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ROLE OF NEUTROPHILS IN ACETIC ACID-INDUCED COLITIS IN RATS[1]

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Abstract—Intrarectal administration of 4% acetic acid produces diffuse inflammation that ultimately results in erosions and ulcerations of the rat colon. Although this model of colitis has been used extensively over the past several years, there are no quantitative data available regarding the relationship between neutrophil infiltration and mucosal injury during times of active inflammation. Therefore, the objective of this study was to define the role of extravasated neutrophils as mediators of mucosal injury and inflammation in acetic acid-induced colitis. We found the intrarectal administration of 4% acetic acid produced an 11-fold increase in colonic mucosal permeability, a 9-fold increase in colonic MPO activity, and a 1.6-fold increase in colon weight at 48 h following administration of acetic acid. In addition, we found significant correlations between colonic MPO activity and mucosal permeability and between colonic MPO activity and colon weight ($P < 0.01$ for both). These data suggested that inflammatory neutrophils may mediate mucosal injury and inflammation in this model of colitis. To assess the role of circulating neutrophils, rats were rendered neutropenic for 48 h by the intraperitoneal administration of antiserum directed toward rat neutrophils (ANS). Although ANS treatment reduced both the number of circulating neutrophils and colonic MPO activity to less than 10% of control values, it did not attenuate the increases in colonic mucosal permeability nor did it attenuate the increases in colon weight produced by acetic acid. Histological inspection confirmed that ANS treatment was not effective in attenuating the injury to the epithelial barrier. These data demonstrate that infiltrating neutrophils do not mediate the mucosal injury and inflammation observed in acetic acid-induced colitis.

INTRODUCTION

Ulcerative colitis (UC) is a diffuse, recurrent inflammation of the colon and rectum of unknown etiology that affects predominantly the colonic mucosa.

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Active episodes of UC are characterized by the infiltration of large numbers of neutrophils into the mucosal interstitium (lamina propria). This enhanced inflammatory infiltrate is accompanied by extensive mucosal injury, including disruption of the extracellular matrix, edema, epithelial cell necrosis, and ultimately erosions and ulcerations (1). The apparent association between neutrophil infiltration and mucosal damage suggests that neutrophils may play an important role in the pathogenesis of UC (2–4). Normally, neutrophils are protective by virtue of their ability to extravasate from the circulation, engulf, and destroy invading microorganisms. However, these phagocytes may become inadvertently activated by the interaction of certain ligands (e.g., immune complex, bacterial products, complement components) with specific receptors on the neutrophil plasma membrane. This metabolic activation results in the production and release of large quantities of potentially cytotoxic reactive oxygen metabolites, such as superoxide, hydrogen peroxide, hypochlorous acid, and *N*-chlorinated derivatives (5). In addition, activated neutrophils secrete a variety of proteases (e.g., elastase, collagenase, gelatinase) and hydrolytic enzymes (e.g., hyaluronidase) capable of degrading the interstitial matrix and epithelial cell membrane (6).

There are several different experimental models of colitis produced in rats, rabbits, and guinea pigs (7, 8). Virtually all of these models require the intraluminal administration of caustic agents (e.g., organic acids, ethanol, formalin) to initiate the inflammatory response. One model that has received substantial attention over the past few years is acetic acid-induced colitis (2, 9–14). This model, originally developed by MacPherson and Pfeiffer (10), uses the intrarectal administration of dilute solutions of acetic acid to produce a diffuse colitis in rats. Although the inflammation produced in this model of colitis is not identical to human ulcerative colitis, it does have some histological similarities to human UC, including epithelial cell necrosis, decreased mucin production, crypt abscesses, and infiltration of large numbers of neutrophils into the mucosal interstitium (10). Furthermore, it has been shown that the pattern of mucosal arachidonate metabolism produced by acetic acid is very similar to that found in biopsies obtained from human UC (12, 15). It has been suggested that inflammatory neutrophils may be responsible for generation of the arachidonate metabolites, leukotriene B_4, and 5-hydroxyeicosatetraenoic acid in this model of colitis (12, 15). Although recent studies suggest an association between neutrophils and mucosal injury, there has been no systematic study to define the role of these phagocytes as mediators of mucosal injury and inflammation in this model of inflammation (2, 9). Therefore, the objective of this study was to define, using quantitative measures of mucosal injury and inflammation, the role of extravasated neutrophils in acetic acid-induced colitis in rats.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 300–350 g were purchased from Harlen Sprague Dawley Inc, Indianapolis, Indiana, and were housed in standard wire-mesh cages in a room carefully controlled for ambient temperature (25°C) and photoperiod (12 : 12 h light–dark cycle). The rats were fed standard laboratory chow and tap water ad libitum.

