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Differential Regulation of β-Defensin Gene Expression during *Cryptosporidium parvum* Infection

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Invasion of enterocytes by pathogenic microbes evokes both innate and adaptive immune responses, and microbial pathogens have developed strategies to overcome the initial host immune defense. β-Defensins are potentially important endogenous antibiotic-like effectors of innate immunity expressed by intestinal epithelia. In this study, the interplay between the enteric protozoan parasite *Cryptosporidium parvum* and host epithelial β-defensin expression was investigated. Using human and murine models of infection, we demonstrated that *C. parvum* infection differentially regulates β-defensin gene expression. Downregulation of murine β-defensin-1 mRNA and protein was observed in both in vitro and in vivo models of infection. Infection of the human colonic HT29 cell line with the parasite resulted in differential effects on various members of the defensin gene family. Partial reduction in human β-defensin-1 (hBD-1), induction of hBD-2, and no effect on hBD-3 gene expression was observed. Recombinant hBD-1 and hBD-2 peptides exhibited significant antimicrobial activity against *C. parvum* sporozoites in vitro. These findings demonstrate that *C. parvum* infection of enterocytes may affect the expression of various defensins in different ways and suggest that the overall outcome of the effect of antimicrobial peptides on early survival of the parasite may be complex.

There is accumulating evidence that the intestinal epithelium protects the host against microbial infection not only by forming a physical barrier, but also by active participation in host innate defense via the production of cytokines, chemokines, and antimicrobial peptides (12, 26). Defensins are small cationic antimicrobial peptides that are increasingly being recognized as important effectors in innate immunity. The mechanism(s) of defensin antimicrobial activity is not fully understood but is likely to be related to the formation of pores in the microbial membrane that result in fatal disruption of integrity and function (41). Several studies have demonstrated that defensins exhibit killing activity against a wide range of organisms, including enteric pathogens such as *Salmonella* spp. (36), *Escherichia coli* (2), and *Giardia* spp. (1).

Based on their pattern of disulfide bonding, mammalian defensins are classified into α, β, and θ subfamilies (15). Six human and 14 murine β-defensins have been characterized, although genome sequences suggest that there are likely to be more (24, 37). Human β-defensin-1 (hBD-1) is constitutively expressed by epithelial cells, whereas expression of hBD-2, -3, and -4 is induced by infection and inflammation (10, 16, 25, 30). Like its human counterpart, murine β-defensin-1 (mBD-1) has been found to be constitutively expressed on mucosal surfaces (3), suggesting that this peptide may play a surveillance-like role in tissue homeostasis. The expression of hBD-1 and another antimicrobial peptide, LL-37, was shown to be markedly reduced during *Shigella* infection, highlighting a potential novel immune evasion mechanism that may facilitate increased persistence of pathogens at mucosal surfaces (13).

The protozoan intracellular parasite *Cryptosporidium parvum* develops in epithelial cells and is an important causative agent of infectious diarrhea in humans and neonatal domestic animals (18). Infection, which is transmitted via the fecal-oral route, begins with the ingestion of oocysts, and following excystation of sporozoites in the gut, the parasites infect the epithelium and undergo asexual reproduction followed by sexual development, which leads to new oocyst formation (6). Cryptosporidiosis may be fatal in AIDS patients (17) and can also be a serious complication among malnourished children in developing countries (22). Immunological elimination of infection involves $CD4^+$ T cells and gamma interferon (IFN-γ) production (7). Innate immune mechanisms are probably also important in early control of parasitic reproduction, and in mice, NK cell production of IFN-γ is a crucial mechanism of innate immunity to the parasite (21). To establish infection, *C. parvum* may also have to overcome the potential effect of endogenous antimicrobial peptides. The mammalian antimicrobial peptide LL-37 was found to have killing activity against *C. parvum* sporozoites in vitro (11). Tarver and colleagues showed increased enteric β-defensin expression in calves infected with *C. parvum* (31), but whether this effect is due to direct invasion of the epithelium or to a general inflammatory response to infection remains unclear.

In the present study, we explored the hypothesis that *C. parvum* actively modulates epithelial β-defensin expression and function, which may allow the parasite to escape early immunosurveillance leading to increased persistence at the gastrointestinal mucosal surface.

MATERIALS AND METHODS

Parasites. *C. parvum* oocysts of the MD isolate (genotype 2, from Moredun Scientific Ltd., Penicuik, United Kingdom) were surface sterilized by suspension in 10% commercial bleach solution (0.55% sodium hypochlorite) and washed in Dulbecco's modified Eagle's medium (DMEM). Sporozoites were prepared by

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TABLE 1. Specific primer sequences used for RT-PCR

