INFECTION AND IMMUNITY, Apr. 1998, p. 1787–1790 Vol. 66, No. 4
0019-9567/98/$04.00+0

Human Intestinal Epithelial Cells Respond to *Cryptosporidium parvum* Infection with Increased Prostaglandin H Synthase 2 Expression and Prostaglandin E_2 and $F_{2\alpha}$ Production

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Received 7 October 1997/Returned for modification 6 November 1997/Accepted 14 January 1998

***Cryptosporidium parvum* is an important cause of diarrhea in humans and several animal species. Prostaglandins play a central role in regulating intestinal fluid secretion in animal models of cryptosporidiosis, but their cellular sources and mechanisms of induction are unclear. Here, we show that *C. parvum* infection directly activates prostaglandin H synthase 2 expression and prostaglandin E_2 and $F_{2\alpha}$ production in human intestinal epithelial cells.**

Cryptosporidium parvum is an important cause of diarrhea in both immunocompetent and immunosuppressed hosts and is responsible for 1 to 2% of deaths in patients with acquired immunodeficiency syndrome (21). The mechanisms underlying *C. parvum*-induced diarrhea in humans are poorly understood. The presence of an enterotoxic activity released by the parasite has been previously reported (8), but these findings are controversial (20). In a porcine model of cryptosporidiosis, diarrhea results from a combination of secretory diarrhea and sodium-glucose malabsorption due to villous atrophy and epithelial cell damage (3). The former is controlled by prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2), which appear to act either directly on intestinal epithelial cell functions or indirectly by activating cholinergic and VIPergic neuronal pathways (1). However, neither the cellular source of PGE_2 and PGI_2 in this model, nor the mechanisms leading to their induction are known. Inflammatory cells in the mucosa, such as macrophages, can produce high levels of prostaglandins and may be important for prostaglandin production after *C. parvum* infection, since infection is often accompanied by some degree of inflammation (7, 17). However, significant diarrhea associated with *C. parvum* infection has been reported in the absence of overt histopathological changes in the gut (14, 18), suggesting that cells normally present in the uninfected mucosa, e.g., epithelial cells or fibroblasts, produce increased prostaglandin levels after infection. Our previous studies suggested that intestinal epithelial cells, which are the predominant host cells infected with *C. parvum* (15), can act as sensors of infection and provide early signals for the initiation of the mucosal inflammatory response by releasing chemoattractant cytokines (12). Furthermore, other studies have shown that infection of epithelial cells with invasive bacteria upregulates prostaglandin production (5). Based on these findings, the present studies tested the hypothesis that intestinal epithelial cells respond to infection with *C. parvum* with increased prostaglandin production.

C. parvum, which was initially isolated from an infected child, was maintained in calves at the Institut National de la Recherche Agronomique, Nouzilly, France, and *C. parvum* oocysts were purified by filtration and diethyl ether sedimentation as described previously (12). The human adenocarcinoma cell lines HCT-8 (ATCC CCL 244) and HT-29 (ATCC HTB 38) were maintained in RPMI 1640 medium–10% heat-inactivated fetal bovine serum (FBS)–2 mM L-glutamine–50 U of penicillin G per ml–50 μg of streptomycin per ml. Infections were performed as described previously (12). Briefly, cells were seeded into six-well Costar tissue culture plates at 2×10^6 cells/well and grown for 24 h. Infections with oocysts were done in a supplemented medium as described by Upton et al. (24). After a 5-h infection period, monolayers were washed, and fresh supplemented medium was added. This time is referred to as 0 h for all experiments. To determine the levels of prostaglandin production, cultures were washed and incubated for 15 min in 1 ml of RPMI 1640 medium per well–10 mM HEPES–2 mg of bovine serum albumin per ml–20 μM arachidonic acid. Levels of the prostaglandins PGE_2 and $PGF_{2\alpha}$ were determined by enzyme immunoassay (Cayman Chemical, Ann Arbor, Mich.). These prostaglandins were chosen for the present studies based on our previous finding that they were the major prostaglandins produced by human intestinal epithelial cell lines (5). To correct for cell loss due to lysis of parasite-infected cells, prostaglandin levels were divided for each culture by the amount of total protein present in the culture at each time point (i.e., the time of the assay). Total protein contents were determined by lysing the monolayers in lysis buffer (150 mM NaCl, 20 mM Tris [pH 7.5], 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg of aprotinin per ml) and by assaying the protein concentrations by the Bradford method (Bio-Rad Laboratories, Hercules, Calif.). The lysates were subsequently used for immunoblot analysis of prostaglandin H synthase 1 (PGHS-1), PGHS-2, and actin expression by using previously published protocols and antibodies (5). Levels of PGHS-2 and β-actin mRNA were determined by quantitative reverse transcription (RT)-PCR with standard RNAs as we described before (5, 10).