Preparation of Antiserum to Rat Polymorphonuclear Leukocytes (Neutrophils). Antiserum to rat neutrophils was raised in New Zealand white rabbits weighing 3 kg as described by Sandler et al. (16). Briefly, rat neutrophils were collected by peritoneal lavage 18–20 h after the intraperitoneal injection of 1% (w/v) oyster glycogen in phosphate-buffered saline (PBS; pH 7.4). The contaminating red cells were removed by hypotonic lysis. The washed neutrophils (2.5×10^8) were suspended in 2 ml PBS, mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly. The rabbits then were given a booster injection using 6×10^7 neutrophils in Freund's incomplete adjuvant three weeks following the primary injection. An antibody agglutination titer against purified rat neutrophils was assessed using serial dilutions of serum prior to sacrifice of the rabbit. When the highest dilution that demonstrated agglutination of five or more cells was 1 : 2048, rabbits were sacrificed (usually day 35). Serum was collected, heat inactivated (30 min at 56°C), and absorbed against pooled rat erythrocytes and mononuclear leukocytes. Immunoglobulins were precipitated by $(NH_4)_2SO_4$ fractionation (50%), the protein dissolved in saline, and dialyzed three times against 6 liters of PBS for 24 h at 4°C. The IgG antineutrophil fraction was stored at −20°C for subsequent use.

Induction of Colitis. Rats were fasted for 24 h with free access to water. A total of 31 rats were randomized into four major groups consisting of a saline control group ($N = 10$), an acetic acid group ($N = 11$), an antineutrophil serum (ANS) + saline group ($N = 4$), and an ANS + acetic acid group ($N = 6$). Diffuse colitis was induced with acetic acid as described previously (14). Briefly, animals were lightly anesthetized with Ethrane and 1 ml of 4% (v/v) acetic acid (pH 2.3) was slowly administered via a 5-cm length of polyethylene tubing (PE-240) fitted onto a 1-ml syringe. After a 30-s period of exposure, excess fluid in the lumen was withdrawn and 1.5 ml PBS was introduced to flush the colon. Saline controls received 1 ml saline followed by 1.5 ml PBS. For some experiments, rats were rendered neutropenic prior to the saline or acetic enema by the intraperitoneal injection of ANS. Eleven rats were given three intraperitoneal injections (0.6 ml each) of ANS. The first injection was given 12 h before the enema, the second one was given immediately following the enema, and the third injection was administered 24 h following the saline or acetic acid enema. Circulating polymorphonuclear leukocytes (neutrophils) were counted using the acetic acid–crystal violet stain and a Welch-Allyn/American Optical "Bright-Line" hemacytometer before the first ANS injection, at the time of the enema, and then at 24 and 48 h following the enema.

