Entamoeba histolytica pathogenesis in the colon occurs in a stepwise fashion. It begins with colonization of the mucin layer, which is followed by stimulation of a proinflammatory response that causes nonspecific tissue damage that may facilitate parasite invasion of the underlying colonic mucosa. Unfortunately, the parasite and/or host factors that stimulate a proinflammatory response in the gut are poorly understood. In this study, we found that live E. histolytica or secretory or proteins (SP) and soluble ameba components (SAP) can markedly increase interleukin-8 (IL-8) mRNA expression and protein production in colonic epithelial cells. The IL-8-stimulating molecule produced by live amebae was identified as prostaglandin E$_2$ (PGE$_2$) as trophozoites treated with cyclooxygenase inhibitors inhibited the biosynthesis of PGE$_2$ and eliminated IL-8 production induced by live parasites or ameba components. Moreover, using specific prostaglandin EP2 and EP4 receptor agonists and antagonists, we found that PGE$_2$ binds exclusively through EP4 receptors in colonic epithelial cells to stimulate IL-8 production. Silencing of EP4 receptors with EP4 small interfering RNA completely eliminated SP- and SAP-induced IL-8 production. These studies identified bioactive PGE$_2$ as a one of the major virulence factors produced by E. histolytica that can stimulate the potent neutrophil chemokine and activator IL-8, which can trigger an acute host inflammatory response. Thus, the induction of IL-8 production in response to E. histolytica-derived PGE$_2$ may be a mechanism that explains the initiation and amplification of acute inflammation associated with intestinal amebiasis.
the cell cycle in amebae. However, the mechanism of IL-8 induction by ameba PGE$_2$ during invasive amebiasis is not known, and it is also not clear if ameba components themselves can directly induce production of this chemokine in the gut. Here, we show that the presence of PGE$_2$ endogenously synthesized by live E. histolytica or the presence of PGE$_2$ in soluble amebic proteins (SAP) or in secretory components or proteins (SP) can induce IL-8 production by a unique pathway involving EP4 receptors on colonic epithelial cells.

**MATERIALS AND METHODS**

*Cells, reagents, and ameba components.* The Caco-2 human adenocarcinoma cell line was obtained from the ATCC and grown to obtain confluent monolayers in minimal essential medium containing 5% fetal bovine serum and 5 mg/ml penicillin-streptomycin. EP receptor-specific agonists and antagonists were obtained from Cayman Chemicals unless indicated otherwise. SAP were prepared by using three cycles of freeze-thaw lysis of phase E. histolytica virulent strain HM1:IMSS (passaged three times in gerbil livers) and were quantified by the bichinchoninic acid protein assay (Pierce). E. histolytica SP were prepared as described previously (18). For transwell studies, trophozoites were added to Corning transwell inserts with a pore diameter of 0.6 μm, with Caco-2 cells in the bottom well.

**Real-time PCR.** Total RNA was extracted with TRIZol reagent (Invitrogen) and quantified. One microgram of RNA was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and oligo(dT) according to the manufacturer’s instructions. One-tenth of the cDNA reaction mixture was used for real-time PCR. Amplification was carried out with a Quantitech SYBR green PCR kit (Qiagen) using the following cycling conditions: 94°C for 15 min, followed by 45 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. The primers used were IL-8 forward (5’ CGTGGCTCTCCTGGGACG3’), IL-8 reverse (5’TCTTTAGCCACTCTTTGCAAAAAC3’), GAPDH forward (5’GAAAGGTGAAGGTCGGA3’), and GAPDH reverse (5’GAAGATGTGATGGATTTC3’). The specificity of amplification was checked by performing a melting curve analysis. IL-8 mRNA expression was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The change compared with control level was determined by using the comparative cycle threshold method as described previously (4).

**Coculturing of epithelial cells with ameba.** Confluent Caco-2 cells (10$^6$ cells) grown in either regular or transwell plates for 7 to 10 days were used for all experiments. For small interfering RNA (siRNA) experiments, subconfluent (40 to 50%) Caco-2 cells grown for 2 to 3 days were used. Transwells with 2 × 10$^6$ amebae were incubated in culture plates containing 10$^6$ epithelial cells for 24 h at 37°C. After incubation, cells were removed from coculture and immediately used for subsequent experiments. Epithelial cells were kept under low-serum conditions (5% fetal bovine serum) during coculturing and subsequent treatments and without antibiotics for siRNA studies.

