INFECTION AND IMMUNITY, Oct. 2006, p. 5497–5505 Vol. 74, No. 10
0019-9567/06/$08.00+0 doi:10.1128/IAI.00153-06

Neutrophils Do Not Mediate the Pathophysiological Sequelae of *Cryptosporidium parvum* Infection in Neonatal Piglets

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Received 30 January 2006/Returned for modification 7 March 2006/Accepted 3 July 2006

***Cryptosporidium parvum* is a minimally invasive protozoal pathogen of intestinal epithelium that results in villus atrophy, mucosal lipid peroxidation, diarrhea, and diminished barrier function. Influx of neutrophils is a consistent feature of human and animal cryptosporidiosis, and yet their contribution to the pathological sequelae of infection has not been investigated. Accordingly, we used an established neonatal piglet model of *C. parvum* infection to examine the role of neutrophils in disease pathogenesis by inhibiting their recruitment and activation in vivo using a monoclonal anti-CD18 antibody. Infected piglets were treated daily with anti-CD18 or isotype control immunoglobulin G and euthanized at peak infection, at which time neutrophil infiltrates, lipid peroxidation, severity of infection, and intestinal barrier function were quantified. *C. parvum* infection resulted in a significant increase in mucosal neutrophil myeloperoxidase activity that was prevented by treatment of piglets with anti-CD18 antibody. Neutrophil recruitment was dependent on mucosal superoxide formation (prevented by treatment of infected piglets with superoxide dismutase). Neutrophils did not contribute to peroxynitrite formation or peroxidative injury of *C. parvum*-infected mucosa and had no impact on the severity of epithelial infection, villus atrophy, or diarrhea. The presence of neutrophils in *C. parvum*-infected mucosa was associated with enhanced barrier function that could not be attributed to mucosal elaboration of prostaglandins or stimulation of their synthesis. These studies are the first to demonstrate that neutrophilic inflammation arising in response to infection by a noninvasive epithelial pathogen results in physiologic rather than pathological effects in vivo.**

Recruitment of leukocytes from the circulation to the lamina propria is a critical event in the inflammatory response of a number of intestinal diseases, including ulcerative colitis, Crohn's disease, celiac disease, reperfusion injury, and infection. Within such injured mucosae, neutrophil integrins become tethered and adhere to receptors expressed by activated endothelium. The leukocyte-specific β_2 integrin family (CD11/CD18 complex) is the most functionally important class of receptors on these cells, and genetic deficiency of β chain expression or function is characterized by an inability of neutrophils to migrate into tissues and to become activated (18). In contrast to enteroinvasive infections, *Cryptosporidium parvum* resides within intestinal epithelial cells and does not invade deeper layers of the mucosa. Nonetheless, *Cryptosporidium* infection results in recruitment of neutrophils to the lamina propria, peroxidation of mucosal lipids, villus atrophy, marked diarrhea, and decreases in barrier function (3, 4, 8–10, 12, 23, 26, 36). The role of neutrophils in mediating these pathological sequelae has never been investigated, perhaps due to the lack of suitable animal models that mimic human disease (35, 39, 41). In the present study, we used a neonatal piglet model of *C. parvum* infection that fully recapitulates human cryptosporidiosis (3) to investigate the role of neutrophils in disease pathogenesis by inhibiting their recruitment and activation using a monoclonal anti-CD18 antibody.

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In this study, we demonstrate for the first time that neutrophils have minimal impact in mediating the pathological sequelae of *C. parvum* infection. Infection of neonatal piglets with *C. parvum* resulted in significant villus atrophy, diarrhea, mucosal lipid peroxidation, and recruitment of neutrophils into the lamina propria. Neutrophil recruitment was dependent on superoxide formation by the mucosa (inhibited by superoxide dismutase [SOD]) and blocked by treatment of piglets with anti-CD18 antibody. Neutrophil depletion did not ameliorate lipid peroxidation or peroxynitrite formation, suggesting that these cells are not a significant source of free radicals in *C. parvum*-infected mucosa. Further, mediators derived from or stimulated by neutrophils did not play a significant role in the genesis of diarrhea, as diarrheal severity, villus atrophy, and epithelial Cl^- secretion were unaffected by neutrophil depletion. Finally, barrier function of *C. parvum*-infected mucosa was significantly worsened, rather than improved, by the absence of neutrophils. These observations suggest that in noninvasive infection of intestinal epithelium, influx of neutrophils promotes physiologic rather than pathological effects in vivo.

MATERIALS AND METHODS

Animals. Experimental animals were 1-day-old crossbred piglets obtained from the College of Agriculture. Piglets were placed into infected and control isolation units and fed a liquid diet by an automated delivery system. An inoculum of 10^8 *C. parvum* oocysts (Bunch Grass Farms, Deary, ID) was given to piglets by orogastric tube on day 3 of life. Control and infected piglets were studied on days 3 to 5 after inoculation, a time span shown previously to be inclusive of peak intestinal infection (3). Piglets were euthanized using sodium pentobarbital given intravenously (i.v.), and sections of ileum, beginning 5 cm

above the ileocecal junction, were taken sequentially for histology, in vitro function testing, and assays. All infected piglets used in the study showed evidence of villus atrophy and organisms adherent to villus enterocytes, whereas control piglets showed normal villus architecture with no evidence of infection. All studies were approved by the Institutional Animal Care and Use Committee.

Morphometric analyses. Sections of ileum were fixed in formalin, paraffin embedded, sectioned at 5 μm, and stained with hematoxylin and eosin for examination by light microscopy. Three sections from each tissue were examined. Three to five well-oriented villi were selected by an examiner blinded to treatment category. Villi were considered well-oriented if the adjacent crypt lumen was patent to the level of the muscularis mucosa. Average villus height (from the crypt opening to the villus tip) and crypt depth were measured using an ocular micrometer, and the percentage of epithelialized villus surface was calculated from linear measurements of epithelialized versus denuded villus perimeter. The total number of villus epithelial cells and total number of intracellular parasites along the perimeter of each of the selected villi were counted.