Gene	Primer type	Sequence (5′→3′)	PCR product size (bp)
mBD-1	Sense	GGC TGC CAC CAC TAT GAA AAC TCA TTA C	147
	Antisense	GAG ACA GAA TCC TCC ATG TTG AAG GCA	
mBD-3	Sense	GTC TCC ACC TGC AGC TTT TAG CAA	132
	Antisense	GCA TTT GAG GAA AGG AAC TCC ACA AC	
hBD-1	Sense	TTG TCT GAG ATG GCC TCA GGT AAC	253
	Antisense	ATA CTT CAA AAGCAA TTT TCC TTT AT	
hBD-2	Sense	CCA GCC ATC AGC CAT GAG GGT CTT G	276
	Antisense	CAT GTC GCA CGT CTC TGA TGA GGG AGG	
hBD-3	Sense	AGC CTA GCA GCT ATG AGG ATC	205
	Antisense	CTT CGG CAG CAT TTT CGG CCA	
GAPDH	Sense	CTA CTG GCG CTG GCA AGG CTG T	359
	Antisense	GCC ATG AGG TCC ACC ACC CTG CTG	

excysting oocysts in DMEM for 1 h at 37°C and purified by passage through a 5-μm polycarbonate filter (Millipore, Bedford, United Kingdom).

In vitro infection of human and murine cell lines. Human colonic (HT29) and murine rectal adenocarcinoma (CMT-93) cell lines (European Collection of Cell Cultures, Salisbury, United Kingdom) were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 1% nonessential amino acids (Invitrogen, Paisley, United Kingdom) in a 5% CO_2 incubator at 37°C. Cells were seeded into 6- or 24-well plastic tissue culture plates (with or without 13-mm-diameter glass coverslips) and grown to confluence over 5 days. Cell monolayers were infected with 4×10^5 *C. parvum* oocysts, and parasite development was quantified after Giemsa staining.

Cytokine stimulation. Human and murine recombinant cytokines (Peprotech, London, United Kingdom) were reconstituted as recommended by the supplier. The range of cytokine concentrations used in the present study exhibited no cytotoxic effects.

In vivo model of infection. Several strains of mice, including BALB/c, C57BL/6, and BALB/c IFN-γ gene knockout (KO) (the last were originally from Jackson Laboratories, Bar Harbor, Maine), were bred under aseptic conditions. During experiments, they were housed in cages with filter lids. All experiments were carried out under license from the United Kingdom Home Office Animal Procedures Committee. Seven-day-old mice were infected by oral inoculation with 10^4 oocysts in 5 μl of phosphate-buffered saline (PBS). Infection was monitored microscopically by acid-fast (Ziehl-Neelsen) staining of oocysts in colonic smears. The number of oocysts in 50 random fields at ×1,000 magnification was calculated.

RNA isolation and RT-PCR. All molecular biological reagents were from Invitrogen, Paisley, United Kingdom. Total cellular RNA was isolated from cell culture and colonic biopsies with a monophasic solution of phenol and guanidine thiocyanate (Trizol), as recommended by the suppliers. To generate cDNA, 2 to 4 μg of total cellular RNA was transcribed at 42°C for 50 min in a 20-μl reaction volume containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 3 mM dithiothreitol, 10 mM deoxynucleoside triphosphate mix, and 0.5 μg of oligo(dT), plus 100 U of Moloney murine leukemia virus reverse transcriptase. PCR amplification was routinely conducted in a 50-μl reaction volume (10 mM Tris [pH 9], 50 mM KCl, 1.5 mM $MgCl_2$, a 200 μM concentration of each of the four deoxynucleoside triphosphates, and 20 pmol of specific 5′ and 3′ primers), plus 1 U of *Taq* polymerase. The amplification profile consisted of denaturation at 94°C for 90 s, annealing at 58°C for 90 s, and extension at 72°C for 90 s for 40 cycles. PCR products were separated on a 2% agarose gel, and band intensities were quantified by densitometry (ID Image Analysis software; Kodak, Rochester, N.Y.). Defensin expression was normalized to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequence-specific primers used in this study and the expected PCR product sizes are listed in Table 1.

β-Defensin peptide expression. Cationic peptide fractions were extracted from control and infected CMT-93 monolayers. Cells were washed with PBS and homogenized in ice-cold 20% acetic acid that contained 1:100 (vol/vol) complete protease inhibitor cocktail (Roche, Lewes, United Kingdom). The extracts were sonicated for 1 min on ice and left with stirring overnight at 4°C. Supernatants were collected by centrifugation (1,500 × *g*, 10 min) and diluted 1:15 (vol/vol) with sterile H_2O, and the pH was adjusted to 7.0 with ammonium hydroxide. The resultant supernatant was mixed with 1:50 (vol/vol) carboxylmethylcellulose (CM) resin (Bio-Rad, Hemel Hempstead, United Kingdom) which had been equilibrated with 10 mM ammonium acetate buffer (pH 6.8). After overnight incubation at 4°C, the resin was collected by centrifugation (1,500 × *g*, 10 min), and unbound proteins were washed with 5 mM ammonium acetate (pH 7.0). The cationic components were eluted in 10% acetic acid.