Mucosal permeability (17, 18). At 48 h following the saline or acetic acid enema, rats were anesthetized with 120 mg/kg of Na-5-ethyl-1(1'-methyl-propyl)-2-thio-barbiturate (Inactin, Byk Gulden Konstanz, Germany). The animals were tracheotomized, and the right femoral artery was cannulated for arterial pressure recording and blood sampling. The right femoral vein was cannulated to inject the radioisotope marker. A midline abdominal incision was performed, and both renal pedicles were ligated to prevent rapid excretion of the radioisotope into the urine. The colon was isolated and cannulated at the splenic flexure and the rectum through the anus using Silastic tubing (Dow Corning, ID, 0.025 and 0.25 in., respectively). The isolated, perfused colon was returned to the abdominal cavity, and abdominal wall was closed to minimize dehydration of the

colon segment during the course of experiment. Luminal contents were removed by perfusion with warm (37°C) modified Tyrode's solution (NaCl 8 g/liter, KCl 0.2 g/liter, NaH_2PO_4 0.33 g/liter, pH 7.4). Body temperature was maintained at 37°C with thermistor-controlled heat lamp. Mucosal permeability was determined using the blood-to-lumen clearance of chromium 51-labeled ethylenediaminetetraacetic acid ([^{51}Cr]EDTA) as described previously (18). One hundred microcuries of [^{51}Cr]EDTA was injected via the femoral catheter followed by a 1-h equilibration period during which time the colon lumen was perfused with solution but no clearance measurements were taken. The perfusate was sampled every 10 min for the appearance of [^{51}Cr]EDTA. Blood samples (0.3 ml) were taken at 40 min for use as reference counts. Blood-to-lumen clearance of [^{51}Cr]EDTA was calculated using the following formula:

$$\text{Clearance (EDTA)} = (C_{per} \times Q)/(C_{bl} \times W)$$

where C_{per} and C_{bl} represent counts per minute per milliliter of [^{51}Cr]EDTA in the luminal perfusate and blood, respecively; Q represents the luminal perfusion rate (0.40 ml/min); and W represents the dry weight of the perfused segment of the colon. Mucosal permeability was determined from the mean of the four clearance values.

Tissue Analyses. After the determinations of mucosal permeability the animals were sacrificed via an overdose of pentobarbital and the perfused colons were excised, opened longitudinally, and gently rinsed with ice-cold saline. The length and weight of the perfused segment then was recorded and the tissue was divided into strips for wet–dry ratio determinations and colonic myeloperoxidase (MPO) activity, respectively. Colonic MPO activity was determined using a minor modification of the method of Grisham et al. (19) in which 3,3′,5,5′-tetramethylbenzidine was used as the electron donating substrate and hexadecyltrimethylammonium hydroxide was used as the detergent. For histological analysis, two random colonic samples were selected from each group and were fixed by immersion in a solution consisting of fresh 2% paraformaldehyde, 1.5% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, at 4°C, and sliced into 2- to 4-mm pieces and fixed overnight. The tissue was then dehydrated to 95% ethanol and embedded in glycol methacrylate (JB-4, Polysciences, Inc., Warrington, Pennsylvania). JB-4 blocks were cured overnight in a vacuum desiccator at room temperature and semi-thin (1- to 2-μm) sections were cut with glass knives and stained with toluidine blue. Samples were examined for mucosal injury in a blinded fashion.

Statistic Analyses. All data are expressed as the mean + standard error of the mean. Statistical differences were determined using one-way analysis of variance procedures with a Bonferoni correction for multiple comparisons. Differences were considered to be significant when the probability of the differences occurring by chance was less than 5% ($P < 0.05$).

RESULTS

The intrarectal administration of 4% acetic acid produced bloody diarrhea in 100% of the animals at 48 h following the enema (data not shown). Gross inspection of the acetic acid-treated colons revealed mucosal hyperemia and severe swelling as well as colonic erosions and ulcerations that were situated from the left portion of transverse colon to the descending colon. Using MPO activity as an index of neutrophil infiltration, colon weight as a generalized index of inflammation, and mucosal permeability as a measure of mucosal (epithelial) injury, we found that the intrarectal administration of acetic acid produced significant increases in all three parameters at 48 h following the enema.