Measurement of lipid peroxidation. Thiobarbituric acid-reactive substances were measured in homogenates of ileal mucosa on the basis of the formation of a colored adduct of malondialdehyde (MDA) with 2-thiobarbituric acid. An 800-μl aliquot of homogenate was added to a reagent solution containing 20% acetic acid (1.5 ml), 8.1% sodium dodecyl sulfate (200 μl), and 0.8% 2-thiobarbituric acid and 0.05% butylated hydroxytoluene (1.5 ml). The mixture was boiled for 1 h in a water bath. After cooling, the MDA products were extracted with 5 ml of *n*-butanol and pyridine (15:1). The absorbance of the butanol layer was measured at a wavelength of 532 nm. A calibration curve was constructed by using malonaldehyde bis(dimethyl acetal) as the standard. Urine samples were obtained by cystocentesis from each piglet at the time of euthanasia and frozen at −80°C. Urine samples were assayed for creatinine and F_2-isoprostanes by use of a commercial kit (8-isoprostane enzyme immunoassay; Cayman Chemical Co.) as described by the manufacturer.

Myeloperoxidase activity. The distal ileum was obtained from each piglet, opened lengthwise, and rinsed in cold Ringer's solution. The epithelium and lamina propria were scraped from the seromuscular layers over ice by using a glass slide and then frozen in liquid nitrogen and stored at −80°C. The mucosal scrapings were thawed and homogenized in 0.5% hexadecyltrimethylammonium bromide buffer (50 mM phosphate buffer, pH 6) to release myeloperoxidase (MPO) from the primary granules of neutrophils. The homogenate was subjected to three cycles of freezing at −80°C, thawed, and sonicated on ice. Samples were centrifuged at 21,000 × *g* at 4°C for 15 min and the supernatant assayed for MPO activity. An aliquot of the supernatant was allowed to react with a solution of tetramethylbenzidine in *N*-dimethylformamide and H_2O_2. The rate of change in absorbance was measured at a wavelength of 655 nm. One unit of MPO activity was defined as that degrading 1 μmol of H_2O_2 per minute at 25°C and was expressed in units per gram (wet weight) mucosa.

Peroxidase and eosinophil granule cytochemistry. For peroxidase cytochemistry, intact ileal mucosa was frozen at −80°C in optimal cutting medium and sectioned at 5-μm thickness. Tissues were fixed in 10% neutral-buffered formalin for 5 min and rinsed in distilled water. Sections were incubated with low-pH liquid diaminobenzidine (DAB) substrate (Biogenex, San Ramon, CA) for 45 s to 1 min and rinsed with distilled water. Sections were incubated with DAB enhancer (Zymed Laboratories, Inc., San Francisco, CA) for 2 min, rinsed in distilled water, and counterstained with methyl green. For identification of peroxidase-positive eosinophils, ileal mucosa was fixed in 10% neutral-buffered formalin for 24 h, embedded in paraffin, and sectioned at 5-μm thickness. Sections were stained with hematoxylin-Biebrich scarlet, 1% acid alcohol, and 0.5% lithium carbonate solutions according to the method of Luna (33). For each piglet, the number of eosinophils present in each of 10 well-oriented villus crypt units was counted using a light microscope.

In vivo inhibition of neutrophil infiltration. Uninfected control and *C. parvum*-infected piglets were paired by litter and body weight and treated daily, beginning at the time of orogastric inoculation with 10^8 oocysts and continuing until 24 h prior to euthanasia on day 4 postinfection. Piglets were sedated by intramuscular injection of ketamine (10 mg/kg of body weight) and xylazine (1 mg/kg) prior to i.v. injection into a lateral ear vein. Neutrophil infiltration, adhesion, and activation were inhibited by treatment with a mouse monoclonal anti-CD18 antibody (43) (IB4, 1 mg/kg i.v.) or isotype control antibody (ChromPure mouse immunoglobulin G [IgG], 1 mg/kg i.v.; Jackson ImmunoResearch Laboratories, West Grove, PA). IB4 antibodies abolished the β_2-integrin-dependent adhesion of porcine neutrophils stimulated by treatment with the phorbol ester phorbol myristate acetate (data not shown). No adverse events were observed when uninfected piglets were treated identically with isotype control IgG or anti-CD18 antibody.

In vivo inhibition of superoxide formation. One-day-old littermate piglets were paired by body weight and treated daily, beginning at the time of orogastric inoculation with 10^8 oocysts and continuing until 24 h prior to euthanasia on day 4 postinfection. Piglets were sedated by intramuscular injection of ketamine (10 mg/kg) and xylazine (1 mg/kg) prior to i.v. injection into a lateral ear vein. Superoxide formation was inhibited by treatment with superoxide dismutase-conjugated polyethylene glycol (PEG-SOD) (5,000 IU/pig i.v.; Sigma Chemical Co., St. Louis, MO) or an equivalent concentration of polyethylene glycol (PEG) alone (3.17 mg/pig i.v.). Drugs were reconstituted in phosphate-buffered saline (pH 7.4) and sterile filtered (0.22 μm) prior to injection.

Immunohistochemistry for nitrotyrosine. Immunohistochemistry for nitrotyrosine (NT) (1:200 polyclonal rabbit anti-NT; Cayman Chemical Co., Ann Arbor, MI) was performed using formalin-fixed, 5-μm sections of mucosae removed from control and infected piglets on days 3 to 5 postinfection. Tissues were deparaffinized by immersion in xylene, rehydrated in a graded series of ethanol (100, 95, 70, and 50%), and hydrated to buffer (phosphate-buffered saline, pH 7.4). Tissues were treated with 3% H_2O_2 in methanol for 10 min at 4°C to quench endogenous peroxidase and blocked for 30 min at room temperature with nonimmune goat serum. A commercial kit was used for blocking endogenous avidin and biotin activity (avidin/biotin blocking kit; Zymed Laboratories, Inc., San Francisco, CA). Tissue sections were incubated for 1 h at room temperature or overnight at 4°C. Sections were immunostained using a commercially available, broad-spectrum streptavidin-biotin-peroxidase system with DAB as the chromogen (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Sections were counterstained with methyl green. Negative-control sections were treated with isotype control primary antibody.

