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Activation of Intestinal Intraepithelial T Lymphocytes in Calves Infected with *Cryptosporidium parvum*

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The objective of this study was to identify disease-related changes in lymphocyte populations within ileal mucosae of calves with cryptosporidiosis. Groups of five neonatal calves were orally infected at 3 days of age with 10^8 oocysts and maintained in enteric-pathogen-free conditions until clinical disease was established or until the animals had recovered from disease. Age-matched uninfected calves were used for comparison. Ileal mucosal lymphocytes were collected, quantitated, and phenotyped to determine whether changes in lymphocyte composition occurred in infected animals. We observed significantly larger numbers of intraepithelial $CD8^+$ T lymphocytes in ileal mucosae from acutely infected calves compared with those from control animals. In addition, a proportion of intraepithelial $CD4^+$ T cells from acutely infected calves coexpressed CD25, whereas there was an absence of coexpressed CD25 on $CD4^+$ T cells from control calves. *Ex vivo* reverse transcriptase PCR of RNA from intraepithelial lymphocytes from control calves showed a cytokine expression pattern consisting of tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), while intraepithelial lymphocytes from calves with cryptosporidiosis expressed IFN-γ but not TNF-α. Together, the results indicate that changes occur in the ileal intraepithelial lymphocyte population coincidently with *Cryptosporidium parvum*-induced enteric disease.

Cryptosporidium parvum is an apicomplexan parasite that infects intestinal epithelium (13, 14). Infection induces acute enteric disease which is self-limiting in immune-competent humans but persistent and life-threatening in immunocompromised individuals (13, 14, 18). Cryptosporidiosis within the human population is of increasing concern, as several recent outbreaks have been associated with contamination of municipal water supplies (15, 19, 24, 30).

Immunodeficient mouse models of *C. parvum* infection have shown that T lymphocytes are important in the prevention of chronic intestinal infection (4, 32, 40) and that $CD4^+$ T lymphocytes and gamma interferon (IFN-γ) are required to terminate persistent infection (2, 33, 41). However, studies with mice have not defined mechanisms in gut mucosa by which immune-competent hosts recover from disease.

C. parvum-infected neonatal calves develop enteric disease resembling that of immune-competent humans. Infection localizes in the small intestine and results in villous atrophy and fusion, with leukocyte infiltration into the intestinal mucosa (18, 39). Although morbidity is high, mortality is low and recovery is spontaneous (13, 18, 39). T-lymphocyte-mediated immune responses have been implicated in the recovery of calves from *C. parvum* infection (42); however, as in the mouse models, the intestinal mucosa has not been extensively studied.

This study was undertaken to identify ileal mucosal lymphocytes which become activated during *C. parvum* infection. The results indicate that affected gut mucosa contains increased numbers of $CD8^+$ T lymphocytes, a substantial proportion of activated $CD4^+$ T lymphocytes, and altered cytokine expression. These changes are confined to the intraepithelial subcompartment.

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MATERIALS AND METHODS

Animals. Newborn bull calves were obtained from the Washington State University dairy facility and maintained for 9 to 22 days in isolation facilities, as previously described (5). A minimum of six fecal samples from each calf were tested for enteric pathogens commonly encountered in calves. These included F41 or F5 pilus-positive *Escherichia coli*, *Salmonella* and *Pseudomonas* spp., rotavirus, and coronavirus. Only animals that consistently tested negative for these agents were used in experiments. Cecal contents were taken at necropsy to confirm the absence of these enteric pathogens at the completion of each experiment.

The calves were maintained according to accepted procedures of the American Association for Accreditation of Laboratory Animal Care. The calves were arbitrarily assigned to one of the following four groups ($n = 5$ for all groups): (i) animals infected at 3 days of age and terminated at 9 to 12 days of age, (ii) age-matched controls terminated at 9 to 12 days of age, (iii) animals infected at 3 days and terminated at 20 to 22 days of age, and (iv) age-matched controls terminated at 20 to 22 days of age. Ileal intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated from all of the calves.