The human intestinal xenograft model used in the present

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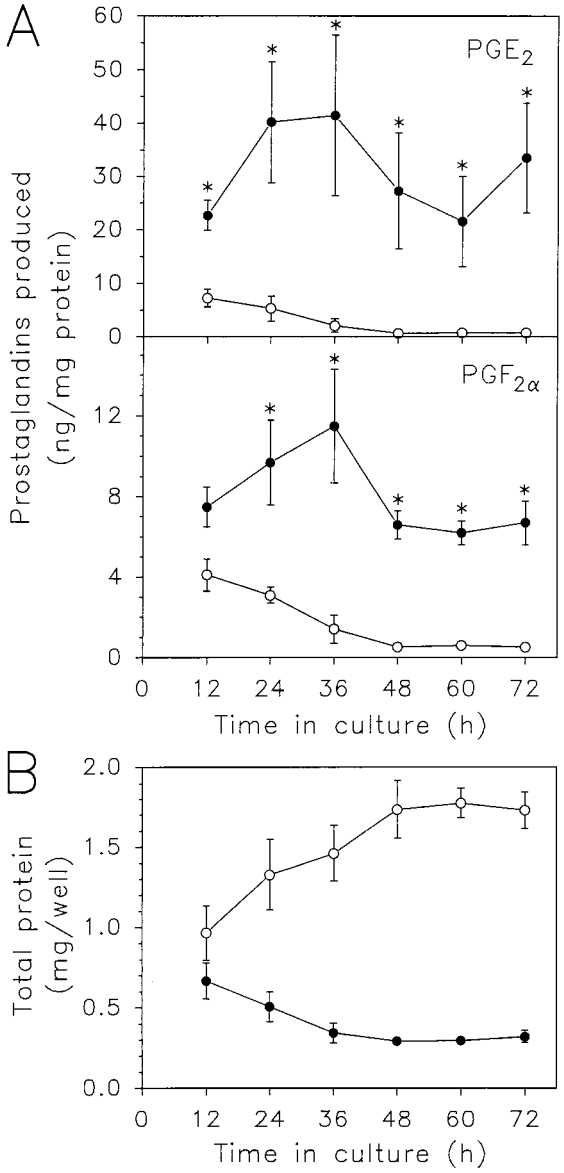


FIG. 1. Time course of increased prostaglandin production after *C. parvum* infection of HCT-8 cells. HCT-8 monolayers in six-well plates were infected with 10^7 oocysts/well (●), of which 40 to 60% were found to excyst, as determined in parallel experiments. Uninfected monolayers were used as a control (○). The amounts of PGE_2 and $PGF_{2\alpha}$ produced (A) were determined after a 15-min incubation period with 20 μM arachidonic acid at the indicated times after infection. Subsequently, monolayers were lysed and the amounts of total protein/well were determined (B). Data are means ± SEM of three to four independent experiments. Asterisks, values that were significantly increased relative to those in the respective uninfected controls ($P \leq 0.05$), as determined by the *t* test.

studies has been described in detail before (12, 19). Briefly, human fetal intestines were transplanted onto the backs of SCID mice and allowed to develop for 10 to 15 weeks. At this time, a differentiated epithelial layer of entirely human origin has developed (19). Xenografts were infected by injecting 10^8 *C. parvum* oocysts in 200 μl of phosphate-buffered saline (PBS) into the lumen. Control xenografts were injected with 200 μl of PBS (sham infection). Tissues were collected 5 days after infection, since this time point was shown in a rabbit intestinal xenograft model to be the earliest after *C. parvum* infection at which there was consistent and widespread epithelial infection

TABLE 1. Effect of PGHS inhibitors on *C. parvum*-induced PGE_2 production by HCT-8 intestinal epithelial cells[a]

Expt and inhibitor added	Amt of PGE_2 produced (ng/mg of cellular protein)	
	Control	With *C. parvum*
1		
None	1.7 ± 0.1	22.4 ± 0.3
Indomethacin	<0.1[b]	0.2 ± 0.1[b]
NS-398	<0.1[b]	0.2 ± 0.1[b]
2		
None	1.0 ± 0.2	125.0 ± 20.5
Indomethacin	0.4 ± 0.1[b]	0.5 ± 0.1[b]
NS-398	<0.1[b]	0.6 ± 0.1[b]