**IL-8 assays.** IL-8 production was measured by using a Titerzyme kit (Assay Designs Inc.) and a monoclonal antibody against human IL-8 according to the manufacturer’s instructions.

**Stimulation of cells with agonists, antagonists, and inhibitors.** Cells were seeded in six-well plates (10$^6$ cells/well) and allowed to attach overnight. Prior to the experiments, cells were fasted in serum-free medium overnight and then stimulated with or without PGE$_2$ and with or without other drugs. Cells were pretreated with AH6809 (an EP2 receptor antagonist) or with AH23845 or L161982 (EP4 receptor antagonists) for 30 to 60 min prior to stimulation with PGE$_2$ or EP receptor-specific agonists. Cells were then processed for RNA extraction by the TRIZol method for real-time PCR analysis or used for other studies. Most compounds were used at a final concentration of 1 μM; the only exception was AH6809, which was used at a concentration of 50 μM. The concentrations of PGE$_2$ and several EP receptor-specific agonists and antagonists used in the experiments were optimal for intestinal epithelial cells, as determined in our previous studies and in studies performed by other workers (5, 7, 14, 28).

**RNA interference.** Wild-type Caco-2 cells were transfected with EP4 siRNA (Smallpool M-005714-00) or control siRNA (D-001210-02 or D-001140-01) obtained from Dharmacon, Inc. (Lafayette, CO) using the manufacturer’s protocol. Briefly, subconfluent (50 to 60%) cells were transfected using the X-Treme Gene siRNA transfection reagent (Roche) for 36 h at an siRNA concentration of 40 nM, and cells were immediately used for experiments.

**RESULTS**

**Induction of IL-8 by E. histolytica.** To determine the kinetics by which E. histolytica induces IL-8 production in colonic epithelial cells, we treated 10$^6$ confluent Caco-2 cells with different concentrations of SP or SAP or with live E. histolytica. Total cellular RNA was extracted by the TRIZol method, and real-time PCR was performed as described in Materials and Methods. The data indicate the changes in mRNA expression compared with controls.

**Graphical representations.** Data are expressed below as means ± standard errors of the means and were analyzed using Student’s t-test for unpaired data using Graphpad Prism software. P values of ≤0.05 were considered significant.

![Graph 1](http://iai.asm.org/)  
**FIG. 1.** E. histolytica components induce IL-8 mRNA expression and protein production in colonic epithelial cells. (A) Confluent Caco-2 cells (10$^6$ cells) were treated with different concentrations of SP or SAP or with 2 × 10$^6$ live E. histolytica cells for 4 h. Live E. histolytica trophozoites were incubated in transwells with colonic cell monolayers. Total cellular RNA was extracted by the TRIZol method, and real-time PCR was performed as described in Materials and Methods. The data indicate the changes in mRNA expression compared with controls. (B) IL-8 production was quantified using enzyme-linked immunosorbent assay kits following treatment of Caco-2 cells with SP or SAP or with live E. histolytica for 12 h. The bars indicate the means and the error bars indicate the standard errors of the means for three different experiments. The asterisks indicate the results of comparisons with the controls (*, P < 0.1; **, P < 0.01; ***, P < 0.001). Eh, E. histolytica.

**FIG. 2.** E. histolytica induces IL-8 mRNA expression and protein production in a time-dependent manner.
mRNA expression occurred after 4 h, and peak IL-8 protein production occurred after 12 h. Interestingly, live E. histolytica trophozoites in transwells separated from colonic cells by membranes also significantly stimulated IL-8 mRNA expression and IL-8 production at 6, 8, and 12 h compared to untreated controls. At these time points in cocultures, about 90% of the amebae were alive as determined by a trypan blue exclusion assay (data not shown).