***C. parvum* infection.** Calves were infected at 3 days of age by oral administration of 10^8 *C. parvum* oocysts as previously described (5). Calves were confirmed as infected when fecal oocysts could be identified and after diarrhea had begun (9 to 12 days of age). These calves shed a mean ± 1 standard error of the mean (SEM) of $1.4 \times 10^9 \pm 6.9 \times 10^9$ oocysts over 2 to 4 days of diarrhea prior to necropsy. Calves were classified as recovered from disease when they no longer shed detectable fecal oocysts and when diarrhea had terminated at least 2 days prior to necropsy (20 to 22 days of age). These animals shed a total of $10 \times 10^9 \pm 4.2 \times 10^9$ oocysts over 3 to 7 days and had diarrhea for 1 to 7 days.

Isolation of IEL and LPL. Each calf was euthanized by intravenous anesthetic overdose. Beginning 1 cm proximal to the ileo-cecal junction, 40 cm of ileum was removed for processing. The ileo-cecal junction, the attached 1 cm of ileum, and a portion of the cecum were fixed in 10% buffered formalin. The tissue was later sectioned and stained with hematoxylin and eosin (H&E) to confirm that gut morphology was consistent with the clinical status of each calf.

The ileum was flushed with cold phosphate-buffered saline and cut into pieces 10 to 14 cm long. Each piece was ligated with string at one end and then everted to expose the luminal surface. Each everted piece was then filled with warm Hank's balanced salt solution (HBSS) (Sigma Chemical Co., St. Louis, Mo.) and

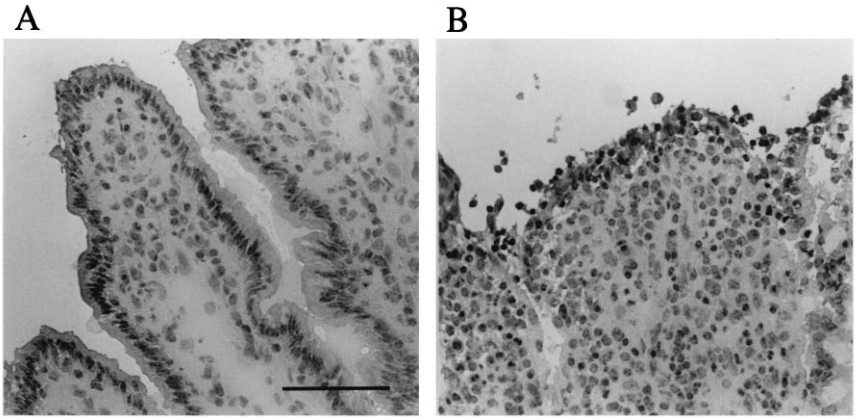

FIG. 1. Comparison of intestinal mucosa before and after removal of IEL. (A) H&E-stained section of ileal mucosa frozen prior to processing to remove IEL. (B) Section of the same ileum after removal of IEL. All ilea were evaluated to confirm maintenance of lamina propria membrane integrity. Bar = 100 μm.

ligated at the opposite end. IEL and LPL isolation has been previously described (43). Briefly, everted ileum lengths were incubated at 37°C in HBSS containing HEPES (GIBCO BRL, Grand Island, N.Y.) and dithiothreitol and then shaken to dislodge epithelial cells and IEL. Collagenase type II (Sigma Chemical Co.) (100 U/10 g of tissue) in HBSS containing HEPES was used to dissociate the lamina propria membrane and obtain LPL. Tissue was incubated in collagenase for 30 min at 37°C. Small pieces of ileum were snap frozen after each step in the processing sequence. These were later sectioned at 10 μm, stained with H&E, and examined to confirm the completeness of the collection procedures. Only IEL preparations in which the lamina propria membrane remained intact and LPL preparations in which underlying continuous Peyer's patch nodules remained intact were included in the results.

IEL and LPL were separated from nonlymphoid cells on discontinuous gradients of Percoll (Sigma Chemical Co.) as previously described (43). Lymphoid cell bands were collected, washed in HBSS containing HEPES, and counted.