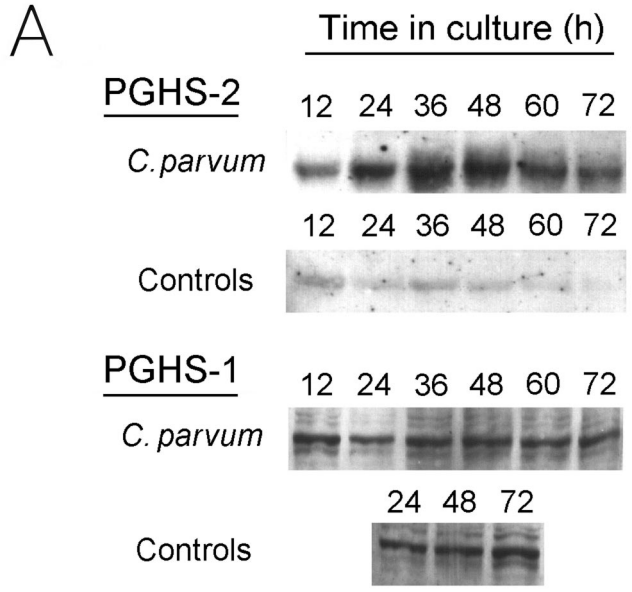
[a] Confluent monolayers of HCT-8 cells in six-well plates were infected with 8×10^6 *C. parvum* oocysts and incubated for 40 h. The PGHS inhibitors indomethacin (10 μM) and NS-398 (10 μM) were added for 1 h, and the amounts of PGE_2 produced were determined after addition of 20 μM arachidonic acid for 15 min. Data are means ± SEM of triplicate cultures.

[b] Significantly different ($P < 0.01$) from values for parallel cultures not treated with a PGHS inhibitor, as determined by the *t* test.

(23). Successful infection was confirmed on paraffin sections stained with hematoxylin and eosin and by transmission electron microscopy as reported previously (12). Total RNA was extracted from mucosal scrapings, and mRNA levels for PGHS-2 and β-actin and levels of 28S rRNA were determined by quantitative RT-PCR (5). The RT-PCR of PGHS-2 and β-actin was specific for the respective human mRNAs, since amplification of mouse spleen RNA or DNA did not yield any PCR products (5). The xenograft studies were performed at the Babraham Institute, Cambridge, United Kingdom, with full approval from the Cambridge Local Ethics Committee and in accordance with the Home Office guidelines specified in the Polkinghorne Report (19).

Infection of HCT-8 cells with *C. parvum* increased PGE_2 production by as much as 50-fold (Fig. 1). The increase was first observed at 12 h after infection, was maximal by 36 h, and was sustained until the end of the observation period (72 h). Production of $PGF_{2\alpha}$ also increased after *C. parvum* infection with a kinetics similar to that of PGE_2 production, although the absolute levels produced and the relative increases after infection were ~5-fold less than those for PGE_2 (Fig. 1). Similarly, *C. parvum* infection of another human intestinal epithelial cell line, HT-29, increased PGE_2 and $PGF_{2\alpha}$ production by >6-fold (controls, <30 pg of PGE_2/mg of total protein and 9 ± 1 pg of $PGF_{2\alpha}$/mg of protein; 36 h after *C. parvum* infection, 204 ± 47 pg of PGE_2/mg of protein and 171 ± 82 pg of $PGF_{2\alpha}$/mg of protein; values are means ± standard errors of the means [SEM] of the results of three independent experiments). In contrast to infection with live *C. parvum* cells, exposure of HCT-8 cells to heat-inactivated *C. parvum* (57°C for 1 h) did not affect PGE_2 or $PGF_{2\alpha}$ production (data not shown).

Prostaglandin production is controlled by the levels of the key enzyme PGHS, which exists in two isoforms, PGHS-1 and PGHS-2. Although both isoforms catalyze the same biosynthetic step, they display considerable differences in regulation and tissue-specific expression and have overlapping as well as unique physiologic functions (25). Both isoforms are expressed in intestinal epithelial cells (5). To define their role in the *C. parvum*-induced increase in PGE_2 and $PGF_{2\alpha}$ production, two PGHS inhibitors were used, i.e., indomethacin and NS-398. Indomethacin, which inhibits both PGHS isoforms, and NS-398, which is a highly specific inhibitor of PGHS-2 (6), com-