Barrier function studies. A 20-cm segment of ileum was opened along the antimesenteric border in an oxygenated Ringer's solution and the seromuscular layers removed. Mucosal sheets were mounted in 1.13-cm^2-aperture Ussing chambers and bathed on both surfaces with a Ringer's solution containing glucose (10 mM, serosal) and mannitol (10 mM, mucosal). Solutions were oxygenated and circulated by gas lift (95% O_2-5% CO_2) and maintained at 37°C by water-jacketed reservoirs. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes by use of a voltage clamp that corrected for fluid resistance. If the spontaneous PD was between −1.0 and 1.0 mV, tissues were current clamped at ±100 μA for 5 s and the PD was recorded. Transepithelial electrical resistance (TER) ($\Omega \cdot cm^2$) was calculated from the spontaneous PD and short-circuit current, which were recorded at 15-min intervals over a 120-min period. Where indicated, the following treatments were added 15 min after tissues were mounted in the Ussing chamber: 16,16-dimethyl-PGE_2 (where PGE_2 is prostaglandin E_2) (10^{-6} M, serosal), the 6a-carba-prostaglandin I_2 (PGI_2) analog carbacyclin (10^{-6} M, serosal), and indomethacin (5×10^{-6} M, serosal and mucosal).

[^{3}H]mannitol and $^{22}Na^+$ flux studies. Isotopic flux studies of mucosal permeability were performed using [^{3}H]mannitol (0.2 μCi/ml) and $^{22}Na^+$ (0.3 μCi/ml) under short-circuit conditions (tissues clamped to 0 mV). Isotope was added to the mucosal ([^{3}H]mannitol) or serosal ($^{22}Na^+$) reservoir 15 min after mounting the mucosa on the chamber. One 60-min flux period (from 60 to 120 min) was performed by taking paired samples from the opposite reservoir. Samples were counted for ^{3}H or $^{22}Na^+$ in a scintillation counter. Flux of mannitol from mucosa to serosa and flux of Na^+ from serosa to mucosa were calculated using standard equations.

PGE_2 assay. For PGE_2 analyses, paired samples were taken from the serosa chamber solution ($t = 120$ min), gassed with N_2, and frozen in liquid N_2. Samples were stored at −80°C prior to assay. Samples were analyzed for concentration of PGE_2 by using a commercial immunoassay according to manufacturer instructions (R & D Systems, Minneapolis, MN).

Data analysis. Data are reported as means ± standard errors. For all analyses, P of <0.05 was considered significant. All data were tested for normality and equal variance by using a statistical software package (SigmaStat; Jandel Scientific, San Rafael, CA). Normally distributed data were analyzed using analysis of variance or Student's t test, whereas nonparametric data were analyzed using a Kruskal-Wallis or Mann-Whitney U test where appropriate. Throughout the report, n represents the number of pigs receiving treatment.

RESULTS

***C. parvum* infection results in villus atrophy and mucosal lipid peroxidation.** Following *C. parvum* infection, ileal mucosa was characterized by marked villus atrophy and organisms present within villus epithelial cells. Uninfected control piglets

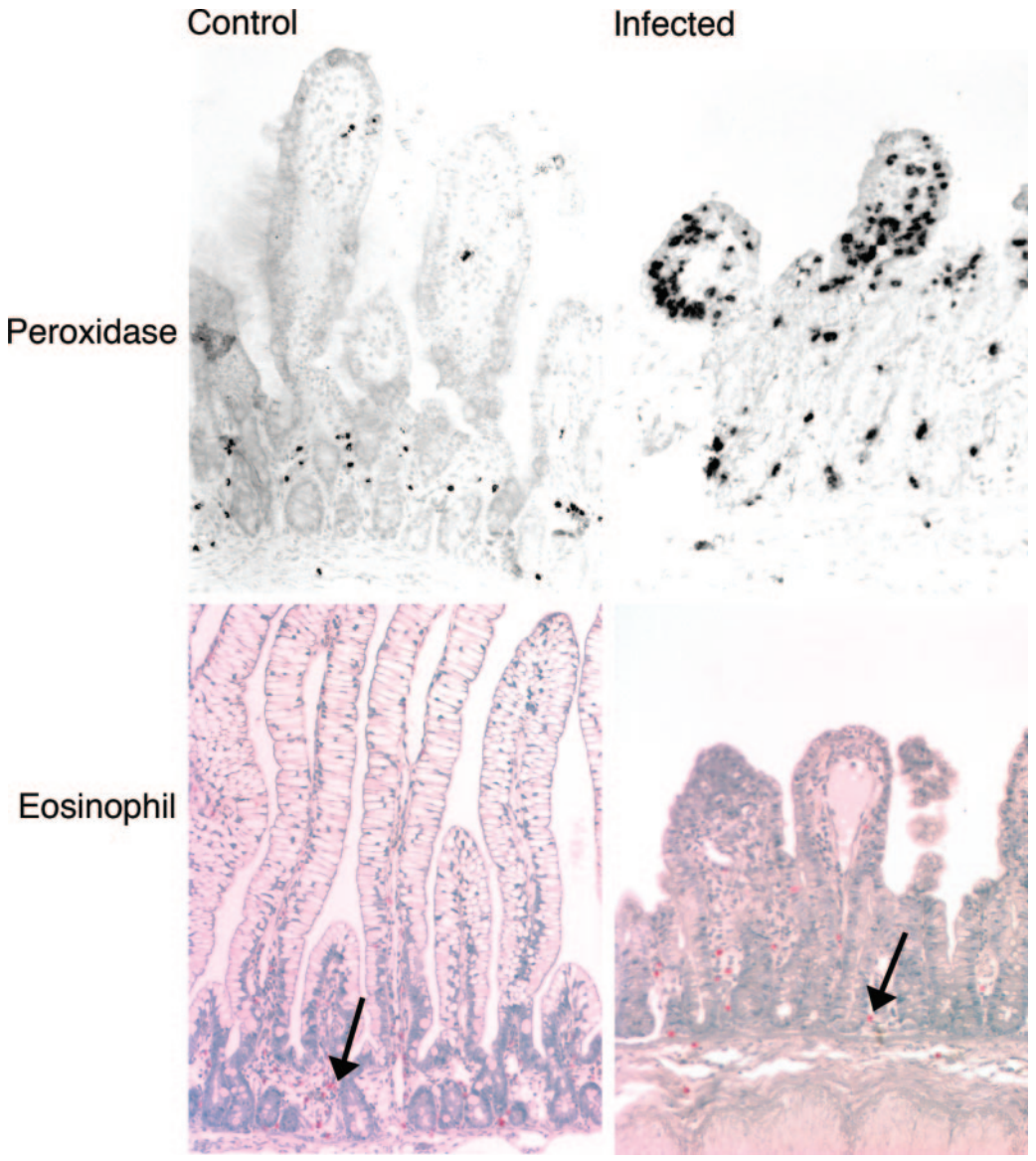

FIG. 1. Appearance of ileal mucosae from uninfected control and *C. parvum*-infected neonatal piglets examined by light microscopy after being stained for the presence of peroxidase-positive and eosinophil granule-containing cells. Control mucosae contained only peroxidase-positive eosinophils (arrows), while infected mucosae contained fewer numbers of eosinophils and numerous peroxidase-positive neutrophils. Peroxidase and eosinophil cytochemistry is shown for a single control and a single infected animal with a frozen and a paraffin-embedded sample, respectively.