The resulting eluate was lyophilized and resuspended in 10 mM acetic acid for further analysis; 10 μg of total protein was subjected to 16.5% Tris–tricine polyacrylamide gel electrophoresis (PAGE) prior to transfer onto a polyvinylidene difluoride membrane with a SemiPhore semidry transfer system (Amersham, Little Chalfont, Bucks, United Kingdom) at 20 V for 40 min. The membrane was fixed with UV cross-linking (5 min) and blocked in 10% skim milk–PBS–0.5% Tween 20 (milk–PBS-T). The blots were incubated with a goat anti-mBD-1 polyclonal antibody (1:2,000; Santa Cruz Biotechnology, Santa Cruz, Calif.) in 5% milk–PBS-T for 2 h at room temperature. After appropriate washes, incubation with a rabbit anti-goat immunoglobulin G-horseradish peroxidase secondary antibody (1:2,000 dilution in 5% milk–PBS-T; Santa Cruz Biotechnology) was performed for 1 h at room temperature. After three washes in PBS-T, the reaction mixture was developed with the ECL Plus detection kit (Amersham).

Antimicrobial assay. Recombinant hBD-1 (rhBD-1) and rhBD-2 (Peprotech, London, United Kingdom) were reconstituted according to the supplier's instructions. *C. parvum* sporozoites (4×10^5) were incubated with or without defensin (10^{-5} M) in 100 μl of 0.01% acetic acid containing 0.2% bovine serum albumin for 60 min at 37°C. Sporozoite viability was then assessed by flow cytometry and by reproduction in CMT-93 cells. For flow cytometric analysis, the sporozoites were resuspended in 100 μl of PBS containing 33 μM CFSE (5[6]-carboxyfluorescein diacetate *N*-succinimidyl ester; Sigma), a nontoxic dye, and incubated at 37°C for 15 min before analysis (5). To determine an appropriate analysis region for viable cells, fresh untreated *C. parvum* sporozoites were studied. The sporozoites were run on a Becton Dickinson FACScan (Cellquest software), and 60 to 70,000 events were recorded per tube. Defensin-treated sporozoite viability was also examined by development in cell culture. Confluent CMT-93 cell monolayers were infected with 4×10^5 defensin-treated or untreated sporozoites, and after 24 h, the number of intracellular parasites was assessed by Giemsa staining.

Statistics. Results were analyzed with a two-tailed Student *t* test of unpaired means.

RESULTS

Murine β-defensin expression during *C. parvum* infection. *C. parvum* development in CMT-93 monolayers was confirmed by Giemsa staining of parasite asexual stages. In a typical experiment, the mean number of intracellular parasites after 24 h of infection was 150 ± 15 parasites/20 fields, with about 20% of

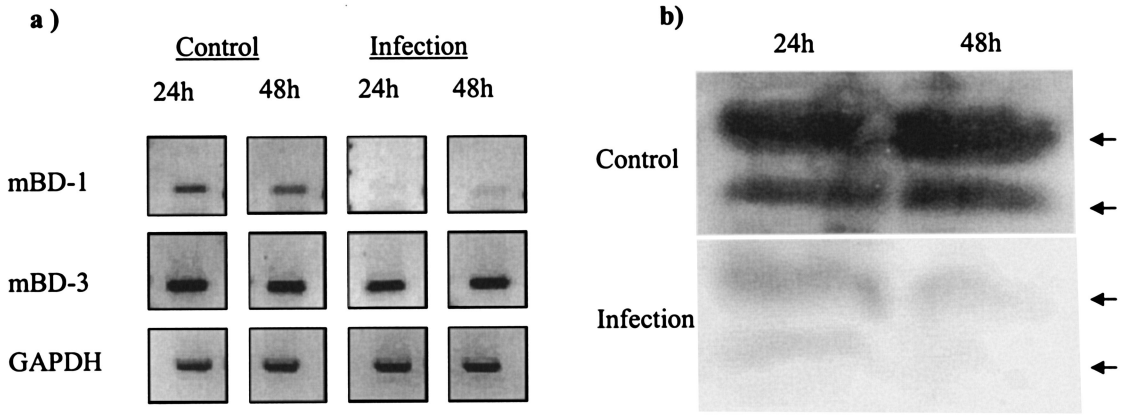

FIG. 1. Effect of *C. parvum* infection on mBD-1 and mBD-3 expression in CMT-93 cells. (a) RT-PCR demonstrated that *C. parvum* downregulated mBD-1 mRNA expression in CMT-93 cells at 24 and 48 h postinfection, with a greater effect observed at 24 h. In contrast, the parasite had no effect on the expression of mBD-3 in CMT-93 cells. (b) Western blots showing downregulation of mBD-1 peptide expression in CMT-93 cells 24 and 48 h after infection with *C. parvum*.

cells being infected. The parasite numbers declined at 48 h (79 ± 10 parasites/20 fields) and 72 h (33 ± 7 parasites/20 fields).

mBD-1 was previously reported to be constitutively expressed by epithelial cells (3, 23). To determine the regulation of mBD-1 gene expression during *C. parvum* infection, RT-PCR was performed on RNA extracted from uninfected and infected CMT-93 cells at 24 and 48 h postinfection. mBD-1 mRNA was constitutively expressed in uninfected control cells, whereas complete inhibition of expression was observed in cells at 24 h postinfection (Fig. 1a). Interestingly, at 48 h postinfection there was partial recovery of expression that was associated with a decrease in the number of developing parasites.