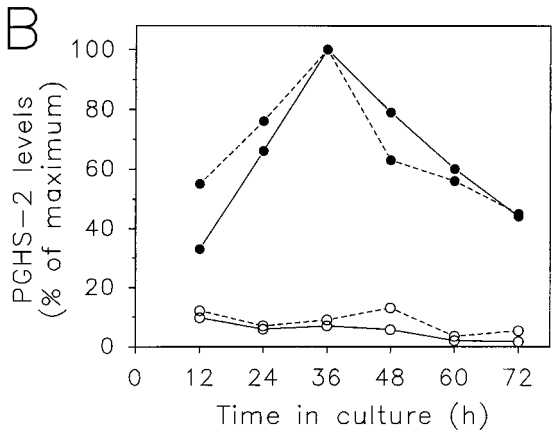


FIG. 2. Immunoblot analysis of PGHS-2 and PGHS-1 expression after *C. parvum* infection of HCT-8 cells. HCT-8 monolayers in six-well plates were infected with 10^7 *C. parvum* oocysts/well or were left uninfected (controls). Cultures were incubated for the indicated periods of time, and cell lysates were prepared, size fractionated, and blotted onto a nitrocellulose membrane. PGHS-2 and PGHS-1 were detected with rabbit anti-human PGHS-2 and PGHS-1 (Oxford Biomedical), respectively, and the ECL system (Amersham). (A) Examples of immunoblots for PGHS-2 and PGHS-1. Scans were obtained with a Bio-Rad GS-670 scanning densitometer and PhotoFinish imaging software. (B) Quantitative densitometric analysis of the PGHS-2 immunoblot shown in panel A (continuous lines), along with data from an additional experiment (dashed lines). PGHS-2 levels were expressed as percentages of the maximum value observed for any of the samples in each experiment (i.e., 36 h after *C. parvum* infection in both experiments). ●, *C. parvum* infected; ○, uninfected controls.

pletely blocked increased production of PGE_2 (Table 1) and $PGF_{2\alpha}$ (data not shown) after *C. parvum* infection of HCT-8 cells. These data indicate that PGHS-2 was mostly responsible for the increase in PGE_2 and $PGF_{2\alpha}$ production after *C. parvum* infection. In agreement with this, PGHS-2 levels increased after *C. parvum* infection of HCT-8 cells (Fig. 2), with time course and relative extent paralleling those of the increase in PGE_2 production (Fig. 1). In contrast, levels of PGHS-1 (Fig. 2) and actin were minimally affected by *C. parvum* infection of HCT-8 cells (the ratios of the levels in infected relative to control cells were 1.3, 1.3, and 0.8 for PGHS-1 at 24, 48, and 72 h after infection, respectively, and 1.3 and 0.8 for actin at 48 and 72 h after infection, respectively, as determined by scanning densitometry of immunoblots). Furthermore, the increase in PGHS-2 protein levels was paralleled by an increase in PGHS-2 mRNA levels in *C. parvum*-infected HCT-8 cells (Fig. 3), although the relative increase in PGHS-2 mRNA levels was less than that of PGHS-2 protein levels (Fig. 2).

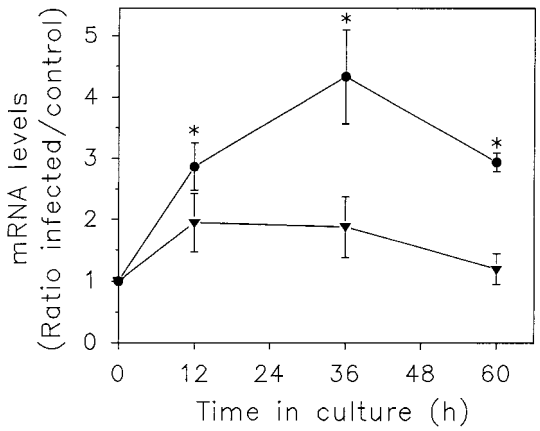


FIG. 3. Increased PGHS-2 mRNA expression in *C. parvum*-infected HCT-8 cells. HCT-8 monolayers in six-well plates were infected with 10^7 oocysts. Levels of mRNAs for PGHS-2 (●) and β-actin (▼) were determined by quantitative RT-PCR with standard RNAs and were expressed as ratios of the values for infected cells to those for controls. Data are the means ± SEM of the results from three independent experiments. Mean mRNA levels in controls were 2.2×10^5 transcripts/μg of total RNA for PGHS-2 and 2.5×10^7 transcripts/μg of RNA for β-actin. Asterisks indicate that values are significantly increased relative to those in the respective uninfected controls ($P \leq 0.05$), as determined by the *t* test.

