American Journal of Pathology, Vol. 130, No. 3, March 1988

Contribution of Oxygen-Derived Free Radicals to Experimental Necrotizing Enterocolitis

DAVID A. CLARK, MD,
DONNA M. FORNABAIO, BS,
HELEN McNEILL, BS,
KEVIN M. MULLANE, PhD,
SALVATORE J. CARAVELLA, MD,
and MARK J. S. MILLER, PhD

From the Departments of Pharmacology and Pediatrics, New York Medical College, Valhalla, New York

Oxygen-derived free radicals, particularly superoxide anion, are considered important mediators of intestinal injury induced by ischemia/reperfusion based on the protective effects of superoxide dismutase and allopurinol. A role for free radicals was investigated in a model of necrotizing enterocolitis (NEC) which was initiated by a luminal, as opposed to a vascular, insult. Intestinal loops of weanling rabbits received either saline (control loops) or a solution of 10 mg/ml casein and 50 mg/ml calcium gluconate acidified to pH 4 with proprionic acid (treated loops). When the animals were sacrificed 3 hours later, severe damage was noted in the treated loops, which included blunting of villi and edema, with all animals surviving. At 16 hours only 5 of 8 rabbits survived, and 3 had hemorrhagic necrosis. Control loops were normal in each case. Intravenous infusion of superoxide dismutase (4 mg/kg/hr), commencing 15 minutes after NEC induction, totally prevented intestinal injury. On the other hand, pretreatment with allopurinol, an inhibitor of xanthine oxidase, for 2 days (30 and 60 mg/kg by mouth) was not protective against intestinal damage. A cellular infiltration in treated loops was not histologically evident in the majority of animals at 3 hours after treatment, a finding confirmed by the minimal accumulation of ^{111}In-labeled leukocytes in damaged and intact intestinal tissue. These results suggest that superoxide generated locally from sources other than xanthine oxidase play a critical and early role in experimental NEC and that superoxide dismutase may prove to be an effective therapy in this devastating neonatal disease. (Am J Pathol 1988, 130:537–542)

NECROTIZING enterocolitis (NEC) is a devastating neonatal disease affecting approximately 6% of all premature infants[1]; 20–40% of these cases are fatal,[2,3] accounting for a major contribution to the mortality of premature infants surviving early respiratory distress syndrome. In addition, surviving babies frequently have postoperative short bowel syndrome or malabsorption. The origin of NEC is uncertain. Numerous studies have proposed a primary vascular insult[4–6]; however, a recent large multicenter study has failed to support any relationship between NEC and the status of the systemic circulation, respiratory distress, or birth asphyxia.[1] On the other hand, a clear relationship was drawn with birth weight; the smaller and more premature the infant, the higher the incidence of NEC.

Based on the inability of systemic cardiovascular events and therefore a primary vascular insult to adequately explain the initiation of NEC, as well as the recognition that NEC is associated with enteral feedings,[6–8] we developed an animal model based on the intraluminal biochemistry of infants with NEC.[9] We postulated that undigested protein in an acidic media (arising from organic acids produced by bacterial fermentation of undigested carbohydrates) initiates an inflammatory response in the bowel. By mimicking the intraluminal contents of infants with NEC in rabbit intestinal loops, the clinical disease can be reproduced.[9,10]

Supported by the Mead Johnson Company.

Dr. Mullane's and Dr. Miller's present address is Pharmaceuticals Division, CIBA-GEIGY Corporation, 556 Morris Avenue, Summit, NJ 07901.

Accepted for publication October 13, 1987.

Address reprint requests to David A. Clark, MD, Department of Pediatrics, New York Medical College, Valhalla, NY 10595.

In the present study, we have addressed the role of oxygen radicals in the inflammatory process associated with NEC. Agents that scavenge superoxide anions (superoxide dismutase) or inhibit their formation by xanthine oxidase (allopurinol) have prevented intestinal injury induced by ischemia and reperfusion.[11,12] Although we propose that NEC results from a luminal-based injury, a beneficial effect of superoxide dismutase and allopurinol similar to that seen with ischemic injury was investigated. Furthermore, because invading inflammatory cells may be a source of oxygen-derived radicals, we examined leukocyte accumulation in the necrotic intestine.