MAbs and flow cytometry. Monoclonal antibodies (MAbs) to bovine leukocyte differentiation molecules were used with single- and dual-fluorescence flow cytometry to evaluate lymphocyte populations. MAbs to bovine CD2 (BAQ95A, immunoglobulin G1 [IgG1]), CD3 (MM1A, IgG1), CD4 (CACT138A, IgG1; ILA11, IgG2a), CD8α (CACT80C, IgG1; CACT130A, IgG3), CD8β (BAT82A, IgG1), TcR1 (GB21A, IgG2a; CACT61A, IgM), surface (s)IgM (BIg73A, IgG1), a non-sIgM B-cell molecule (BAQ44A, IgM), MHC class II (TH14B, IgG2a), and interleukin 2 (IL-2) receptor α (CD25) (CACT116A, IgG1; CACT108A, IgG2a) were obtained from W. C. Davis, Washington State University, Monoclonal Antibody Center (Pullman, Wash.) (16, 17, 20, 27). The percentage of cells expressing a given molecule was determined from the average of two to four sample replicates.

Cells were labeled for dual-fluorescence flow cytometry as previously described (16, 44, 45), using indirect label with goat anti-mouse immunoglobulin isotype-specific antibodies conjugated with either fluorescein or phycoerythrin (CalTag, South San Francisco, Calif.). Labeled cells were evaluated with a FACScan flow cytometer and LYSYS II analysis software (Becton Dickinson Cytometry Systems, San Jose, Calif.). Forward scatter and side scatter gating excluded dead cells and clumps. A minimum of 3,000 events was collected per sample.

Data analysis. The total number of cells expressing a given molecule was determined by multiplying the number of IEL or LPL recovered by the percentage of positive cells derived by flow cytometry. Means and SEMs were determined, and pooled one-tailed *t* tests for significance (7) were performed.

RNA isolation. RNA was isolated by lysing 7.5×10^6 cells per ml of TRIzol reagent (GIBCO BRL), followed by extraction with chloroform (1:5, vol/vol) and then isopropanol (1:2, vol/vol). Pellets were washed with 75% ethanol and then dried and resuspended in distilled water. RNA concentration was determined spectrophotometrically.

Reverse transcriptase PCR analysis. Cytokine mRNA was detected in PCR after reverse transcription of 0.25 μg of total RNA. For reverse transcription, RNA in 12.5 μl of diethyl pyrocarbonate (DEPC)-treated water (heated at 75°C for 3 min; cooled to 4°C) was mixed with 1 μl of oligo$(dT)_{12–18}$, or random hexamers (both from GIBCO BRL), 1 μl of 10 mM deoxynucleoside triphosphates (Perkin-Elmer Cetus, Foster City, Calif.), 4 μl of 5× first-strand buffer, 0.5 μl of 1-U/μl RNase inhibitor, 1 μl of 0.1 M dithiothreitol, and 1 μl of 200-U/μl Moloney murine leukemia virus reverse transcriptase (all from GIBCO BRL) in a total reaction mixture volume of 21 μl (42°C, 1 h; 94°C, 2 min; cooled at 4°C). For PCR amplification, cytokine-specific primer pairs were designed, using Oligo 4.1 Primer Analysis Software (National Biosciences, Inc., Plymouth Minn.) from sequences deposited in GenBank. The following primer pairs were used: for bovine IL-2, CAG TTG CTT TTG GAG AA (141 to 157) and GAG TAG ATG CTT TGA CA (472 to 456) (8); for bovine IL-4, CTG GTC TGC TTA CTG GT (97 to 113) and TCT TGG CTT CAT TCA CA (400 to 384) (25); for bovine IL-10, GTT GCC TGG TCT TCC TGG CYG (23 to 43) and TAT GTA GTT GAT GAA GAT GTC (504 to 484) (23); for IFN-γ, CCT CAA AGA TAA CCA GGT C (339 to 357) and CGC TTT CTG AGG TTA GAT T (549 to 530) (9); and for bovine tumor necrosis factor alpha (TNF-α), CGG TAG CCC ACG TTG TA (5214 to 5230, of exon 3) and TGG CCT CAG CCC ACT CT (5828 to 5812, of exon 4) (12). β-Actin primers were determined from the rat β-actin sequence, as used in cattle (26), and were included to confirm mRNA integrity. The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). Prior to use, they were tested on plasmids containing relevant inserts (plasmids for IL-2, IL-4, IFN-γ, and TNF-α were generously provided by H. F. Seow, Commonwealth Scientific and Industrial Research Organisation, Parkville, Victoria, Australia; the IL-10 plasmid was developed by A. C. Rice-Ficht and W. C. Brown. The fragment sizes amplified were as follows: for IL-2, 332 bp; for IL-4, 304 bp; for IL-10, 483 bp; for IFN-γ, 211 bp; for TNF-α, 283 bp; for β-actin, 422 bp. PCR performed using genomic DNA confirmed that IL-2, IL-4, IL-10, and TNF-α primers amplified pieces larger those observed with mRNAs (greater than 700 bp), while IFN-γ and β-actin primers amplified pieces with sizes similar to those observed with IFN-γ and β-actin mRNAs.