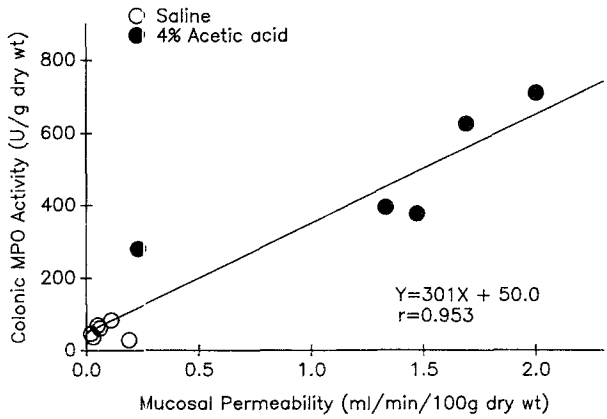

Fig. 1. Relationship between colonic myeloperoxidase (MPO) activity and mucosal permeability at 48 h following the saline or acetic acid enema. Each data point represents one animal.

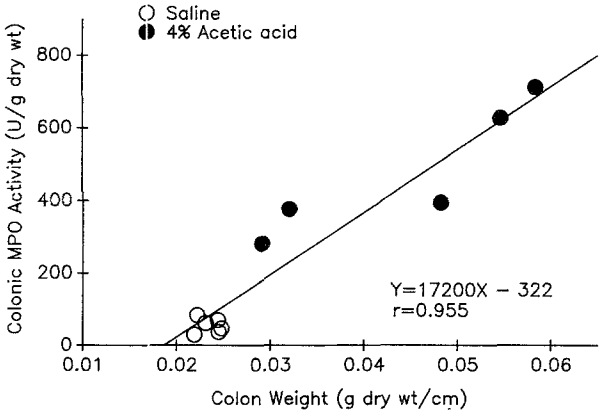

Fig. 2. Relationship between colonic myeloperoxidase (MPO) activity and colon weight at 48 h following the saline or acetic acid enema. Each data point represents one animal.

Using the individual data points obtained from the saline controls and acetic acid-treated animals, we found a significant correlation between colonic MPO activity and mucosal permeability (Figure 1) ($P < 0.01$) and a significant correlation between colonic MPO activity and colon weight as expressed per gram dry weight (Figure 2) ($P < 0.01$). These data suggested a relationship between neutrophil infiltration and mucosal injury and inflammation at 48 h after the enema.

In order to define the role of inflammatory neutrophils (colonic MPO activity) as mediators of mucosal injury (mucosal permeability) or colon weight gain (inflammation), we depleted circulating neutrophils by the intraperitoneal injec-

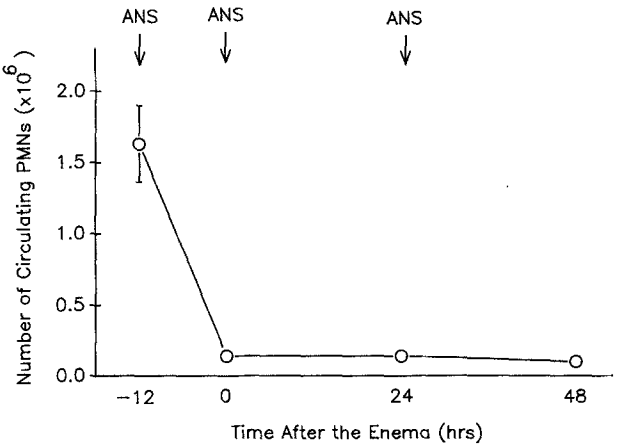

Fig. 3. Effect of antineutrophil serum (ANS) on the number of circulating polymorphonuclear leukocytes (neutrophils). Arrows indicate the time of each ANS injection.