Materials and Methods

Induction of NEC in Rabbits

We have previously described the model of NEC,[9] which is based on a rabbit intestinal loop model of Kasai and Burrows.[13] Briefly, weanling New Zealand white male rabbits were fasted for eight hours prior to anesthesia induction with ketamine (50 mg/kg) and xyalazine (10 mg/kg) intramuscularly. The abdomen was shaved and cleansed for surgery. The peritoneum was opened, and a ligature placed just distal to the ligament of Treitz. The intestine was then flushed with warm sterile saline and ligated into a series of four intestinal loops of approximately 10 cm in length, with particular care taken to preserve blood supply. The intestinal loops were then injected, using a 26-gauge needle, with 1 ml/2 cm of intestine of saline or test (acidified protein + calcium) solution. The osmolarity of saline and test solutions were, respectively, 300 and 310 mOsm/l. Only one loop received the test solution. The control (saline) loop was not positioned immediately adjacent to the loop containing the test solution (treated loop), so that we could avoid complications which may arise from extension of the inflammatory process from damaged intestine to adjacent, nontreated areas. The section of intestine separating control and treated loops was therefore designated as "interloop." The peritoneum was closed, and after 3 or 16 hours the animals were reanesthetized for sacrifice and tissue collection. The test solution, which induces intestinal necrosis, consisted of 10 mg/ml bovine casein in saline titrated to pH 4.0 with an organic acid (proprionic acid) and calcium gluconate, 50 mg/ml. At the time of sacrifice, sections of each intestinal loop were placed in neutral buffered formalin prior to histologic preparation.

In those animals receiving superoxide dismutase (SOD), an intravenous infusion of SOD (4 mg/kg/hr) was commenced 15 minute after intestinal loop preparation, ie, NEC induction. This infusion was continued until the animal was sacrificed. Additional groups of animals received allopurinol (30 mg/kg or 60 mg/kg by mouth) for 2 days prior to NEC induction.

Accumulation of ^{111}In-Labeled Leukocytes

Buffy-coat leukocytes were collected from citrated blood from donor rabbits. Contaminant erythrocytes were destroyed by hypotonic lysis, and the remaining leukocytes were washed in normal saline. The leukocyte count was determined on a hemocytometer, and the cells were then resuspended at 10^7 cells/ml in Hanks' balanced salt solution (HBSS), pH 7.4. ^{111}In-labeling was achieved by adding ^{111}In-oxine (200 μCi) to the leukocyte suspension in a dropwise manner, followed by a 30-minute incubation at room temperature. Leukocyte viability after labeling was tested by trypan blue exclusion. After the incubation period, the leukocyte preparation was pelleted by centrifugation at 900g for 10 minute and gently resuspended with HBSS. The cells were centrifuged and washed with HBSS twice; after the final wash and centrifugation, cells were gently resuspended in 10 ml of autologous platelet- and leukocyte-poor plasma. ^{111}In-labeled leukocytes were infused into a peripheral ear vein 30 minutes before intestinal loop preparation. Peripheral venous blood samples were collected at selected time intervals (1, 5, 10, and 30 minutes) before NEC induction, to ensure a stable circulating level. The degree of leukocyte sequestration in the intestine and reference tissues was determined at autopsy by counting the radioactivity per milligram tissue wet weight. Intestinal leukocyte sequestration was expressed as a percentage of the radioactivity recovered in the myocardium, a noninvolved reference tissue. In 5 of 6 animals circulating leukocyte levels (cpm/ml) were taken at sacrifice, and the results validated a comparative analysis between heart and intestine.

Histologic analysis of intestinal morphology was performed on representative sections examined by light microscopy. Evidence for necrosis was classified according to the following grading system: Grade 0, villi completely intact; Grade 1, villous tip necrosis with preservation of villous crypts; Grade 2, necrosis of villous tips and crypts with loss of mucosal and submucosal architecture; Grade 3, necrosis extending into the muscularis; Grade 4, transmural necrosis. The presence of edema, lymphatic dilatation, hemorrhage, and immune/inflammatory cell infiltrates was also noted.