The amplification reaction mixtures contained cDNA from the reverse transcription reaction, primers, optimum Mg^{2+}, 10× PCR buffer, deoxynucleoside triphosphate mix, and AmpliTaq Polymerase (all from Perkin-Elmer Cetus) diluted in water to a total volume of 100 μl. The mixtures were heated to 94°C for 4 min. Annealing temperatures were as follows: IL-2, 45°C; IL-4, IFN-γ, and β-actin, 55°C; TNF-α and IL-10, 60°C. Each cycle consisted of 30 s at 94°C, a 30-s annealling, and 30 s at 72°C. Thirty cycles were run, after which the mixtures were held at 72°C for 7 min and then cooled to 4°C. To each reaction mixture, 200 μl of 100% ethanol was added, and the tubes were held at −20°C for at least 1 h. Precipitates were centrifuged, dried, and resuspended in 10 μl of water. Agarose (2%) gels containing ethidium bromide were loaded and run at 97 V, and profiles were recorded with an IS-1000 Digital Imaging System (Alpha Inotech Corp., San Leandro, Calif.).

RESULTS

Evaluation of ileum during mucosal lymphocyte collections. Because subsequent interpretation of immunophenotype data depended upon confirmation that mucosal lymphocytes were not contaminated by large numbers of cells from underlying locations, histologic sections of the ileum pieces that had been snap frozen in liquid nitrogen at each step were examined by light microscopy. Fig. 1 shows histologic sections of tissue from a representative ileum taken from an uninfected control calf. The section of ileum frozen prior to removal of the epithelial layer shows intact villi. The same ileum after removal of the epithelial layer retains an intact lamina propria membrane, with an intact underlying LPL compartment. The villi appear somewhat shortened, because nearly all of the IEL and most of the epithelial cells have been removed.

After enzymatic digestion of the lamina propria membrane, numerous LPL were released; however, because of the rela-

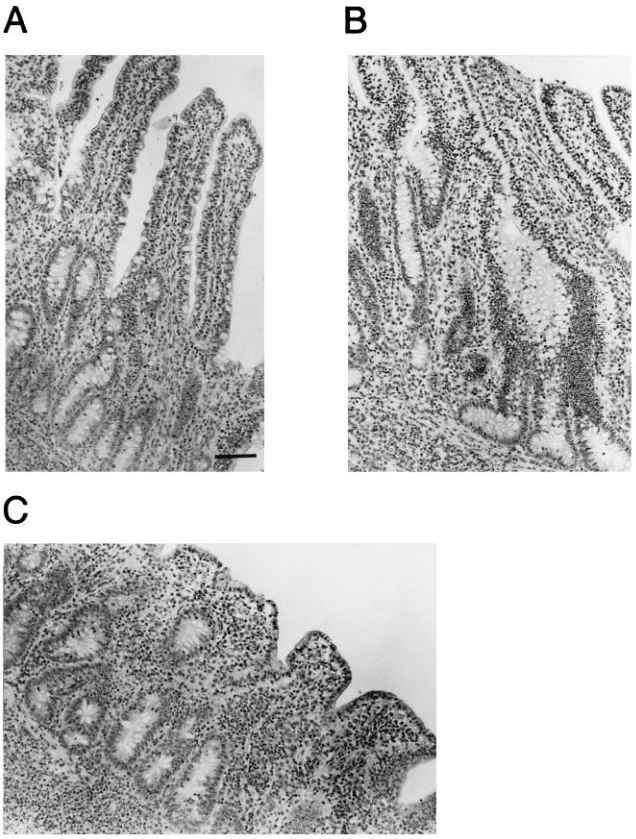

FIG. 2. Small intestine lesions in *C. parvum* infection. Histologic sections from 1.5-week-old control calf (A), 3-week-old calf recovered from enteritis (16 days postinoculation) (B), and 1.5-week-old infected calf with enteritis (7 days postinoculation) (C). Bar = 100 μm.

tively short digestion time, approximately 50% of LPL remained attached (data not shown). This procedure resulted in retention of intact underlying continuous Peyer's patch nodules, as well. Thus, the isolated mucosal cell populations contained only the cells normally found in those locations in the ileum.