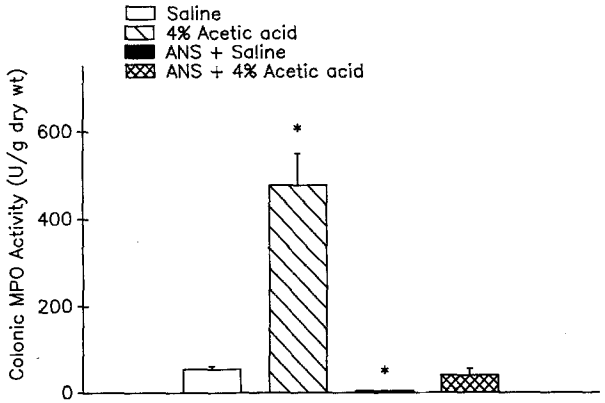

Fig. 4. Effect of antineutrophil serum (ANS) on the acetic acid-induced increase in colonic myeloperoxidase (MPO) activity. Data represent the mean ± *SEM*.* $P < 0.05$ compared to saline control group.

tion of ANS at three different times. Figure 3 illustrates the number of circulating neutrophils in the ANS + saline and ANS + acetic acid groups. The numbers of circulating neutrophils before the ANS injection, at the time of the saline or acetic acid enema, and at 24 and 48 h following the enemas were 1.63 ± 0.27, 0.14 ± 0.05, 0.14 ± 0.05, 0.10 ± 0.05 × 10^6 cells/ml blood, respectively. Figure 4 demonstrates that acetic acid produced a 9-fold increase in colonic MPO activity when compared to saline controls (477.0 ± 73.1 vs. 54.9 ± 7.8 units/g dry weight, respectively, $P < 0.05$). Rendering the animals neu-

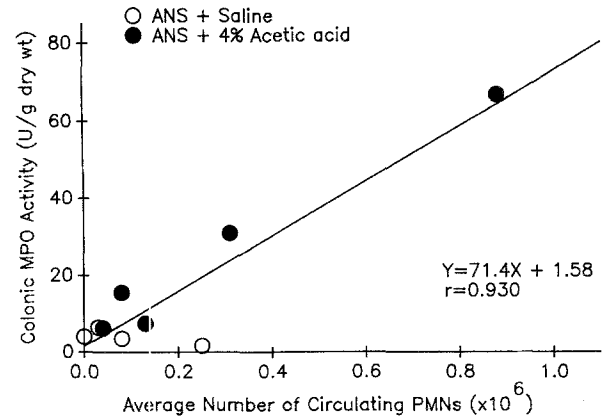

Fig. 5. Relationship between colonic myeloperoxidase (MPO) activity and the average number of circulating neutrophils. Each data point represents one animal.

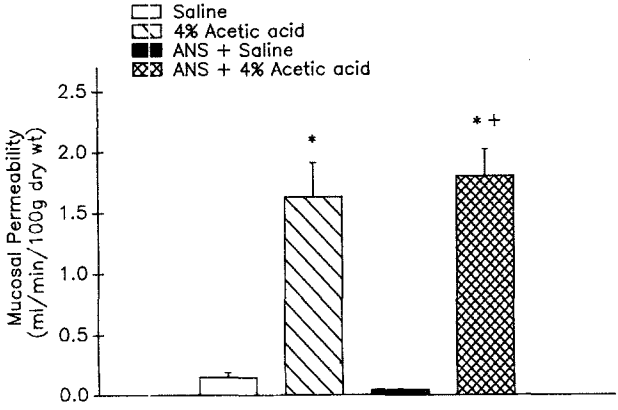

Fig. 6. Effect of antineutrophil serum (ANS) on the acetic acid-induced increase in mucosal permeability at 48 h following the enema. Data represent the mean ± *SEM*. $^{*}P < 0.05$ compared to saline control group. $^{+}P < 0.05$ compared to ANS + saline group.

tropenic prior to the acetic acid enema completely suppressed the increased colonic MPO activity associated with acetic acid. In addition, ANS treatment significantly reduced basal colonic MPO activity in the ANS + saline group compared to saline controls ($P < 0.05$). Furthermore, we found that the reduction in circulating neutrophils correlated significantly with reductions in colonic MPO activity (Figure 5) ($P < 0.01$).