To confirm the cell line findings in vivo, we used a human intestinal xenograft model (19). This model has the advantage that normal human intestinal epithelial cells can be infected with *C. parvum* under controlled conditions in the absence of potentially confounding coinfections, which are commonly encountered when samples from *C. parvum*-infected patients are used. We showed previously that the intestinal xenograft model can be successfully infected with *C. parvum* and can be used to characterize cytokine responses to infection (12). As shown in Table 2, levels of human PGHS-2 mRNA increased by 10-fold when they were assayed 5 days after *C. parvum* infection of intestinal xenografts. As a control, levels of 28S rRNA and β-actin mRNA changed by <2-fold after infection.

TABLE 2. Increased expression of human PGHS-2 mRNA in *C. parvum*-infected human intestinal xenografts[a]

RNA species	No. of RNA transcripts/μg of total RNA		Ratio of infected/control
	Control	With *C. parvum*	
PGHS-2	1.3×10^4	1.3×10^5	10.0
28S rRNA	4.4×10^8	8.0×10^8	1.8

[a] Human intestinal xenografts were infected with 10^8 *C. parvum* oocysts in PBS by transcutaneous injection. Controls were injected with PBS alone (i.e., sham infected). Tissues were collected 5 days later, and total RNA was extracted from mucosal scrapings of each individual xenograft. Equal amounts of RNA were pooled from five to eight different xenografts in each group, and levels for PGHS-2 mRNA and 28S rRNA were determined by quantitative RT-PCR. mRNA levels for β-actin that changed by <2-fold after infection (12) are not shown.

Intestinal epithelial cells are the most abundant human cell type in the xenografts (19) and are likely the only cells in this model that become infected with *C. parvum* (12). Moreover, the RT-PCR used in these studies is specific for human PGHS-2 mRNA (5). Together, these findings indicate that intestinal epithelial cells most likely are responsible for the increase in PGHS-2 mRNA levels after *C. parvum* infection in the intestinal xenograft model.

Intestinal epithelial cells respond to *C. parvum* infection, as shown here, with increased PGHS-2 expression and PGE_2 and $PGF_{2\alpha}$ production. This epithelial response can provide a partial explanation for the observation that PGHS products, and PGE_2 in particular, are important for regulating increased fluid secretion in response to *C. parvum* infection, as shown in piglets (2). Nonetheless, it is difficult at present to assess the relative contributions of epithelial cells and other cells, such as macrophages and subepithelial fibroblasts, to *C. parvum*-induced mucosal PGE_2 production at different stages of the infection. In the normal unstimulated state, epithelial PGE_2 production represents only a relatively small fraction of total mucosal PGE_2 production in the rabbit colon (4, 13). However, based on the present findings, the epithelial contribution appears to increase substantially after infection. Regardless of the absolute amount of PGE_2 produced by intestinal epithelial cells after *C. parvum* infection, epithelial cell-derived PGE_2 is likely to be more efficient than PGE_2 produced by cells in the underlying mucosa in affecting epithelial functions in a paracrine or autocrine manner, due to reduced diffusion distances. In contrast to PGE_2, epithelial production of $PGF_{2\alpha}$ is relatively high compared with total mucosal $PGF_{2\alpha}$ production in the normal rabbit colon, even in the unstimulated state (4, 13). However, $PGF_{2\alpha}$ may be less important than PGE_2 in controlling epithelial ion transport, since it was shown to be much less effective in inducing chloride secretion by polarized human intestinal epithelial cell monolayers (5).

Increased epithelial PGHS-2 expression and PGE_2 production after *C. parvum* infection may have functions other than regulating epithelial chloride and, concomitantly, fluid secretion. For example, PGE_2 upregulates epithelial mucin expression (16). Increased mucus production is observed after *C. parvum* infection of suckling mice (9), which may be a protective host response against further infection by the extracellular stages of the parasite (22). PGE_2 has also been shown to downregulate inflammatory cytokine production by activated macrophages (11), which could explain why, in some cases, diarrhea can develop after *C. parvum* infection in the apparent absence of mucosal inflammation.

We thank Roselyne Mancassola for expert technical help.

This work was supported by National Institutes of Health grant DK35108, a fellowship from the DRI of the Institut National de la Recherche Agronomique, France (F.L.), and a Career Development award from the Crohn's and Colitis Foundation of America (L.E.).

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Editor: J. M. Mansfield