Lesion development during infection. Histologic sections of ilea from all animals in the study were examined to confirm that *C. parvum* infection of intestinal epithelium induced villous atrophy that resolved upon recovery from disease. Figure 2 shows representative sections of the ilea from a 1.5-week-old control calf, an infected calf, and a calf that had recovered from infection. Villous atrophy and blunting are apparent in the histologic section from the infected calf, compared with the sections from the control and recovered calves, which is consistent with clinical disease.

Composition of mucosal lymphocytes obtained from infected and control calves. The IEL and LPL obtained from calves with cryptosporidiosis and from age-matched control calves were immunophenotyped to determine the composition of the populations. Table 1 presents the mean lymphocyte numbers for IEL and LPL isolated from each calf group. Overall, similar numbers of IEL were obtained from ilea of infected calves and from ilea of age-matched control calves. The majority of IEL were T lymphocytes, although B cells were present in the intraepithelial subcompartment in all animals.

The total number of LPL obtained from ilea of infected calves was also similar to that for controls. As seen for IEL, T lymphocytes predominated, although B lymphocytes were present. For both IEL and LPL, there were no statistically significant differences in either the number of T cells or the number of B cells isolated from the affected mucosae compared with tissues from the age-matched control calves.

TABLE 1. Descriptive statistics for isolated mucosal lymphocytes

Surface molecule	No. (mean ± SEM [10^6]) of IEL or LPL expressing surface molecule[a]			
	IEL		LPL	
	Infected	Control	Infected	Control
CD3	39 ± 8	28 ± 5	37 ± 10	14 ± 5
sIgM	9 ± 3	18 ± 10	9 ± 4	5 ± 2
MHC II[b]	20 ± 6	30 ± 10	16 ± 3	21 ± 14
Total[c]	53 ± 14	63 ± 16	41 ± 10	44 ± 26

[a] $n = 5$.
[b] MHC II, major histocompatibility complex class II.
[c] Total number of IEL or LPL recovered.

Immunophenotype of mucosal T lymphocytes in diseased and control calf ilea. Differences between intraepithelial T-lymphocyte subsets from diseased ilea of infected calves and ilea of age-matched control calves were observed (Fig. 3). Similar numbers of $CD4^+$, total $TcR1^+$, and $TcR1/CD2^+$ T lymphocytes were obtained from diseased ilea and control ilea. In contrast, substantially larger numbers of $CD8^+$ T lymphocytes were isolated from diseased ilea compared with those isolated from controls. Indeed, significantly increased numbers of CD8αβ heterodimer-expressing T lymphocytes were isolated from diseased ilea compared with those isolated from controls ($P < 0.0366$).

Intraepithelial T lymphocytes were evaluated for CD25 (IL-2rα) coexpression (Fig. 4), as an indicator of activation. IEL from diseased ilea had substantially increased numbers of activated $CD8^+$ T lymphocytes and had significantly increased numbers of activated $CD4^+$ T lymphocytes compared with control IEL ($P < 0.025$). Activated $CD4^+$ T cells comprised approximately 30 to 50% of the total $CD4^+$ T-lymphocyte subset in infected calves.