To determine whether *C. parvum* infection also caused a reduction in translation of mBD-1, peptide levels were analyzed by Western blotting. mBD-1 peptide levels were decreased as a result of infection compared to the control, and there appeared to be a greater reduction in expression at 48 h than at 24 h postinfection (Fig. 1b). The antibody used for Western blotting revealed that mBD-1 was a doublet, suggesting that partial degradation of mBD-1 may have occurred during the peptide extraction procedure.

mBD-3, a homologue of hBD-2, was previously reported to be upregulated in response to bacterial infection (4, 29). In the present study, we observed high constitutive expression of mBD-3 in uninfected control cells with no further effect during infection (Fig. 1a).

Downregulation of mBD-1 expression occurs in vivo following infection. Studies were performed to investigate defensin gene regulation in a murine model of *C. parvum* infection. In neonatal BALB/c mice, oocyst production became patent on day 4, the peak of infection was observed on day 7, and recovery was achieved by day 21 (Fig. 2a). Constitutive expression of mBD-1 mRNA was observed in all uninfected control mice (Fig. 2b). In contrast, all infected mice showed significant downregulation of defensin expression as early as day 4 postinfection, proceeding to complete inhibition by day 7. As the mice approached recovery (day 14), expression was detectable in two of four mice (Fig. 2b). Densitometric analysis (Fig. 2c) of the PCR data confirmed the extent of downregulation of mBD-1 expression.

In studies with C57BL/6 mice, the pattern of infection was similar to that observed in BALB/c mice and downregulation of mBD-1 expression was obtained. However, the complete inhibition of mBD-1 expression consistently observed with BALB/c mice at the peak of infection was not found in C57BL/6 mice (results not shown).

IFN-γ indirectly regulates mBD-1 expression infection in vivo. The crucial role of IFN-γ in immunity to *C. parvum* is now well established (21, 35, 40). To elucidate if IFN-γ regulates host innate immune responses at the site of invasion via modulation of defensin gene regulation, studies were conducted with BALB/c IFN-γ KO mice. These mice developed an acute infection often accompanied by diarrhea (which was not observed in wild-type animals) and while wild-type mice had recovered by day 21 (Fig. 2a), the IFN-γ KO mice developed a low-grade chronic infection that lasted up to at least day 42 (Fig. 3a). This pattern of infection was similar to that reported previously for adult BALB/c IFN-γ KO mice (40).

Analysis of defensin gene expression showed substantial downregulation of mBD-1 in three out of five mice around the peak of infection on day 7, and on day 14 there was no significant difference in defensin expression between infected and uninfected mice (Fig. 3b and c). The variable observations on day 7 were reproducible in another experiment, suggesting that the downregulation of mBD-1 mRNA expression is less pronounced in IFN-γ KO animals than in BALB/c wild-type mice, in which expression at this time was eliminated (Fig. 2b). These results suggest that IFN-γ may play a role in vivo in the observed downregulation of mBD-1 expression during *C. parvum* infection. To investigate further the regulatory role of IFN-γ-mediated effects on mBD-1 expression, CMT-93 cells (which express the IFN-γ receptor) were cultured in the presence of IFN-γ (100 U/ml) for 24 h, and mBD-1 expression was quantified. No significant difference was obtained in mBD-1 expression between IFN-γ-stimulated and unstimulated cells, suggesting that IFN-γ does not directly modulate mBD-1 expression.

***C. parvum* infection has various effects on human β-defensin gene expression.** Expression of human β-defensins during *C. parvum* infection was investigated with HT29 cells, in which