had normal villus architecture and no evidence of epithelial infection. MDA, a by-product of lipid peroxidation, was significantly elevated in ileal mucosae from *C. parvum*-infected piglets (17.4 ± 1.8 nmol/mg protein for uninfected piglets [$n = 5$]; 36.3 ± 6.4 nmol/mg protein for infected piglets [$n = 6$]; $P < 0.05$). Urinary excretion of F_2-isoprostanes (free radical-catalyzed peroxidation products of polyunsaturated fatty acids [32]) was also increased in piglets infected with *C. parvum* (411 ± 121 pg of 8-isoprostane/mg creatinine for uninfected piglets [$n = 6$]; 1,871 ± 773 pg of 8-isoprostane/mg creatinine for infected piglets [$n = 5$]; $P = 0.03$).

***C. parvum* infection results in neutrophilic inflammation.** Human cryptosporidiosis is characterized by an influx of neutrophilic leukocytes into the intestinal mucosa (8, 9, 23). Mucosal infiltration of neutrophils has also been identified as characteristic of *C. parvum* infection in the present neonatal piglet model (3). To quantify neutrophilic infiltrates, ileal mucosae from uninfected control and infected piglets were assayed for activity of the neutrophil enzyme myeloperoxidase. Myeloperoxidase activity was significantly increased in ileal mucosae from *C. parvum*-infected piglets (activity per gram tissue was 7.6 ± 1.2 for uninfected piglets and 23.5 ± 3.0 for infected piglets [$n = 6$ each]; $P = 0.002$). Peroxidase-positive cells and eosinophils were visualized in sections of ileal mucosa by means of histochemistry. In uninfected mucosa, peroxidase-positive cells were identified as eosinophils. In infected mucosa, peroxidase-positive cells were predominantly neutrophils admixed with fewer numbers of eosinophils (Fig. 1). As eosino-

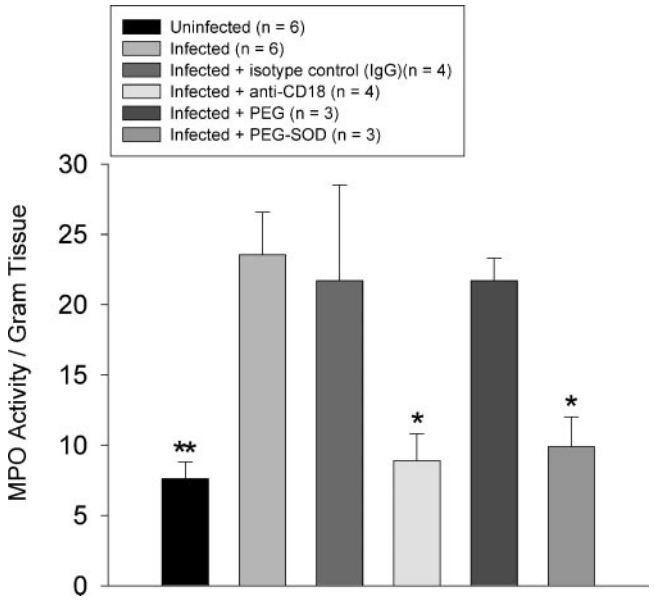

FIG. 2. Activities of the neutrophil enzyme MPO in ileal mucosae from uninfected control and *C. parvum*-infected piglets at time of peak infection. Piglets were treated daily with (i) isotype control antibody, (ii) anti-CD18 antibody to inhibit neutrophil infiltration, (iii) PEG vehicle, or (iv) PEG-SOD to scavenge superoxide. MPO activity was significantly lowered by treatment of infected piglets with anti-CD18 antibody or PEG-SOD (*, P of <0.05 versus infected mucosa; **, P of <0.01 versus infected mucosa).

phil peroxidase can contribute to measures of tissue myeloperoxidase activity, the numbers of eosinophils residing in control and infected mucosae were enumerated. The number of eosinophils present was significantly less in infected than in control mucosa, suggesting that eosinophils were not responsible for increases in MPO activity after infection (average number of eosinophils per villus crypt unit was 11 ± 1.8 for uninfected piglets [$n = 5$] and 4.6 ± 1.3 for infected piglets [$n = 6$]; $P = 0.03$).

Treatment of *C. parvum*-infected piglets with anti-CD18 antibody results in decreased infiltration of mucosal neutrophils. Piglets were infected with *C. parvum* (day 0) and treated daily with a mouse monoclonal anti-CD18 antibody to inhibit neutrophil influx and activation (IB4, 1 mg/kg i.v.) or isotype control antibody (ChromPure mouse IgG, 1 mg/kg i.v.; Jackson ImmunoResearch Laboratories, West Grove, PA). At peak infection (day 4), mucosal MPO activity was assayed to determine the effectiveness of neutrophil influx inhibition. The rise in MPO activity resulting from *C. parvum* infection was decreased to uninfected control levels by treatment of piglets with anti-CD18 antibody (Fig. 2). Neutrophils were not observed in mucosae of piglets treated with anti-CD18 antibody.

Neutrophils do not mediate mucosal lipid peroxidation in *C. parvum* infection. To determine if lipid peroxidation could be attributed to infiltration of neutrophils in *C. parvum* infection, levels of MDA and F_2-isoprostane formation were measured in infected piglets treated with anti-CD18 or isotype control antibody. Neither MDA nor F_2-isoprostane formation was significantly mitigated by neutrophil depletion (levels of F_2-isoprostanes were 5,513 ± 2,047 pg/mg creatinine for the *C. parvum* plus isotype control group and 3,908 ± 645 pg/mg creatinine for the *C. parvum* plus anti-CD18 group [$n = 4$ each]; levels of MDA were 9.8 ± 0.79 nmol/mg protein for the *C. parvum* plus isotype control group [$n = 5$] and 9.6 ± 0.70 nmol/mg protein for the *C. parvum* plus anti-CD18 group [$n = 6$]).