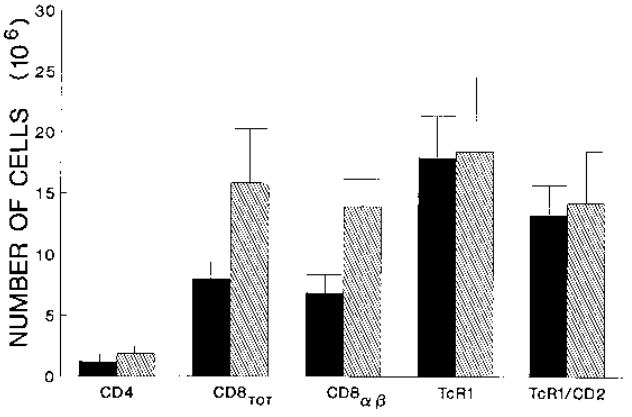

FIG. 3. Composition of intraepithelial T lymphocytes collected from ilea from calves with cryptosporidiosis and from control calves. Mean cell numbers for T lymphocytes from control ilea ($n = 5$; solid bars) and from infected ilea ($n = 5$; hatched bars) are shown. Error bars represent 1 SEM above the mean cell number for each T-lymphocyte subset. Total $CD8^+$ T lymphocytes are labeled $CD8_{TOT}$, and the $CD8^+$ T lymphocytes expressing the CD8αβ heterodimer are labeled $CD8_{\alpha\beta}$. γδ TCR^+ cells are labeled TcR1.

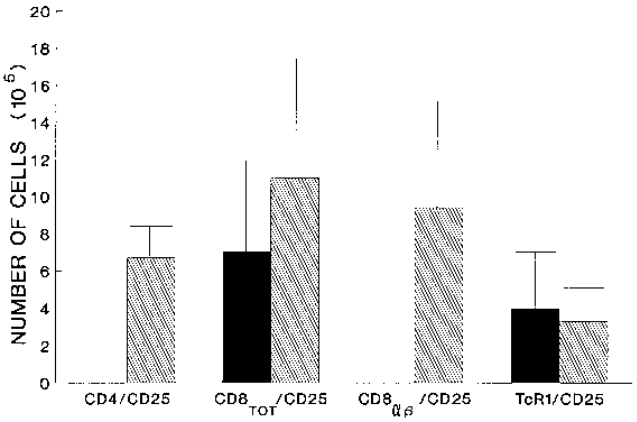

FIG. 4. CD25 coexpression on intraepithelial T-lymphocyte subsets isolated from ilea from calves with cryptosporidiosis and from control calves. Mean cell numbers for $CD25^+$ T lymphocytes from control calves ($n = 5$; solid bars) and from calves with cryptosporidiosis ($n = 5$; hatched bars) are shown. Error bars represent 1 SEM above the mean cell number for each T-cell subset. Total $CD8^+$ T lymphocytes are labeled $CD8_{TOT}$, and the $CD8^+$ T lymphocytes expressing the CD8αβ heterodimer are labeled $CD8_{\alpha\beta}$. γδ TCR^+ cells are labeled TcR1.

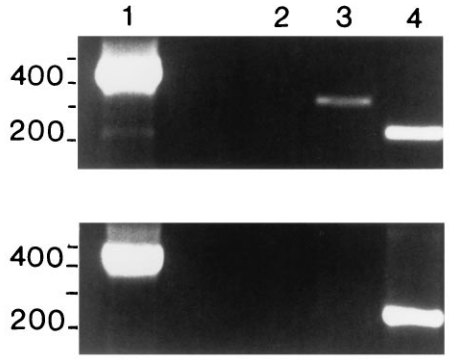

FIG. 5. Cytokine-specific mRNA in isolated ileal IEL. Reverse transcriptase PCR products are shown for mRNA from IEL isolated from a control calf (top panel) and from a calf with cryptosporidiosis (bottom panel). In the left margin are molecular size markers in base pairs. Lane 1, β-actin; lane 2, IL-2; lane 3, TNF-α; lane 4, IFN-γ. This experiment was repeated twice for each starting RNA and was done with RNA from an additional two control calves and two infected calves with similar results.

When LPL were compared, however, no major differences were found between diseased and control ilea. The total numbers of $CD4^+$, total $CD8^+$, $CD8\alpha\beta^+$, and $TcR1^+$ T cells were similar for the two groups (data not shown). Further, there were no T-cell subsets that were significantly activated to CD25 coexpression in diseased ilea compared with the controls (not shown). Thus, the changes in lymphocyte composition that occurred in ileal mucosae coincidently with lesions in calves with cryptosporidiosis were located intraepithelially.