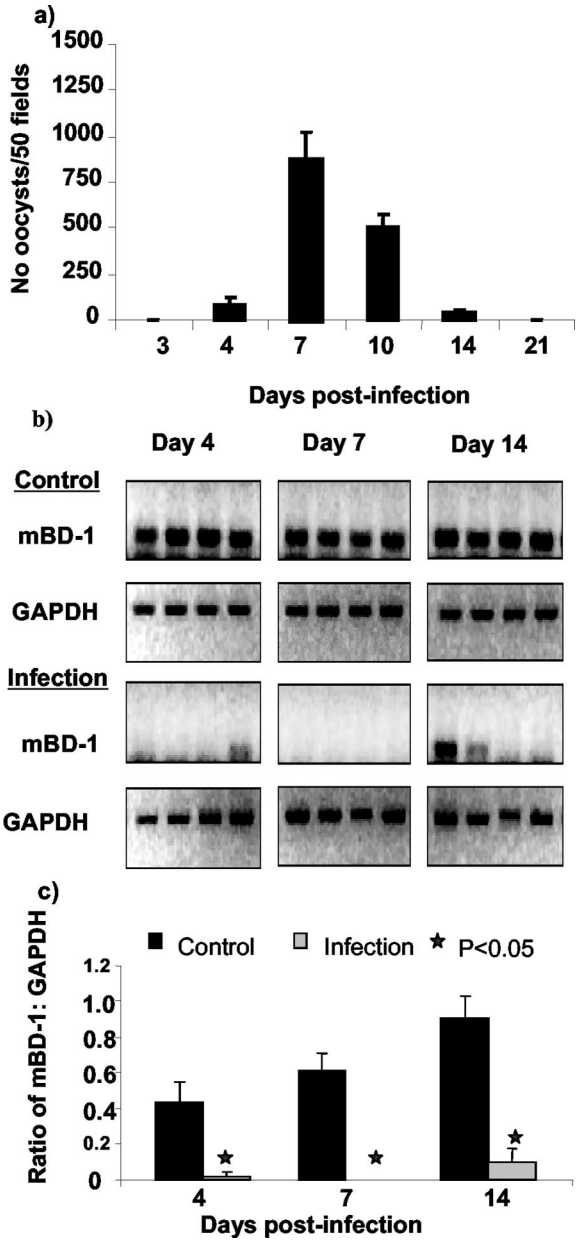

FIG. 2. Effect of *C. parvum* infection on mBD-1 expression in the intestine of mice. (a) Measurement of *C. parvum* oocyst production in infected neonatal BALB/c mice. Oocyst excretion first became patent on day 4, the peak of infection occurred around day 7, and recovery was achieved by day 21. (b) Results of RT-PCR showing dynamic changes in colonic mBD-1 mRNA expression in infected BALB/c mice. There was marked downregulation on day 4, complete inhibition on day 7, and expression recovery in some mice (two of four) on day 14 postinfection. (c) Densitometric analysis of RT-PCR data showing significant downregulation of mBD-1 mRNA expression on days 4 and 14 ($P < 0.02$), and no defensin expression was observed on day 7.

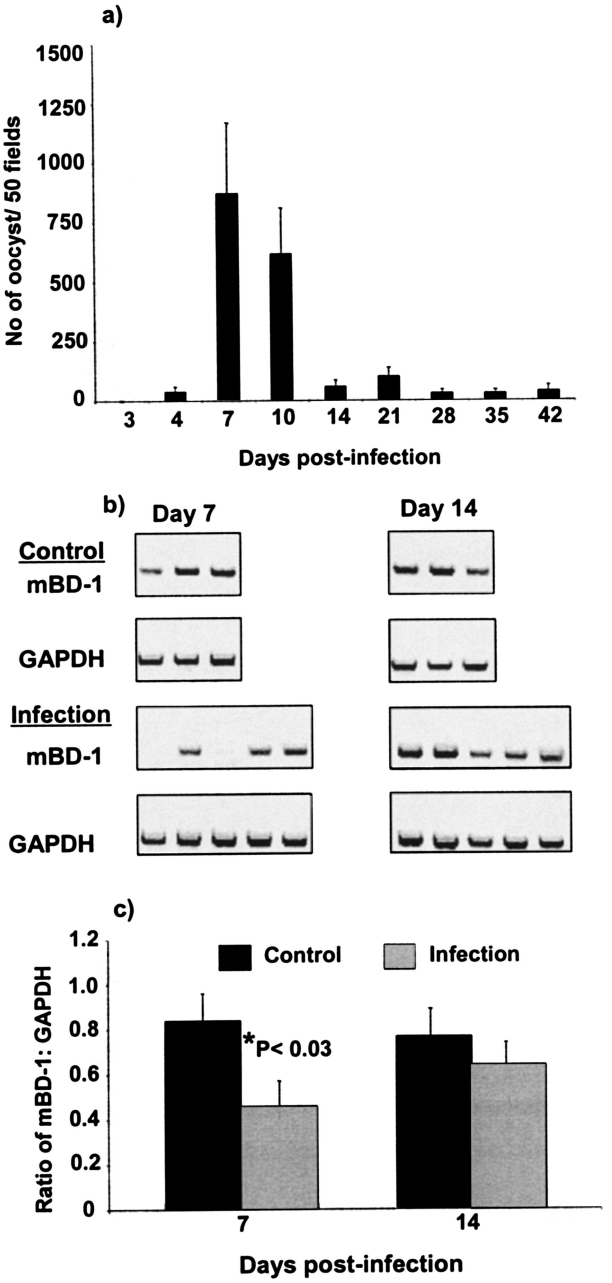

FIG. 3. Role of IFN-γ in downregulation of mBD-1 expression by *C. parvum* infection. (a) Oocyst production by *C. parvum*-infected neonatal BALB/c IFN-γ KO mice. The mice developed an acute infection that peaked around day 7, followed by a low-level chronic infection lasting until at least day 42. (b) RT-PCR analysis showed downregulation of mBD-1 mRNA at the peak of infection (day 7), but expression had recovered by day 14. (c) Densitometry analysis of data showing significant inhibition of mBD-1 at day 7 ($P < 0.03$) but not at day 14.

parasite development was confirmed by microscopic examination. The expression of hBD-1 was found to be constitutive in uninfected control cells, and, as observed in the murine model, significant downregulation of expression had occurred 24 h after infection (Fig. 4a). No expression of hBD-2 was detectable in uninfected cells, but there was expression in response to *C. parvum* infection (Fig. 4b). Whereas expression of hBD-1 and -2 was modulated by *C. parvum* infection, the parasite had no effect on the expression of hBD-3 mRNA in this cell line. IFN-γ (40 ng/ml)-stimulated HT29 cells were included as a positive control in this experiment because this cytokine is known to induce expression of hBD-3 (Fig. 4c). Densitometric

