-infected epithelial cells, Caco-2 cells were cultured as polarized monolayers in Transwell chambers. The impermeability of monolayers to IL-8 was established by the addition of a relatively high dose of IL-8 (10 ng/ml) to the basolateral compart-
ment of uninfected monolayers. This resulted in the appearance of only 3% of the added IL-8 in the apical compartment 24 h later. After apical infection with C. parvum, the medium in the top and bottom compartments of Transwell chambers was changed daily and the IL-8 and GRO α levels, as well as LDH release, in the apical and basolateral compartments were determined. As shown in Fig. 3, 80% or more of IL-8 and GRO α was found in the basolateral compartment. This was not related to a reduced barrier function of infected monolay-
ners, since LDH was released predominantly into the apical compartment (Fig. 3) and the electrical resistance across monolayers decreased only slightly after infection (430 to 495 Ω × cm² in controls and 380 and 270 Ω × cm² at 24 and 48 h, respectively, after infection at an oocyst/epithelial cell ratio of 3). Thus, the secretion of IL-8 and GRO α in C. parvum-infected epithelial cells occurs predominantly from the basolat-
eral surface. In controls, polarized Caco-2 monolayers stimu-
lated with IL-1α for 24 h secreted 110-fold more total IL-8 than did controls and 84% of IL-8 was found in the basolateral compartment.

Increased IL-8 and GRO α expression by intestinal epithe-

cells in vivo in a human intestinal xenograft model infected with C. parvum. We next asked whether C. parvum infection induces IL-8 and GRO α expression in intestinal epithelial cells in vivo. To infect human intestinal epithelial cells with C. parvum, we used a human fetal intestinal xenograft model in which human fetal intestine (gestational age, 10 to 14 weeks) is transplanted subcutaneously into SCID mice. Such xenografts develop a fully differentiated epithelial layer of entirely human origin over a 10- to 20-week period (29). To establish that human intestinal xenografts could be infected with C. parvum, xenografts were injected intraluminally with C. parvum oocysts and tissues were removed 5 days later and analyzed on hema-
toxyn- and eosin-stained paraffin sections and by transmission electron microscopy. Infection showed a patchy distribution, with C. parvum localized to the luminal surface of intestinal epithelial cells (Fig. 4A). The characteristic developmental stages of C. parvum were observed by transmission electron microscopy (Fig. 4C), indicating that C. parvum infection proceeded normally in xenografts. Furthermore, infection elicited an inflammatory host response, since small cell infiltrates with mostly neutrophils were observed beneath the epithelium (Fig. 4B). Based on the nuclear morphology, infiltrating neutrophils appeared to be of mouse origin.

To determine chemokine expression in C. parvum-infected xenografts, RNA was extracted from mucosal scrapings and analyzed by quantitative RT-PCR. As shown in Table 2, the levels of human IL-8 and GRO α mRNAs increased by >20-

fold after C. parvum infection of intestinal xenografts, whereas β-actin mRNA levels were only slightly increased after infec-
tion (4.2- and 1.5-fold in two separate experiments). Since intestinal epithelial cells are the most abundant human cell type in the mucosa of the intestinal xenograft model and only those cells were infected with *C. parvum*, these data suggest that epithelial cells were most likely responsible for the increases in IL-8 and GROα expression after infection.

**DISCUSSION**

*C. parvum* infects and undergoes its life cycle within epithelial cells that line the human intestinal mucosa but does not invade deeper layers of the human gastrointestinal mucosa (5, 31). Nonetheless, moderate-to-severe infection of the gastrointestinal tract with *C. parvum* is often associated with prominent infiltration of neutrophils and mononuclear phagocytes in the lamina propria, which underlies the epithelium, and intraepithelial neutrophils (11, 22). The mechanisms by which an infection with a pathogen, such as *C. parvum*, that is confined to epithelial cells results in subepithelial inflammation are not well understood. The present studies used in vitro and in vivo models to demonstrate that intestinal epithelial cells infected with *C. parvum* upregulate the expression and polarized basolateral secretion of the C-X-C chemokines IL-8 and GROα, which are known chemoattractants and activators of neutrophils (2). This epithelial response to *C. parvum* appears to be well suited for contributing to the accumulation of neutrophils at the site of infection by establishing a chemotactic gradient within the underlying mucosa and to the activation of these cells.