Ileal mucosal lymphocyte composition after recovery from disease. The IEL and LPL obtained from the ilea of calves that had recovered from disease and resolved intestinal lesions had compositions similar to those from the ilea of age-matched control calves. Means ± SEMs of $1.9 \times 10^8 \pm 0.7 \times 10^8$ and $1.4 \times 10^8 \pm 0.4 \times 10^8$ IEL were obtained from the ilea of recovered and age-matched control calves, respectively. T lymphocytes predominated, although B lymphocytes were present in both intraepithelial and lamina propria subcompartments ($8.8 \times 10^7 \pm 3.2 \times 10^7$ and $5.0 \times 10^7 \pm 1.4 \times 10^7$ intraepithelial T lymphocytes and $4.5 \times 10^7 \pm 1.8 \times 10^7$ and $4.6 \times 10^7 \pm 1.8 \times 10^7$ intraepithelial B lymphocytes from the ilea of recovered and control calves, respectively; $4.3 \times 10^7 \pm 2.5 \times 10^7$ and $9.8 \times 10^6 \pm 2.0 \times 10^6$ lamina propria T lymphocytes and $8.8 \times 10^6 \pm 3.8 \times 10^6$ and $5.4 \times 10^6 \pm 4.0 \times 10^6$ lamina propria B lymphocytes from the ilea of recovered and control calves, respectively). T lymphocytes from ileal mucosae from which lesions had resolved did not show elevated CD25 coexpression compared with those from controls (data not shown). Thus, coincidentally with resolution of intestinal lesions, mucosal lymphocytes reverted to the normal phenotype.

Cytokine expression in ileal IEL and LPL. Reverse transcriptase PCR was used to analyze mRNA isolated from ileal IEL and LPL for IL-2, IL-4, IL-10, IFN-γ, and TNF-α expression. In the absence of exogenous stimulation, IEL obtained from control ilea expressed IFN-γ and TNF-α. In contrast, IEL obtained from diseased ilea expressed IFN-γ but had downregulated TNF-α expression (Fig. 5). IL-2, IL-4, and IL-10 were not detected in the absence of exogenous stimulation. After lesions had resolved, the normal expression pattern consisting of IFN-γ and TNF-α had returned (data not shown).

IFN-γ and TNF-α, but not IL-2, IL-4, or IL-10, were expressed by LPL from diseased ilea as well as by LPL from control tissue, and no changes were detected after lesions had resolved (data not shown). Thus, altered cytokine expression occurred in IEL but not LPL isolated from diseased ilea. The IEL cytokine expression pattern returned to normal coincidently with the end of disease.

DISCUSSION

These experiments document changes in intestinal mucosal lymphocytes that occurred in conjunction with intestinal lesions and clinical disease following infection with *C. parvum*. Neonatal calves were used, because unlike immunocompetent mice, they develop enteritis in a manner similar to that of humans (13, 14). The ileum was chosen as the tissue source because it is preferentially infected by *C. parvum* (5, 38).

Interesting observations were obtained with IEL isolated from control calf ilea. On average, <10% of the total $CD8^+$ IEL coexpressed CD25. Dual-fluorescence analysis in which CD8α and CD8β, CD8α and CD25, or CD8β and CD25 antibodies were compared showed that the $CD25^+$ IEL expressed CD8α but not CD8β. In cattle, $CD8^+$ T cells express either CD8αα homodimers or CD8αβ heterodimers (29, 43). No additional $CD8^+$ T-cell subsets have been identified, indicating that the activated $CD8^+$ cells expressed the CD8αα homodimer. These activated $CD8\alpha\alpha^+$ IEL appeared to be γδ T lymphocytes. In neonatal calves, $CD8\alpha\alpha^+$ IEL are largely γδ T cells, and $CD8\alpha\beta^+$ IEL are αβ T cells (43). Dual-fluorescence analysis showed that CD25 was coexpressed on a small proportion of γδ T cells from control ilea (on average, <3% of total γδ T cells). Thus, γδ T cells likely accounted for CD25 expression on normal $CD8^+$ IEL.

IEL from control calves also expressed the cytokines IFN-γ and TNF-α in the absence of exogenous stimulation. IFN-γ and TNF-α are inflammatory cytokines involved in cytotoxic immune responses. Both cytokines have been implicated in the destruction of transformed epithelial cells (1, 31, 34), with synergistic effects on abnormal target cells (10, 35). Thus, expression of IFN-γ and TNF-α by control calf IEL suggests that a cytotoxic response occurs within the ileal mucosa.