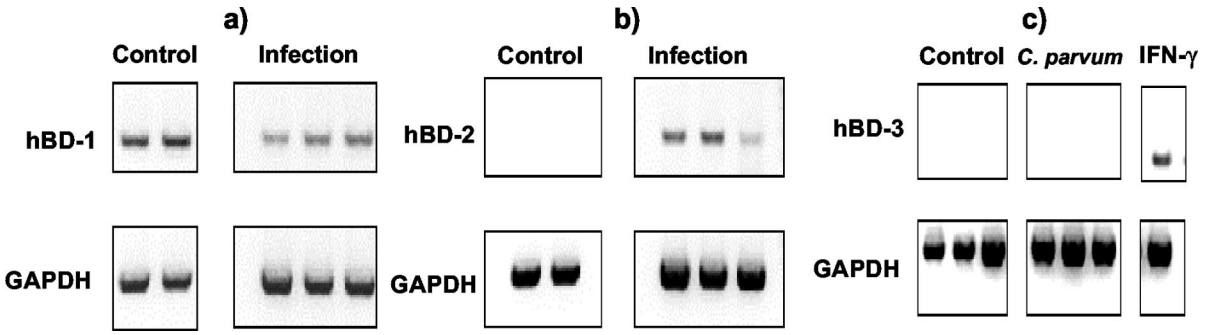

FIG. 4. Regulation of human β-defensin gene expression in HT29 cells during *C. parvum* infection. (a) RT-PCR analysis showing that *C. parvum* infection downregulated hBD-1 mRNA 24 h after infection. (b) Infection upregulated hBD-2 mRNA expression in HT29 cells 24 h after infection. (c) Infection did not induce hBD-3 mRNA expression in HT29 cells, although cells stimulated with IFN-γ (40 ng/ml) did express hBD-3.

analyses of the effect of *C. parvum* infection on human defensin expression confirmed the results described here (data not shown).

Proinflammatory cytokines have varied effects on expression of human β-defensins. As *C. parvum* infection induces a strong inflammatory response in the intestine (28, 35), we investigated the effect of a number of proinflammatory cytokines on defensin expression. Our data indicated that the constitutive expression of hBD-1 was not affected by any of the cytokines tested, IFN-γ, interleukin-1β or interleukin-6 (Fig. 5). In contrast, hBD-2 was upregulated in interleukin-1β-stimulated cells, and expression of hBD-3 was induced by all three cytokines. These results demonstrate the varied effects of cytokines on expression of members of the β-defensin family and suggest that, in vivo, the local inflammatory response as well as the parasite may influence expression.

β-Defensins exhibit killing activity against *C. parvum* sporozoites. Experiments were performed to determine the effect of rhBD-1 and rhBD-2 peptides on intracellular development of *C. parvum* sporozoites and also the structural integrity of the sporozoites. After exposure to 10^{-5} M rhBD-1 or hBD-2 for 1 h, parasite viability was determined by reproduction in CMT-93 cells. After 24 h of infection initiated with rhBD-1- or rhBD-2-treated sporozoites, the average number of intracellular parasites was reduced by 43% ± 2.3% and 58% ± 3.8%, respectively, compared with untreated sporozoites ($P < 0.05$) (Fig. 6). To determine if this reduction in parasite viability could be explained in part by defensin-mediated damage to parasites, sporozoites treated with either peptide were stained with CFSE and analyzed by flow cytometry. CFSE itself did not appear to reduce sporozoite viability, as preliminary experiments showed that the parasites were able to invade host cells and undergo intracellular development (data not shown). Live and dead parasites were gated (Fig. 7, control, right and left, respectively), and a reduction in the percentage of viable sporozoites was observed following treatment with rhBD-1 (20%) or rhBD-2 (40%) compared with controls (Fig. 7). These results indicate that both hBD-1 and hBD-2 have the potential to contribute significantly to the host's armory of innate responses by directly damaging sporozoites and reducing their capacity to develop intracellularly.

DISCUSSION

The regulation of epithelial β-defensin expression during *C. parvum* infection was investigated to further our understanding of host-parasite interactions at the site of invasion. Constitutive expression of mBD-1 mRNA and protein in CMT-93 cells was inhibited during infection. Similarly, expression of mBD-1 in BALB/c mice was severely impaired around the peak of infection. Hence, downregulation of defensin expression by *C. parvum* infection may be an immune escape strategy used by the parasite.