Superoxide promotes mucosal influx of neutrophils in *C. parvum* infection. Because lipid peroxidation could not be attributed to influx of neutrophils in *C. parvum* infection, we sought to determine if formation of superoxide by the mucosa served as a stimulus for neutrophil influx. Piglets were infected with *C. parvum* (day 0) and treated daily with the superoxide scavenger PEG-SOD (5,000 IU/pig i.v.) or PEG alone (3.17 mg/pig i.v.). At peak infection (day 4), mucosal MPO activity was assayed to determine the effect of SOD on neutrophil influx. MPO activity was reduced to uninfected control levels in piglets treated with PEG-SOD (Fig. 2). Histochemical analysis of ileal mucosae from piglets treated with PEG-SOD revealed the presence of resident eosinophils only and not neutrophils. These results suggest that superoxide formation is a cause, rather than a consequence, of neutrophil influx in *C. parvum*-infected mucosa.

Peroxynitrite is generated independently of neutrophil influx in *C. parvum* infection. We have shown previously that the potent oxidant peroxynitrite is generated along the villus tips in *C. parvum* infection and in association with induction of inducible nitric oxide synthase (iNOS) expression (10). Peroxynitrite arises from the reaction of nitric oxide (NO) with superoxide radical. Neutrophils are the usual source of superoxide for peroxynitrite formation in vivo, and peroxynitrite formation is limited in a site-specific manner to areas of high superoxide generation (14). To determine whether neutrophils are the source of superoxide for peroxynitrite formation in *C. parvum* infection, we examined whether peroxidase-positive cells localize in proximity to the infected epithelium, where iNOS and protein tyrosine nitration (a footprint of peroxynitrite action) predominate. In mucosae from *C. parvum*-infected piglets, peroxidase-positive cells varied in location, from predominantly beneath the infected villus epithelium to predominantly within the lamina propria surrounding the crypts. When immunohistochemistry for localization of nitrotyrosine was performed on these same tissues, peroxynitrite formation remained greatest in proximity to the infected epithelial cells along the villus tips regardless of the variation in location of peroxidase-positive cells (Fig. 3). Further, inhibition of neutrophil influx did not attenuate NT formation by the intestinal mucosae of piglets infected with *C. parvum*.

Neutrophils do not contribute to control of epithelial infection or mucosal pathology in *C. parvum* infection. The impact of neutrophilic inflammation in limiting the extent of cryptosporidial infection is largely unknown. Therefore, it is of interest to determine whether inhibition of neutrophil influx alters the severity of epithelial infection or mucosal pathology. Neutrophil depletion of piglets infected with *C. parvum* had no significant effect on the severity of epithelial parasitism or villus atrophy (Table 1). There were also no differences in epithelial disruption between treatment groups (average percentage [±standard error] of villus denuded was 0 ± 0 for the uninfected control [$n = 10$], 6 ± 0.3 for the *C. parvum* plus isotype control group [$n = 13$], and 4 ± 0.1 for the *C. parvum* plus anti-CD18 group [$n = 13$]).