E. histolytica induces IL-8 production through a lipid molecule.

To determine the biochemical characteristics of the ameba-derived component(s) that stimulated IL-8 production, we boiled SP, SAP, and PGE2 for 30 min or delipidized the ameba components with chloroform-methanol (2:1, vol/vol) (21) prior to testing. As shown in Fig. 3, SP, SAP, PGE2, and live E. histolytica stimulated robust IL-8 production 27-, 44-, 57-, and 37-fold, respectively, after 4 h compared to untreated controls. However, following boiling or delipidization of the ameba components, IL-8 production was completely eliminated (P > 0.001). In parallel, we determine that proteinase K treatment of ameba components had no effect on inhibition of IL-8 production (data not shown). These data clearly suggest that lipid or glycolipid molecules may be responsible for stimulating IL-8 production in colonic cells.

Effect of COX-1/2 inhibitors on IL-8 production. Based on the results shown in Fig. 3, we thought that PGE2 produced by amebae was a likely candidate for the molecule that was responsible for stimulating IL-8 production. We have previously shown (9) that E. histolytica synthesizes PGE2 by using a novel COX-like enzyme that was inhibited by high concentrations (1 mM) of the nonselective COX inhibitor aspirin but not by other COX-1/2-specific inhibitors. Moreover, live E. histolytica amebae incubated with 100 μM arachidonic acid, the precursor of PGE2, produced high levels of bioactive PGE2 in a time-dependent manner (9). Thus, to determine the biological function of ameba-derived PGE2 in stimulation of IL-8 production in colonic cells, live amebae were treated with a COX-1/2-specific inhibitor (indomethacin), a COX-2-specific inhibitor (nimesulide), and a nonspecific COX-1/2 inhibitor (aspirin) for 16 h. As shown in Fig. 4, only treatment with aspirin significantly inhibited (eightfold; P > 0.001) IL-8 production in Caco-2 cells following exposure to live amebae in transwells compared to the results obtained with untreated amebae or amebae treated with a COX-1/2-specific inhibitor. Furthermore, SAP and SP derived from aspirin-treated live amebae also significantly inhibited IL-8 production after 12 h (sevenfold for both; P > 0.001) compared to the results obtained with untreated amebae.

FIG. 2. Induction of IL-8 mRNA expression and protein production. Confluent Caco-2 cells (10⁶ cells) were treated with 40 μg/ml of SP, 100 μg/ml of SAP, or 2 × 10⁶ live E. histolytica cells for different times as described in the legend to Fig. 1. (A) Increases in IL-8 mRNA expression compared with controls were determined by real-time PCR as previously described. (B) IL-8 production was quantified using an enzyme-linked immunosorbent assay kit. The bars indicate the means and the error bars indicate the standard errors of the means for three different experiments. The asterisks indicate the results of comparisons and the error bars indicate the standard errors of the means for three different experiments. *, P < 0.1; **, P < 0.01; ***, P < 0.001. Eh, E. histolytica.

FIG. 3. Involvement of lipid mediators (PGE2) in IL-8 production. Confluent Caco-2 cells (10⁶ cells) were treated with 40 μg/ml of SP, 100 μg/ml of SAP, 1 μM PGE2, or 2 × 10⁶ live E. histolytica cells for 12 h, and IL-8 production was measured. All components were boiled for 30 min or delipidized before treatment, and IL-8 production was assayed. Ctl, control (no treatment). The bars indicate the means and the error bars indicate the standard errors of the means for three different experiments. ***, P < 0.001. Eh, E. histolytica.

FIG. 4. Role of COX inhibitors in inhibiting E. histolytica-induced IL-8 production. Confluent Caco-2 cells were treated with 40 μg/ml of SP, 100 μg/ml of SAP, or 2 × 10⁶ live E. histolytica cells for 12 h, and IL-8 production was quantified. For inhibition studies, live amebae were pretreated with the COX-1/2 inhibitor indomethacin (50 μM), the COX-2-specific inhibitor nimesulide (40 μM), and the nonspecific COX-1/2 inhibitor aspirin (1 mM) for 16 h, and SP and SAP were prepared from drug-treated amebae as previously described. Caco-2 cells were treated with SP and SAP components, and IL-8 production was quantified by an enzyme-linked immunosorbent assay. Ctl, control (no treatment). The bars indicate the means and the error bars indicate the standard errors of the means for three different experiments. ***, P < 0.001. Eh, E. histolytica; Indo, indomethacin; NS, nimesulide; ASA, aspirin.
untreated controls or cells treated with a COX-1/2-specific inhibitor. These data suggest that PGE₂, synthesized by the COX-like enzyme in amebae, was responsible for IL-8 induction in colonic epithelial cells.