The secretion of IL-8 and GROα after *C. parvum* infection occurred mostly from the basolateral surface in polarized monolayers of intestinal epithelial cells. These data suggest that increased basolateral IL-8 secretion did not simply result from cell lysis, since LDH (as a marker of cell lysis) was found predominantly in the apical compartment after *C. parvum* infection, a finding that is consistent with previous observations (13). Similar to *C. parvum*-infected epithelial cells, polarized Caco-2 cells stimulated with the proinflammatory agonist IL-1α and polarized T84 human colon epithelial cells stimulated with the agonist tumor necrosis factor alpha (8) predominantly secreted IL-8 at the basolateral surface. In general, secreted proteins that are not specifically targeted to the apical surfaces of polarized epithelial cells appear to be predominantly secreted at the basolateral surfaces of those cells (27). Although this has not been specifically documented for IL-8 or other chemokines, a similar default mechanism is likely re-

![FIG. 4. *C. parvum* infection of human small intestinal xenografts. Human intestinal xenografts in SCID mice were infected with 5 × 10⁶ *C. parvum* oocysts by transcutaneous injection. Xenografts were collected 5 days after infection, and paraffin sections were prepared and stained with hematoxylin and eosin (A and B). In parallel, sections were prepared for transmission electron microscopy (C). (A) Two atrophied villi with multiple *C. parvum* parasite stages (arrows) at the apical surface of the intestinal epithelium; original magnification, ×400. (B) Damaged villi with several *C. parvum* parasite stages (arrows with solid arrowheads) and a small cell infiltrate containing mostly neutrophils (arrows with open arrowheads); original magnification, ×400. (C) Three parasite stages of *C. parvum* at the apical surfaces of three intestinal epithelial cells; original magnification, ×5,000. On the right side, a schizont containing several merozoites can be seen. In the middle, a trophozoite is visible, and on the left either the remains of a schizont which released its merozoites or a schizont whose merozoites were not sectioned is shown. Epithelial microvilli and tight junctions between epithelial cells are also shown.](image-url)
TABLE 2. Increased chemokine mRNA levels after C. parvum infection of human small intestinal xenografts

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>mRNA species</th>
<th>No. of mRNA transcripts/µg of total RNA</th>
<th>Infected/control ratio</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>IL-8</td>
<td>&lt;5 × 10^5 &lt;1.1 × 10^4</td>
<td>&gt;22</td>
</tr>
<tr>
<td></td>
<td>GROα</td>
<td>&lt;5 × 10^5 &lt;1.0 × 10^4</td>
<td>&gt;20</td>
</tr>
<tr>
<td>2</td>
<td>IL-8</td>
<td>1.0 × 10^5 6.0 × 10^4</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>β-Actin</td>
<td>8.0 × 10^5 1.2 × 10^4</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Human intestinal xenografts were infected with 5 × 10^5 and 5 × 10^6 C. parvum oocysts in experiments 1 and 2, respectively. Paired xenograft samples derived from the same donors were kept uninfected as controls. Total cellular RNA was extracted from mucosal scrapings of individual xenografts obtained 5 days after infection, and equal amounts of RNA were pooled from five to eight xenografts. mRNA levels for IL-8, GROα, and β-actin were determined by quantitative RT-PCR with internal standards.

and Caco-2 cells appeared to be due to the production of IL-8 and GROs by infected cells themselves and was not amplified by mediators released from C. parvum-infected cells.

Our data suggest that epithelial cells play an active role in the host response to C. parvum infection. After infection, increased epithelial chemokine production may attract inflammatory cells into the subepithelial region, and those cells may subsequently migrate into and across the epithelium (21, 22, 25). In this regard, neutrophils in mucosal secretions have previously been shown to retain the ability to phagocytose and kill pathogens (7). In addition, neutrophils may function to regulate other aspects of the inflammatory response at the site of C. parvum infection through the secretion of cytokines (18).

Human small intestinal xenografts were shown to be a good model for studying important aspects of the host response to C. parvum infection. This model has several advantages over clinical studies, since it is possible to characterize the response of normal human intestinal epithelial cells to the infection in a controlled and easily accessible environment (32). In contrast, clinical studies of immunocompetent patients are difficult to conduct since cryptosporidiosis in these patients is generally transient (6); for this reason, such patients are rarely biopsied. Moreover, immunodeficient patients with C. parvum infection often have multiple other opportunistic pathogens, which complicates the selection of appropriate controls and the interpretation of findings. The xenograft model consists of a chimeric mixture of human and mouse cells (29). Nonetheless, our finding that mouse neutrophils accumulated under the human epithelium after C. parvum infection suggests that epithelial cell-derived chemotactic signals were active on mouse cells. Consistent with this notion, human IL-8 has previously been shown to activate and chemoattract mouse neutrophils (28).

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