IFN-γ production by T cells and NK cells plays an important part in clearance of *C. parvum* infection (21). In contrast to observations with wild-type BALB/c mice, only a modest decrease in mBD-1 mRNA was observed in IFN-γ KO mice, suggesting that IFN-γ may be partly involved in the downregulation of mBD-1 expression. Why IFN-γ might have this effect on expression of an antimicrobial peptide is unclear at this stage, but the finding that the cytokine had no effect on the expression of mBD-1 in CMT-93 cells suggests that, in vivo, it

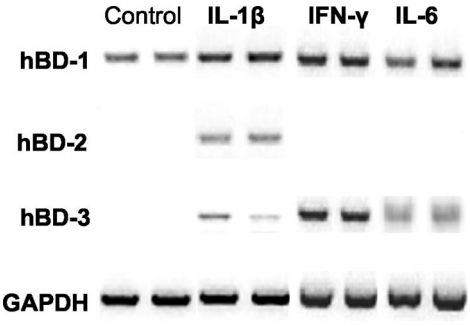

FIG. 5. Effect of proinflammatory cytokines on human β-defensin gene expression in HT29 cells. Cells were incubated with recombinant human interleukin-1β (IL-1β) (20 ng/ml), IFN-γ (40 ng/ml), or interleukin-6 (IL-6) (40 ng/ml) for 24 h. RT-PCR was performed on RNA extracted from control and stimulated cells. There was constitutive expression of hBD-1 mRNA in unstimulated cells, and this was not affected by stimulation with proinflammatory cytokines. In contrast, hBD-2 mRNA expression was upregulated after stimulation with interleukin-1β but not with IFN-γ or interleukin-6. hBD-3 mRNA was not detected in unstimulated cells but was upregulated in response to all three cytokines.

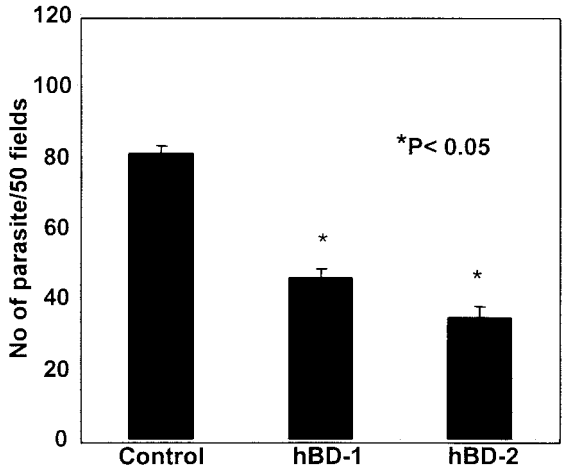

FIG. 6. Effect of treatment of *C. parvum* sporozoites with recombinant human β-defensins on in vitro intracellular development. Purified *C. parvum* sporozoites (4×10^5) were exposed to 10^{-5} M rhBD-1 or hBD-2 or medium only for 1 h and then added to CMT-93 cell monolayers in a 24-well plate. After 24 h, the cells were fixed and stained with Giemsa stain, and the number of intracellular parasites was determined by microscopic examination. Treatment with either defensin reduced the viability of sporozoites compared with that of sporozoites incubated in medium only ($P < 0.05$).

acts indirectly through activation of other cells. Studies have suggested that mBD-3 mRNA is only weakly, if at all, expressed in enterocytes under basal conditions, but expression is upregulated in response to infection and inflammation (29). Unexpectedly, a high level of constitutive expression of mBD-3 mRNA was observed in CMT-93 cells, which was unaffected by infection. This level of expression of mBD-3 may be a peculiar feature of this murine cell line.

In experiments with the human enterocyte cell line HT29, *C. parvum* was shown to downregulate hBD-1 mRNA expression, although the level of inhibition appeared to be less than for mBD-1. This difference might be partly attributable to variation between cell lines in susceptibility to infection. Another study has shown downregulation of hBD-1 and LL-37 expression in colonic mucosa obtained from patients with *Shigella* infection, and bacterial plasmid DNA was identified as a mediator of this immune evasion mechanism (13). The expression of hBD-2 mRNA has previously been reported to be upregulated in various epithelia by inflammatory stimuli or other infections (25). The present study showed that a protozoan parasite, *C. parvum*, can induce hBD-2 mRNA expression. The expression of hBD-3 has been shown to occur not only in epithelial cells but also in other cells, such as leukocytes, heart cells, and skeletal muscle cells (9). *C. parvum* infection did not induce expression of hBD-3 mRNA. The variance between the effect of *C. parvum* on hBD-2 and -3 might reflect requirements for different stimuli and activation of specific signaling pathways for expression. *C. parvum* infection of enterocytes is known to activate NF-κB (20), and whereas the hBD-2 gene has been demonstrated to be a target for NF-κB (25), induction of hBD-3 expression has been reported to involve NF-κB-independent mechanisms (8).

The proinflammatory cytokines IFN-γ, interleukin-1β, and interleukin-6 had varied effects on human defensin gene expression. None of the cytokines affected constitutive expression of hBD-1, only interleukin-1β induced hBD-2 expression, and all three cytokines induced expression of hBD-3. Tumor necrosis factor alpha is upregulated in the intestine during