Figure 6 demonstrates that the intrarectal administration of acetic acid produces an 11-fold increase in mucosal permeability when compared to saline controls (1.63 ± 0.28 vs. 0.15 ± 0.04 ml/min/100 g dry weight, respectively;

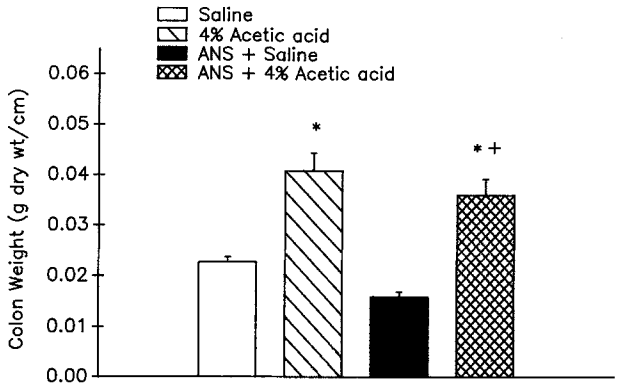

Fig. 7. Effect of antineutrophil serum (ANS) on the acetic acid-induced increase in colon weight at 48 h following the enema. Data represent the mean ± *SEM*. $^{*}P < 0.05$ compared to saline control group. $^{+}P < 0.05$ compared to ANS + saline group.

$P < 0.05$). Pretreatment with ANS did not significantly attenuate the increase in mucosal permeability associated with acetic acid nor did it significantly reduce the basal permeability of the ANS + saline group (Figure 6). Similarly, acetic acid enema produced significant increases in colon weight compared to saline controls (Figure 7) ($P < 0.05$) and pretreatment with ANS failed to attenuate the increase in the colon weight produced by an acetic acid enema. Histological inspection revealed massive epithelial destruction at 48 h following exposure of distal colon to 4% acetic acid (Figure 8A). Although the damage was variable, portions of the bowel always exhibited total exfoliation of the epithelium. In addition, the intrarectal administration of acetic acid produced a massive influx of leukocytes that were predominantly neutrophils. Intrarectal administration of saline to neutropenic rats had no effect on the epithelium, resulting in an entirely normal-appearing mucosa (Figure 8B). Rendering the animals neutropenic with ANS prior to the acetic acid enema resulted in substantial mucosal injury, including a total break of the epithelial barrier. There did appear to be some sparing of the deep crypt epithelial cells (Figure 8C). Figure 9 shows that there were no significant differences in wet-to-dry ratios among the four groups.

DISCUSSION

Neutrophils and neutrophil-derived oxidants have been implicated as important mediators of vascular and mucosal injury in a variety of different models of gastrointestinal inflammation. For example, extravasated neutrophils