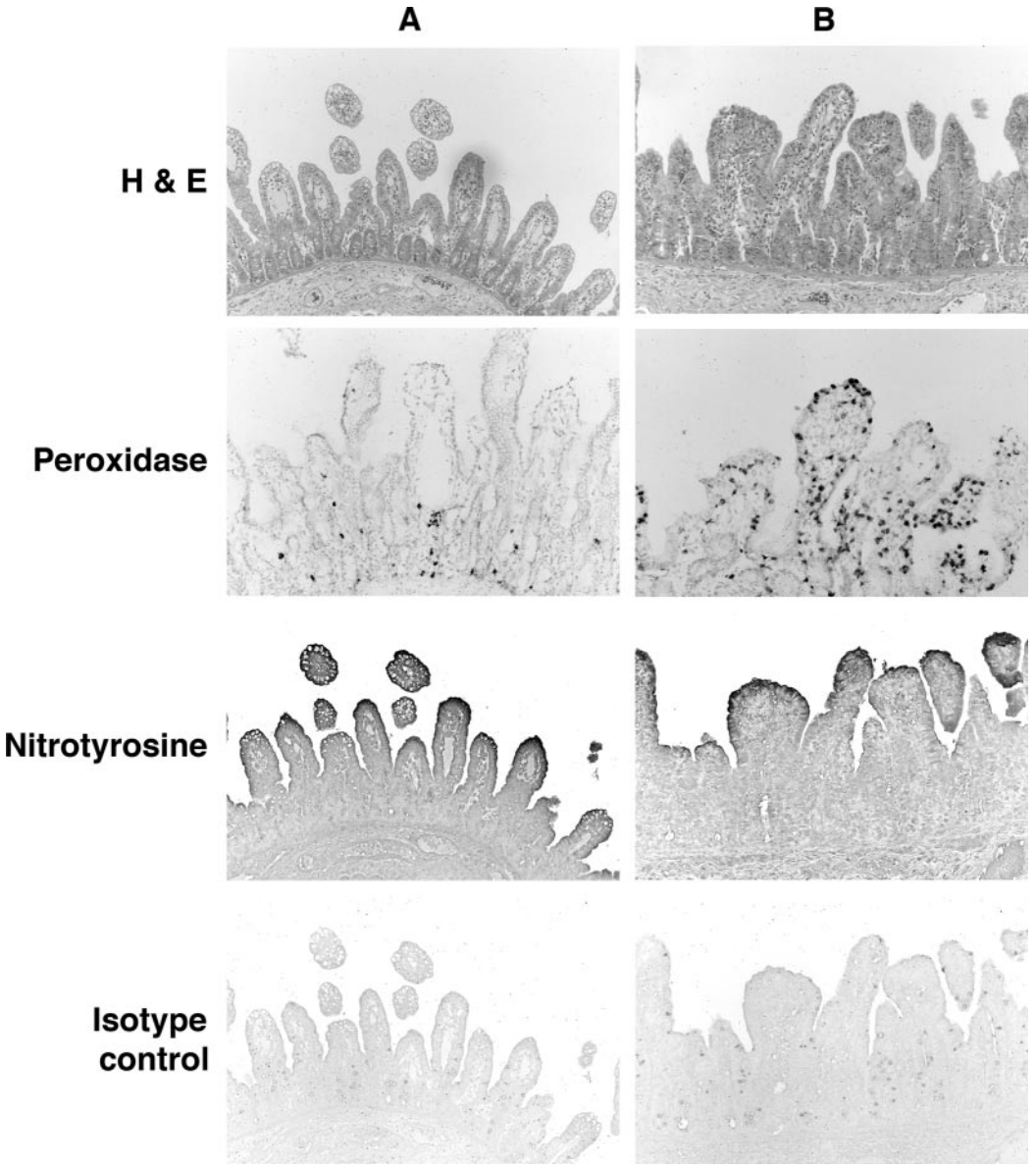

FIG. 3. Immunohistochemistry for tyrosine-nitrosated proteins (nitrotyrosine, a stable "footprint" of peroxynitrite action) was performed using ileal mucosae obtained from *C. parvum*-infected piglets having a mild pericryptal peroxidase-positive cellular infiltrate (A) or a predominantly subepithelial peroxidase-positive cellular infiltrate (B). Peroxynitrite formation was greatest in proximity to infected enterocytes along the apical villus regardless of the variation in location of peroxidase-positive cells or apparent degree of epithelial infection. The counterstain used was methyl green. H & E, hematoxylin and eosin.

Neutrophil-derived mediators are not responsible for diarrhea in *C. parvum* infection. Neutrophils have been purported to be the source of a variety of mediators that may promote diarrhea in *C. parvum* infection. In infected piglets, neutrophil depletion had no effect on severity of diarrhea, maintenance of body weight (a parameter indicative of diarrheal severity in this model [11]) (Table 1), or intestinal epithelial short-circuit current (an indirect measure of Cl^- secretion in this tissue) (average $\mu A \cdot cm^2$ [±standard error] was 40 ± 5.9 for the *C. parvum* plus isotype control group [$n = 9$] and 41 ± 4.6 for the *C. parvum* plus anti-CD18 group [$n = 10$]).

Mucosal neutrophil influx promotes barrier function in *C. parvum* infection. We have shown previously that *C. parvum* infection is associated with a decrease in paracellular barrier function (12). To determine whether neutrophils mediate physical or biochemical effects on barrier function in *C. parvum* infection, infected piglets were treated in vivo with isotype control or anti-CD18 antibody daily, beginning at the time of infection and continuing until peak infection severity (day 4), at which time mucosa from each piglet was mounted in Ussing chambers for measurement of TER and flux of $^{22}Na^+$ and [^{3}H]mannitol. Inhibition of neutrophil influx decreased TER significantly, indicating a decrease in barrier function of the infected mucosa (Fig. 4). Transepithelial flux of $^{22}Na^+$ and [^{3}H]mannitol was also increased in mucosae from piglets treated with anti-CD18; however, sample sizes did not allow

TABLE 1. Indices of severity of *C. parvum* infection in the ilea of neonatal piglets treated with anti-CD18 antibody or isotype control antibody (IgG)[a]

Treatment group (*n*)	Villus ht (μm)	Crypt depth (μm)	No. of epithelial cells per villus	No. of parasites per villus	No. of parasites per epithelial cell	Body wt (kg) at peak infection[c]
Uninfected control (10)[b]	638 ± 39	133 ± 8	208 ± 14	0	0	ND
C. parvum plus IgG (13)	204 ± 33	142 ± 6	106 ± 17	49 ± 9	0.7 ± 0.2	3.05 ± 0.26*
C. parvum plus anti-CD18 (13)	241 ± 44	130 ± 8	118 ± 19	53 ± 13	0.7 ± 0.2	3.08 ± 0.30*

[a] Daily treatments began at time of infection and continued until euthanasia at peak infection (day 4). Values are means ± standard errors. *P* values are as follows: for villus height, 0.16; for crypt depth, 0.44; for number of epithelial cells per villus, 0.30; for number of parasites per villus, 0.37; for number of parasites per epithelial cell, 0.48; and for body weight at peak infection, 0.37.

[b] Data for the uninfected control group were published previously (10).

[c] ND, not done. *, $n = 9$.

for an unequivocal statistical comparison. In contrast to results with infected mucosae, a significant decrease in TER was not observed with ileal mucosae obtained from uninfected control piglets treated identically with isotype control or anti-CD18 antibody (Fig. 4, inset).

Neutrophils mediate barrier function independently of effects on mucosal prostaglandin synthesis. An increase in endogenous prostaglandin synthesis has been demonstrated previously to promote paracellular barrier function in *C. parvum* infection (2, 12). Therefore, we sought to determine whether neutrophils mediate barrier function of *C. parvum*-infected mucosa by serving as a source or stimulus of mucosal prostaglandin synthesis. For assay of mucosal PGE_2 synthesis, sheets of ileal mucosae from anti-CD18 antibody- or isotype control antibody-treated piglets were mounted in Ussing chambers. After 120 min of incubation, the serosal bath was assayed for PGE_2. No significant difference in PGE_2 synthesis between piglets receiving isotype control or anti-CD18 antibody was observed (1,216 ± 442 pg/ml for *C. parvum* plus isotype control and 990 ± 181 pg/ml for *C. parvum* plus anti-CD18 [$n = 5$ each]). Ex vivo inhibition of cyclooxygenase (5×10^{-5} M indomethacin) decreased barrier function of both isotoype control and neutrophil-depleted mucosae. Exogenous addition of PGE_2 and PGI_2 to mucosae from piglets treated with anti-CD18 did not result in rescue of barrier function ($\Omega \cdot cm^2$ was 59 ± 3 for the *C. parvum* plus anti-CD18 group, 48 ± 3 for the *C. parvum* plus anti-CD18 plus indomethacin group [$P \leq 0.05$], and 61 ± 5 for the *C. parvum* plus anti-CD18 plus PGE_2/PGI_2 group [$n = 6$ each]).