Together, the data from the control calf IEL are consistent with the idea advanced by others (6, 21, 28) that mucosal lymphocytes, especially γδ T cells, perform epithelial cell surveillance functions. Epithelial cell surveillance in the small

intestine could include a cytotoxic response directed against transformed epithelial cells or against epithelial cells infected by invasive variants of gut flora (22). If epithelial cell surveillance occurs within the intestinal mucosa, then in neonatal calves, perhaps this function is mediated by small numbers of endogenously activated intraepithelial CD8$\alpha\alpha^+$ $\gamma\delta$ T cells that make IFN-γ and TNF-α.

When IEL from calves with *C. parvum*-induced enteritis were compared with IEL from uninfected calves, several differences were apparent. First, there was a significant increase in the number of intraepithelial CD8$\alpha\beta^+$ T lymphocytes isolated from diseased ilea. Second, a substantial proportion of CD4$^+$ T lymphocytes in diseased tissue were activated, as indicated by CD25 coexpression. Third, the normal cytokine pattern was altered such that TNF-α expression was downregulated. It is noteworthy that after the calves recovered from clinical disease, ceased shedding oocysts, and resolved intestinal lesions, all changes in the IEL subcompartment observed during disease also resolved. Thus, the number of intraepithelial CD8$^+$ T lymphocytes was no longer elevated, CD25 expression was modulated, and the *ex vivo* cytokine expression pattern consisting of IFN-γ and TNF-α was reestablished.

The increased number of intraepithelial CD8$\alpha\beta^+$ T lymphocytes and the activation of a proportion of CD4$^+$ T cells suggest the occurrence of a T-lymphocyte-mediated immune response during enteric disease. Participation of these cells in a gut immune response is not unexpected. CD8$\alpha\beta^+$ IEL isolated from mice with *Toxoplasma gondii* infections are cytotoxic for *T. gondii*-infected enterocytes (11). Further, CD4$^+$ T lymphocytes have been shown in several mouse models to be critical to recovery from *C. parvum* infection (2, 33, 41).

However, downregulation of cytokine expression in IEL from diseased ilea suggests alteration of a mucosal immune response during *C. parvum*-induced enteric disease. While a substantial proportion of CD4$^+$ IEL from diseased ilea coexpressed CD25 (approximately 35% of CD4$^+$ cells), no IL-2 message could be amplified from these IEL. IL-2 message can be amplified from phytolectin-stimulated bovine peripheral blood cells with our primers and reaction conditions (42a). Thus, the absence of IL-2 message in IEL from diseased ilea was not attributable to technical failure. In mice, IEL activated by T-cell receptor stimulation with antireceptor MAbs synthesize IL-2 and other cytokines, such as IFN-γ and TNF-α (3). They can also express CD25 (37), indicating that IEL can respond to antigenic stimuli in many of the same ways as peripheral lymphoid-tissue T cells. Thus, the absence of IL-2 in the presence of CD25 coexpressing CD4$^+$ T lymphocytes in *C. parvum*-infected ilea suggests perturbation of a normal autocrine process of activated IEL.

A second observation that suggests disruption of an IEL-mediated immune response during *C. parvum*-induced enteritis is the absence of TNF-α message, especially with continued expression of IFN-γ. It has been suggested that IFN-γ synergizes with TNF-α primarily by upregulating TNF-α receptors on target cells. Binding of TNF-α to TNF-α receptors on target cells mediates apoptotic cell death (10; reviewed in reference 36). If indeed TNF-α is the critical cytokine required to kill infected epithelial cells, then downregulation of TNF-α expression might have important survival implications for *C. parvum* within intestinal epithelia.

In order to evaluate the roles of mucosal T lymphocytes and the cytokines they produce in *C. parvum* infection, it will be necessary to examine the events that occur in the intestinal mucosa after infection but prior to the onset of lesions and clinical disease. Since this study has shown that intraepithelial CD8$\alpha\beta^+$ T lymphocytes, CD4$^+$ T lymphocytes, and TNF-α made by IEL are affected during cryptosporidiosis, these components can form the focus for an examination of the events that occur in the intestinal mucosa early in *C. parvum* infection.

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