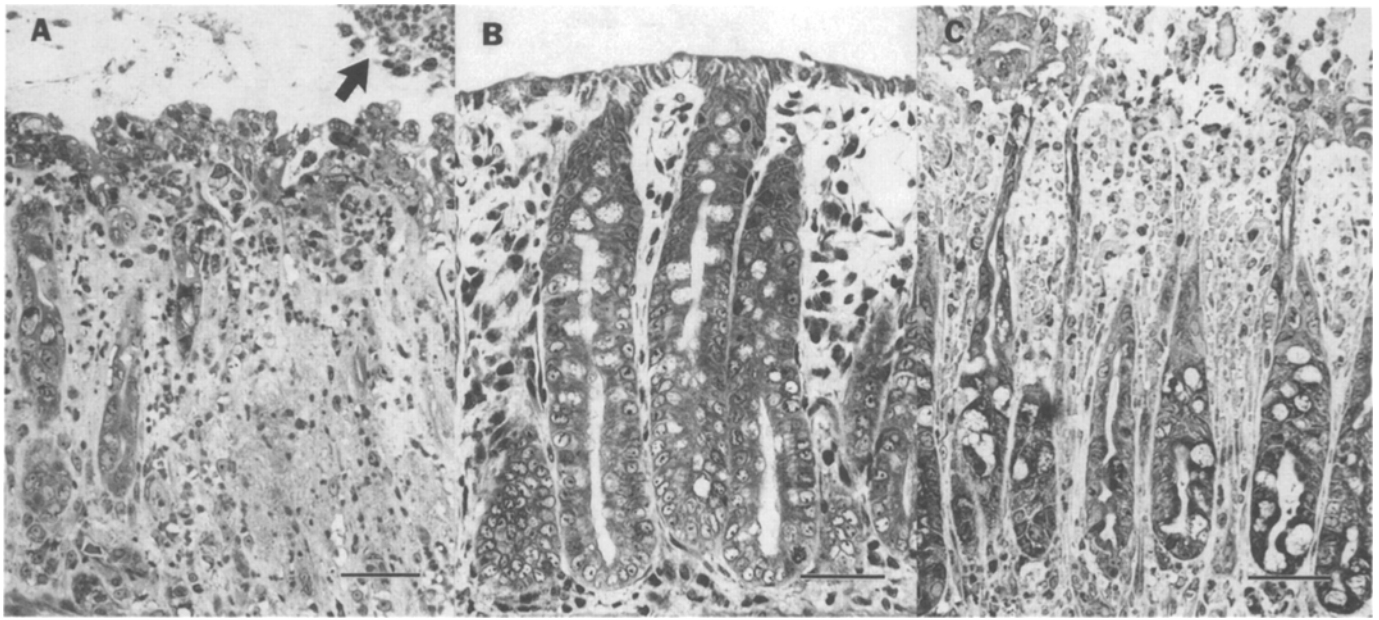

Fig. 8. Rat distal colon 48 h after treatment. Two- to three-micrometer plastic sections stained with toluidine blue. Scale marker equals 50 μm. (A) Rat colonic tissue exposed to 4% acetic acid. The mucosal injury is variable, but all samples exhibit regions of total epithelial exfoliation. The connective tissue is edematous and filled with inflammatory cells, especially neutrophils. A neutrophilic exudate is evident in the lumen (arrow). (B) Exposure of neutropenic animals to luminal saline has no effect on the colonic mucosa. (C) Exposure of neutropenic animals to 4% acetic acid results in substantial mucosal injury, including a total breach of the epithelial barrier.

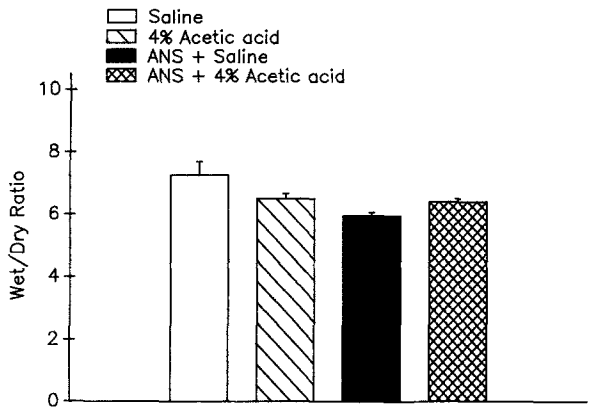

Fig. 9. Effect of antineutrophil serum (ANS) on the wet-to-dry ratio at 48 h following the enema.