DISCUSSION

Infection of neonatal piglets with *C. parvum* resulted in significant recruitment of peroxidase-positive neutrophils to ileal mucosa as determined by cytochemistry and increases in activity of the neutrophil enzyme myeloperoxidase. Myeloperoxidase activity has been used extensively as a means to quantify neutrophil infiltrates in intestinal mucosa. Eosinophil peroxidase may also contribute to measures of MPO activity; however, the number of eosinophils present in ileal mucosa was significantly less in infected than in control mucosa. Neutrophil recruitment into *C. parvum*-infected mucosa was entirely inhibited by treating piglets with anti-CD18 antibody insofar as MPO activity was reduced to levels observed for uninfected control piglets and neutrophils were not observed in histologic sections. Residual MPO activity measured in mucosae from uninfected piglets and those treated with anti-CD18 was attributed to the presence of resident, peroxidase-positive eosinophils, whose identity was established by cytochemistry.

The mechanism by which a noninvasive epithelial pathogen such as *C. parvum* elicits subepithelial inflammation is not well understood. In xenografts of human intestine and model epithelium, *C. parvum* infection results in expression of the chemokines interleukin-8, tumor necrosis factor alpha (TNF-α), and GROα (7, 16, 21, 22, 25, 38). These chemokines are hypothesized to signal neutrophil influx into intestinal mucosa in *C. parvum* infection. Superoxide has been identified as a key mediator of neutrophil recruitment in intestinal injury induced by reperfusion, irradiation, dextran sodium sulfate, acetic acid, and trinitrobenzene sulfonic acid (13, 19, 27, 37, 40, 42). Because neutrophil depletion did not attenuate lipid peroxidation of mucosae from *C. parvum*-infected piglets, we considered the possibility that neutrophil recruitment was mediated by superoxide. Accordingly, treatment of *C. parvum*-infected piglets with superoxide dismutase inhibited the rise in mucosal MPO activity resulting from *C. parvum* infection, demonstrating that neutrophil recruitment was mediated by superoxide-dependent mechanisms. Thus, formation of superoxide is identified here as a cause, rather than a sole consequence, of neutrophilic inflammation in *C. parvum*-infected intestine. Probable cellular sources of superoxide for neutrophil recruitment in *C. parvum* infection include activated macrophages (28), intestinal epithelium, or vascular endothelium. A significant influx of macrophages has been demonstrated in the present model (17), and peroxynitrite, a reaction product of nitric oxide and superoxide, is generated at epithelial sites in *C. parvum*-infected mucosa (10). Inhibition of peroxynitrite formation is unlikely to be the means by which SOD inhibited neutrophil influx into *C. parvum*-infected mucosa, however, as superoxide reacts with NO at rates that exceed dismutation by SOD (31). It has been shown with patients with ulcerative colitis that increased superoxide production can be attributed to both invading monocytes and the vascular endothelium (30). In vascular endothelium, cytokines induce superoxide-dependent expression of adhesion molecules, such as ICAM-1, which increase the transmigration of neutrophils into inflamed intestine (5, 27, 31, 34). Importantly, our finding that superoxide formation is required for neutrophil recruitment in *C. parvum* infection does not negate the importance of epithelial-derived cytokines and chemokines in initiating activation of those lamina propria cells responsible for superoxide formation. Such a role has already been demonstrated for TNF-α (5, 34). The