**Effect of EP receptor-specific agonist and antagonist on IL-8 production.** PGE₂ exerts its biological effects by coupling and signaling through EP2 or EP4 receptors to induce intracellular cyclic AMP production. Cyclic AMP responsive element (bp 828 to 835) regulatory sequences have been identified in the 5' untranslated region of the IL-8 gene (15). Therefore, to unequivocally demonstrate that PGE₂ derived from amebae was responsible for the induction of IL-8 production in colonic cells, we treated cells with PGE₂, SP, SAP, live E. histolytica, and several EP2 or EP4 receptor-specific agonists or antagonists and quantified IL-8 production. As shown in Fig. 5, PGE₂, the EP4 receptor-specific agonist ONO-AE1-329, SP, SAP, and live E. histolytica induced IL-8 production 29-, 26-, 9-, 15-, and 13-fold compared with the results obtained for untreated control Caco-2 cells. The EP2 receptor-specific agonist butaprost and the antagonist AH6809 did not induce IL-8 production. Remarkably, pretreatment of cells with the EP4 receptor-specific antagonist AH23848 or L161982 (EP4 receptor antagonists) for 60 min prior to treatment with PGE₂, SP, or SAP for 12 h, and IL-8 production was measured by an enzyme-linked immunosorbent assay. The bars indicate the means and the error bars indicate the standard errors of the means for three different experiments. ***, $P < 0.001$.

Interestingly, silencing of EP2 receptor expression by EP2 siRNA did not inhibit PGE₂-induced IL-8 production, suggesting that the EP2 receptor plays a minimal role in this process (data not shown). Using green fluorescent protein as a control, the transfection efficiency was routinely found to be between 65 and 75% (data not shown). During these silencing experiments with EP2 siRNA and EP4 siRNA, more than 90% of transfected Caco-2 cells were alive as determined by a trypan blue exclusion assay. Taken together, these data clearly demonstrate that EP4 receptors have an important role in PGE₂-induced IL-8 production in colonic epithelial cells.

**DISCUSSION**

E. histolytica invades the intestinal mucosa and causes amebic colitis and severe ulceration. Analysis of the inflammatory response during intestinal amebiasis in human and animal models of the disease has revealed an important regulatory role for chemokines and cytokines. Recruitment and activation of inflammatory cells can also be modulated by secreted amebic factors, such as amebapores and monocyte locomotion inhibitory factor. Several Th1/2 cytokines, such as IL-6 and IL-4, and regulatory cytokines, like IL-10 and transforming growth factor β, have been shown to be associated with the development of amebiasis (13).

Recent studies have provided evidence that chemokines, such as IL-8, are crucial mediators in inflammation and in tissue injury in intestinal inflammation. IL-8 is a small, 8- to
11-kDa secreted protein and may participate in immune and inflammatory responses through chemotraction and activation of neutrophils or leukocytes (1). The precise nature of the IL-8 signaling pathway related to epithelial cell signaling has not been defined yet. However, the initial signaling events during inflammation ultimately lead to activation and translocation of various transcription factors that control the transcription of genes encoding the various chemokines and cytokines secreted by epithelial cells (24). It has been found that both the C-X-C and C-C members of the chemokine family of proteins, as well as the proinflammatory cytokines IL-1β, IL-6, and tumor necrosis factor alpha and the cell growth factor GM-CSF, are released by epithelial cells after bacterium-endocyte interactions (27). Recently, we showed that in colonic epithelial cells, monocyte chemoattractant protein 1 is secreted in response to soluble ameba components via the phosphatidylinositol 3-kinase/P65 pathway (16). The release of this cytokine in vivo by the epithelium would be an effective means of initiating a mucosal inflammatory response. Cytokines, particularly IL-8, growth-related oncogene α, and monocyte chemoattractant protein 1, are potent chemoattractants for neutrophils and monocytes, while GM-CSF prolongs the survival of these cells and increases their response to proinflammatory agonists (3).