have been shown to participate in the gastric vascular and mucosal injury associated with hemorrhagic shock, as well as the mucosal injury produced by ethanol or indomethacin administration (20–23). It also has been demonstrated that the microvascular and mucosal dysfunction induced by reperfusion of the ischemic small intestine is mediated by circulating and extravasated neutrophils (3, 19). Finally, neutrophils have been shown to mediate disruption of the ileal epithelium when the lumen of the bowel is perfused with the proinflammatory bacterial tripeptide *N*-formyl-methionyl-leucyl-phenylalanine (18). Although much work has been done on the mediators of inflammation and mechanisms involved in tissue injury in these models of stomach and small bowel inflammation, very little is known regarding the mechanisms of inflammation-induced mucosal injury in the various models of colonic inflammation. There is some circumstantial evidence to suggest that neutrophils may be involved in the pathogenesis of colitis produced by acetic acid. For example, it has been demonstrated that pretreatment with certain prostaglandin analogs or leukotriene B_4-receptor antagonists significantly inhibited inflammation (neutrophil infiltration) and the mucosal injury produced by acetic acid (2, 13). In addition, other investigators suggest that in the inflamed mucosa of acetic acid-treated rat colons, formation of proinflammatory mediators is mediated by extravasated neutrophils (12). Indeed, data obtained in the present study demonstrate a statistically significant relationship between neutrophil accumulation and mucosal injury and inflammation in the acetic acid model of colitis (Figures 1 and 2). Therefore, it was of interest to determine whether neutrophil infiltration was a cause or an effect of mucosal injury in this acetic acid model of colitis.

Previous studies with this model of colitis have demonstrated maximum inflammation at 48 h after enema (11, 13, 14). In the present study we found

that the intrarectal administration of 4% acetic acid produced significant increases in MPO activity (neutrophil accumulation), mucosal permeability (epithelial cell injury), and colon weight at 48 h following the enema (Figures 4, 6, and 7). Administration of three intraperitoneal injections of antiserum directed toward rat neutrophils (ANS) produced a remarkable neutropenia that lasted throughout the course of the experiment (Figure 3). In addition, treatment with ANS produced a significant reduction in basal mucosal MPO activity and prevented the increase in colonic MPO following the acetic acid enema (Figure 4). Furthermore, this decrease in circulating neutrophils correlated significantly with reductions in colonic MPO activity, suggesting that the source of colonic MPO is the circulating pool of neutrophils (Figure 5). Rendering the animals neutropenic did not reduce the increases in mucosal permeability nor did it attenuate the increase in colon weight produced by acetic acid, suggesting that circulating neutrophils do not mediate epithelial cell injury nor do they contribute to the increases in colon weight (Figures 6 and 7). In an analogous series of experiments, Sekizuka et al. found that infiltrating neutrophils did not mediate the hyperemia associated with acetic acid induced colitis (11). Taken together, these data suggest that the mechanisms responsible for acetic acid-induced mucosal injury, inflammation, and hyperemia occur independently of neutrophil adherence and extravasation. The precise mechanisms by which intrarectal acetic acid produces inflammation and mucosal injury remain only speculative. It is known that the protonated form of the acid (CH_3COOH) is required for the production of colitis, since intrarectal administration of either HCl at the same pH (2.3) or sodium acetate (pH 7.0) does not produce colonic inflammation (8). It may be that significant amounts of the lipophilic acid gain access to the intracellular compartment of the epithelial cell where it equilibrates to generate large quantities of its conjugate base (acetate anion) and protons:

$$CH_3COOH \rightarrow CH_3COO^- + H^+ \ (pKa = 4.76)$$

This massive intracellular acidification would injure the epithelial cells, thus initiating the inflammatory response. Therefore, the inflammation observed at 48 h after enema would be considered a result of mucosal injury and not a cause. It is also quite likely that any model of colitis requiring the use of mucosal irritants or caustic agents such as formalin (7, 8), ethanol (24, 25, 26), or organic acids (10) will produce inflammation in response to mucosal injury. Data obtained in the present study suggest that although this model may be useful in studying those events that occur at the time of inflammation, it may not represent the most physiologically appropriate model for investigating the pathogenesis of ulcerative colitis. Therefore, we suggest that the interpretation of data obtained to assess the protective or antiinflammatory effects of certain drugs should be made with caution. It could be that a drug found to be efficacious in

this model may simply represent the ability of the drug to inhibit the initial insult produced by acid rather than by attenuating the inflammatory reaction.

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