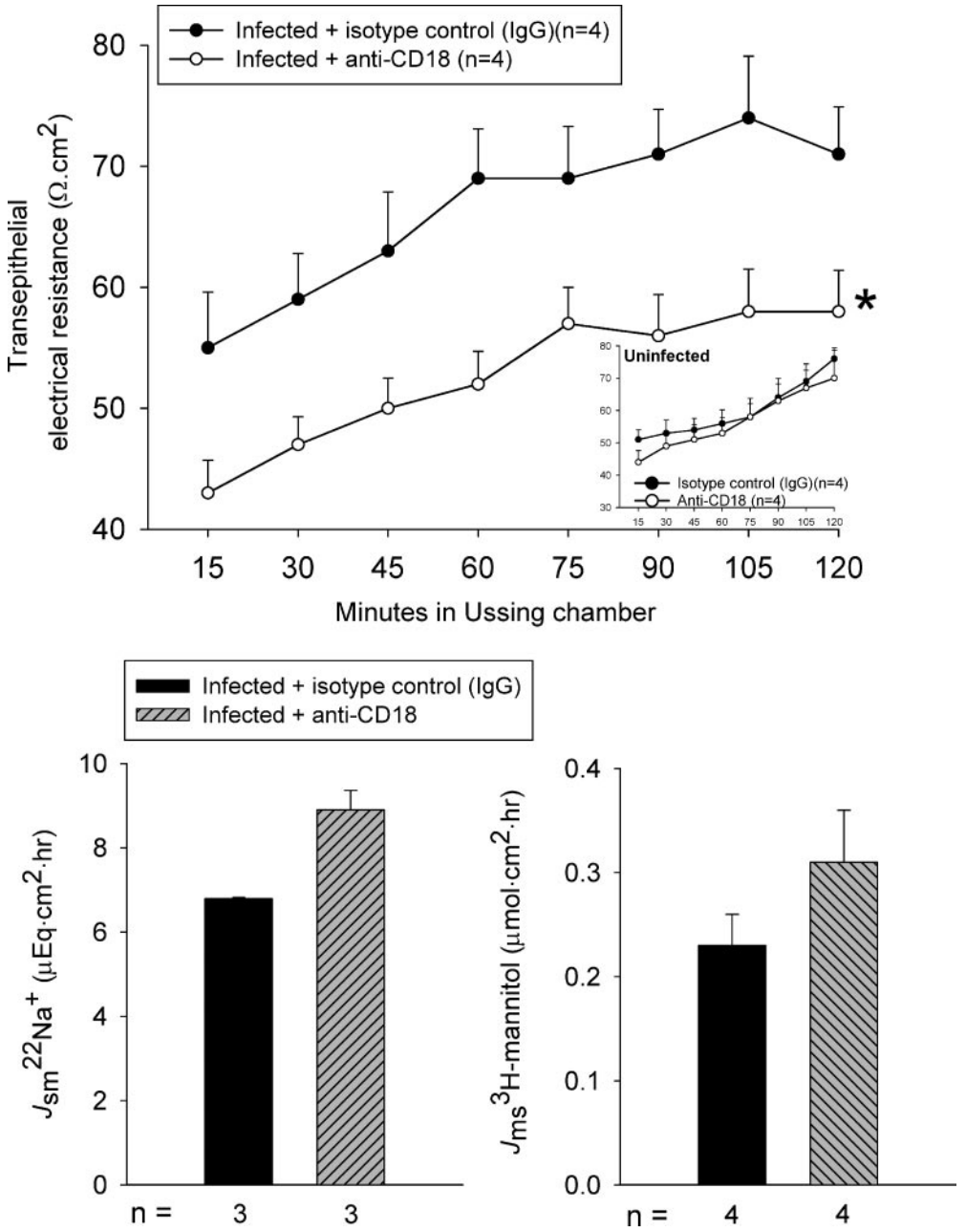

FIG. 4. TER and permeability of *C. parvum*-infected ileal mucosae from neonatal piglets treated with anti-CD18 antibody to inhibit neutrophil influx and activation or isotype control antibody. Mucosae were mounted in Ussing chambers and TER recorded over a 120-min period. Passive flux of $^{22}Na^{+}$ from serosa to mucosa (J_{sm}) and passive flux of [^{3}H]mannitol from mucosa to serosa (J_{ms}) were measured between the time points of 60 and 120 min. TER of mucosae from neutrophil-depleted piglets was significantly lower than that for piglets treated with isotype control antibody (*, P of <0.05 by Friedman repeated-measures analysis of variance on ranks), and this finding was supported by an increase in flux of $^{22}Na^{+}$ and [^{3}H]mannitol. A significant decrease in TER was not observed in ileal mucosae obtained from uninfected control piglets treated identically with isotype control or anti-CD18 antibody (inset).

relatively modest neutrophilic inflammation observed with *C. parvum* compared to enteroinvasive infection may be attributed to the enhanced ability of the intestine to dismutate superoxide and the absence of xanthine oxidase activity in young piglets (6).

We have shown previously that peroxynitrite is generated along the villus tips in *C. parvum* infection and in association with induction of epithelial iNOS expression (10). Peroxynitrite arises from the diffusion-limited reaction of superoxide radical with NO. Because the cellular concentration of superoxide is 1,000 times less than that of NO, peroxynitrite formation is limited in a site-specific manner to areas of high superoxide generation (14). Therefore, we examined whether peroxidase-positive cells, as putative sources of superoxide, localize in proximity to the infected epithelium, where iNOS and protein tyrosine nitration (a footprint of peroxynitrite ac-

tion) predominate. Our results demonstrate that neutrophils are not consistently found in proximity to the infected epithelium, nor are they required for peroxynitrite formation. These observations suggest that superoxide arises from another cell type residing near the villus tips, such as macrophages or intestinal epithelium. Efforts were made, using five different antibodies, to determine the location of macrophages along the villus-crypt axis of control and infected mucosae; however, unambiguous staining of cells could not be obtained. As a consequence, any effect of anti-CD18 antibody treatment on macrophage infiltration cannot be determined by the present study.

It is of interest to determine whether inhibition of neutrophil influx alters the severity of epithelial infection or mucosal pathology. In AIDS patients with cryptosporidiosis, no correlation between neutrophilic inflammation and intensity of infection was found (23). On the other hand, antioxidants have been shown in several studies to exacerbate *C. parvum* infection (10, 15, 44). Whether neutrophils are the source of these antiparasitic oxidants has not been determined. In the present study, neutrophil depletion had no effect on severity of *C. parvum* infection. In addition to the lack of effect of neutrophil depletion on measures of lipid peroxidation and peroxynitrite formation, these findings suggest that neutrophils are not a significant source of free radicals in *C. parvum*-infected mucosa. This may be attributed to a paucity of neutrophil activational factors released as a consequence of the minimally invasive infection. For example, in studies of transgenic mice, the induced expression of interleukin-8 by intestinal epithelium is alone sufficient to recruit neutrophils into intestinal mucosa; however, they do not become activated or result in intestinal injury (20). Results of the present study do not identify the cellular source of peroxidative injury in *C. parvum* infection, although activated macrophages are a likely possibility.

The mechanism(s) by which *C. parvum* infection results in diarrhea remains speculative. Diarrhea has been attributed both to villus atrophy-associated malabsorption and to mucosal inflammation-induced alteration in intestinal water and electrolyte transport. Neutrophils are a source of mediators, including reactive oxygen metabolites (4), TNF-α (17), 5′-AMP (24), and prostaglandins (1, 2, 4, 12), that may directly or indirectly promote epithelial secretion and diarrhea in *C. parvum* infection. Neutrophil depletion of *C. parvum*-infected piglets had no effect on clinical severity of diarrhea or epithelial Cl^- secretion, suggesting that mediators derived from or stimulated by neutrophils do not play a significant role in the genesis of diarrhea in the infection.

Although neutrophilic inflammation was not responsible for severity of villus atrophy or loss of epithelial continuity in *C. parvum*-infected mucosa, neutrophils may biochemically and physically compromise intestinal barrier function by disrupting epithelial tight junctions (29). We have shown previously that *C. parvum* infection results in a loss of barrier function that is compensated for by an increase in mucosal synthesis of prostaglandins (12). To determine if neutrophils are the cause of this defect in barrier function, transepithelial electrical resistance and flux of $^{22}Na^+$ and [^{3}H]mannitol were measured using ileal mucosae from neutrophil-depleted and isotype control-treated piglets infected with *C. parvum*. Barrier function was significantly worsened, rather than improved, by the absence of neutrophils. This effect could not be attributed to a deficiency in mucosal prostaglandin synthesis. Perhaps more interesting than the barrier-sparing effect of neutrophils is the fact that neutrophil depletion had no effect on mortality, mucosal injury, or clinical severity of disease. These observations suggest that barrier function is well-maintained in *C. parvum* infection and that the barrier-sparing effects of neutrophils may not be clinically significant. The latter is suggested by the sustained ability of the mucosal epithelium from anti-CD18 antibody-treated piglets to restrict permeation by [^{3}H]mannitol. Although the cause of the barrier-sparing effect of neutrophils is not disclosed by the present study, neutrophils may mediate this effect by stimulating the release of cytokines that modulate tight junction function or by removing factors that contribute to loss of barrier integrity.

ACKNOWLEDGMENTS

This work was supported by public health service grants DK-02868 and DK-070883 from the National Institute of Diabetes and Digestive and Kidney Diseases.

We thank Sandra Horton and Monica Mattmuller for excellent technical assistance.

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Editor: J. F. Urban, Jr.