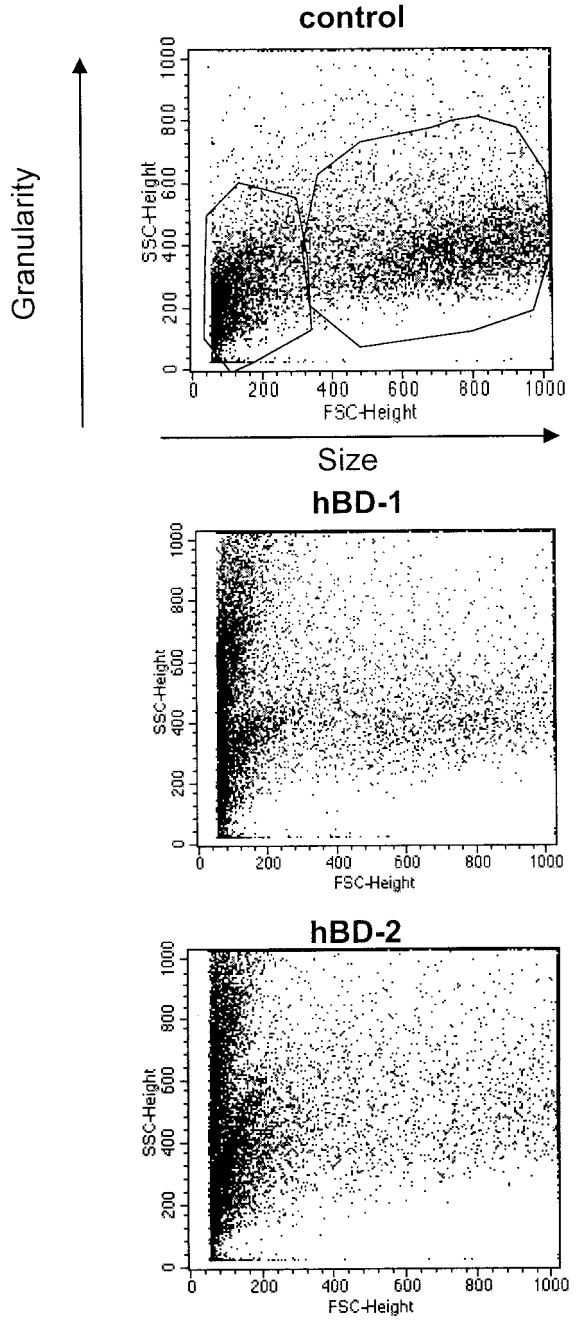

FIG. 7. Flow cytometry measurement of in vitro killing of *C. parvum* sporozoites by recombinant human β-defensins. Purified *C. parvum* sporozoites were exposed to 10^{-5} M rhBD-1 or rhBD-2 or incubation medium only for 1 h and then stained with CFSE. The representative scatter plot analyses were obtained after the following treatments: the upper plot is an untreated sample, with the right gate representing viable sporozoites as determined by size and granularity and the left gate representing dead parasites; the middle plot shows sporozoites treated with rhBD-1; the bottom plot shows sporozoites treated with rhBD-2. Analysis of flow cytometry data indicated that exposure to rhBD-1 or rhBD-2 reduced the percentages of viable parasites by 20 and 40%, respectively.

C. parvum infection (27), but this cytokine was previously found not to affect expression of human β-defensins (25). Overall, however, the results from the present study suggest that during *C. parvum* infection, the inflammatory response by enterocytes or local immune cells might affect β-defensin expression. Previous studies have shown that induction of hBD-2 in skin during bacterial infection required interleukin-1 production by dendritic cells or macrophages (16) and NF-κB activation (32). The present study offers the first data demonstrating the regulation of hBD-3 mRNA expression in intestinal cells. Interestingly, hBD-3 mRNA has been reported to be expressed constitutively at a low level in a keratinocyte cell line, and in contrast to our results with intestinal cells, expression was not affected by interleukin-1α or -6, although it was upregulated by IFN-γ stimulation (9). These observations may suggest that expression of hBD-3 is differentially regulated at different epithelial sites.

How *C. parvum* downregulates β-defensin-1 expression is unknown. The fact that only a moderate percentage of cells became infected in vitro might suggest that the effect of the parasite is indirect. *C. parvum*-infected enterocytes upregulate the expression of proinflammatory mediators (14, 19), and some of these may modulate defensin expression via autocrine stimulation. Alternatively, the parasite releases numerous soluble factors during infection which are potentially involved in virulence (33), and these may also modulate defensin expression and function.

Induction of hBD-2 during *C. parvum* infection could be important not only for its microbicidal but also for its chemotactic activity, allowing recruitment of dendritic and T cells to the site of infection (39). Furthermore, defensins can upregulate T-cell responses by effects on dendritic cells, inducing production of proinflammatory cytokines such as interleukin-12 (38), which plays an important role in immunity to *C. parvum* (34).

Defensins have been reported to have a broad-spectrum killing activity against a wide range of bacteria, fungi, and viruses. The protozoan parasite *Giardia lamblia*, which colonizes the small intestinal lumen, was previously reported to be sensitive to α-defensin peptides in vitro (1), and the cathelicidin peptide LL-37 had killing activity against *C. parvum* sporozoites (11). In the present study, sporozoites were susceptible to killing by both hBD-1 and hBD-2, with hBD-2 appearing to be more efficient in this activity than hBD-1. These results suggested that defensin peptides might actively participate in intestinal innate immunity against *C. parvum* extracellular stages (sporozoites, merozoites, and microgametes). Hence, downregulation of innate immune mechanisms such as defensin expression by the parasite could facilitate its survival in a potentially hostile environment.

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