In this study, we established that endogenously synthesized PGE2 present in soluble and secretory ameba components could induce robust IL-8 production in colonic epithelial cells via activation of the EP4 receptor. During amebic invasion, the epithelium responds by mounting a protective inflammatory response. This may cause release of epithelial cytokines and chemokines, such as CXCL1, CXCL8, CCL2, CCL3, CCL5, IL-6, GM-CSF, gamma interferon, and tumor necrosis factor alpha. Altogether, histological analyses of human biopsies have shown that there is mild infiltration of neutrophils, accompanied by hyperplastic lymphoid aggregates with macrophages and dendritic cells, in the submucosa at the beginning of amebic ulceration along with neutrophils, macrophages, and T cells as the infection progresses (11). A member of the transmembrane kinase family, phagosome-associated TMK96, is required for amebic infection (6). Since neutrophils predominantly recruit to the submucosa during amebic infection, in vivo experiments have shown that neutrophils are not capable of killing the parasite, probably because parasite superoxide dismutases and oxidoreductases are produced, which may inhibit the neutrophil respiratory burst (8). Furthermore, neutrophil depletion in murine models of infection resulted in more severe ulceration of susceptible strains than of resistant strains (2). Moreover, ameba trophozoites interact with β2 integrins on the surface of neutrophils, induce their apoptosis through the phosphatidylinositol 3-kinase-mediated pathway (23), and activate intracellular signaling (19). Interestingly, the monocyte locomotion inhibitory factor is an anti-inflammatory oligopeptide produced by *E. histolytica* that inhibits locomotion of human monocytes (19).

In previous studies, it was shown that neutrophils play a major protective role in resolving hepatic *E. histolytica* infection in mice (25). Thus, IL-8 may play an important role in chemotraction and activation of neutrophils during the onset of amebic colitis, which may help control ameba infection. Our current findings are of considerable interest, as it is not known how *E. histolytica* triggers a host inflammatory response in the gut in the absence of cellular contact. We have shown previously that the SP and SAP components are able to induce IL-8 production in colonic epithelial cells (28); however, the identity of the ameba component responsible for the induction of IL-8 production was not determined. Here we found that ameba-derived PGE2 is responsible for stimulating IL-8 production through EP4 receptors in colonic epithelial cells. Thus, it is not surprising that silencing EP4 receptor expression completely eliminated SP- and SAP-induced IL-8 production. All attempts to date to knock down the in vitro expression of the COX-like gene by using an antisense strategy or by silencing the gene using siRNA treatments have failed. As amebae died slowly in culture following these treatments, it appears that the COX-like enzyme responsible for PGE2 biosynthesis may play a critical role in the ameba cell cycle. These findings showed that PGE2 produced in the gut by amebae is a major player in the initiation of inflammation because it induces IL-8 production in the pathogenesis of intestinal amebiasis.

The local induction of production of chemokines, such as IL-8, by epithelial cells could explain the histopathological findings for *E. histolytica* infection and thus may provide a mechanism for initiation and exacerbation of the inflammation seen during intestinal amebiasis. Moreover, modulation of host chemokines like IL-8 could perhaps be used as a virulence marker for *E. histolytica*. In summary, we show here that PGE2 produced by *E. histolytica* stimulates IL-8 mRNA expression and protein production in human colonic epithelial cells through an EP4 receptor signaling mechanism in a contact-independent manner. This observation is very important for understanding ameba pathogenic mechanisms as EP4 antagonists targeted against EP4 receptors have the potential to become therapeutically important in the treatment of amebiasis. Perhaps directly targeting the production of IL-8 or blocking EP4 receptors might alter the course of invasive amebiasis, but this is only speculation at this time.

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12. Reference deleted.


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