Results

In this model of NEC, severe intestinal damage is evident within 3 hours. This damage is characterized by a dramatic blunting of villi lymphatic dilatation and edema (Figures 1 and 2). Treated loops were swollen and turbid, in contrast to saline-treated loops, indicative of a net movement of fluid into the intestinal lumen. When examined 16 hours after NEC induction, only 5 of 8 rabbits had survived, and damage was generally more severe than at 3 hours, but confined to the treated loop. Further, in some animals (3 of 8) hemorrhagic necrosis of the treated loop was noted. Intravenous infusion of superoxide dismutase completely prevented intestinal necrosis in each 3-hour experiment (Figure 3). When the degree of damage was quantified on a score basis, the appearance of treated loops of rabbits receiving superoxide dismutase was indistinguishable from control, undamaged loops (Table 1). The protection offered by superoxide dismutase in the 3-hour protocol was not examined with a 16-hour insult. Allopurinol at both low and high doses was ineffective in preventing intestinal damage (Table 1). Furthermore, allopurinol appeared to have a direct toxic effect on the intestinal epithelium, as evidenced by damage to control and interloops.

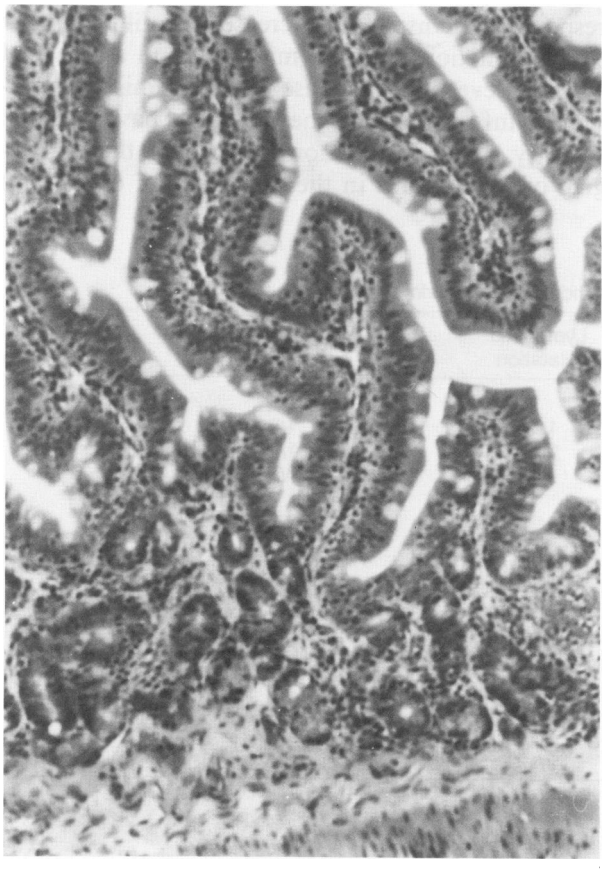

Figure 1—Light-microscopic appearance of a control loop (saline-treated) from a rabbit 3 hours after NEC induction.

Figure 2—Light-microscopic appearance of a treated intestinal loop 3 hours after NEC induction. Note the loss and blunting of villi.

NEC-induced intestinal damage at 3 hours was not usually associated with a cellular infiltration in remaining villous and crypt structures (Figure 2). This finding was confirmed with the use of ^{111}In-leukocytes; intestinal sequestration of leukocytes was minimal at 3 hours and similar in control and treated intestinal loops (Figure 4). However, 16 hours after NEC induction in two of four experiments, spikes of leukocyte accumulation were evident in normal tissue adjacent to necrotic intestine (interloops). In the remaining two experiments, a high level of radioactivity was recovered in the treated loop, which may have been the result of hemorrhagic necrosis in these animals. The amount of radioactivity in these hemorrhagic loops was disproportionate to the degree of hemorrhage; ie, the amount of extravasated blood could not account for the levels of radioactivity in these loops, suggesting active accumulation of leukocytes in the treated loop as well as a possible contribution of leukocytes to this hemorrhagic response. In each 16-hour experiment radioactivity was higher

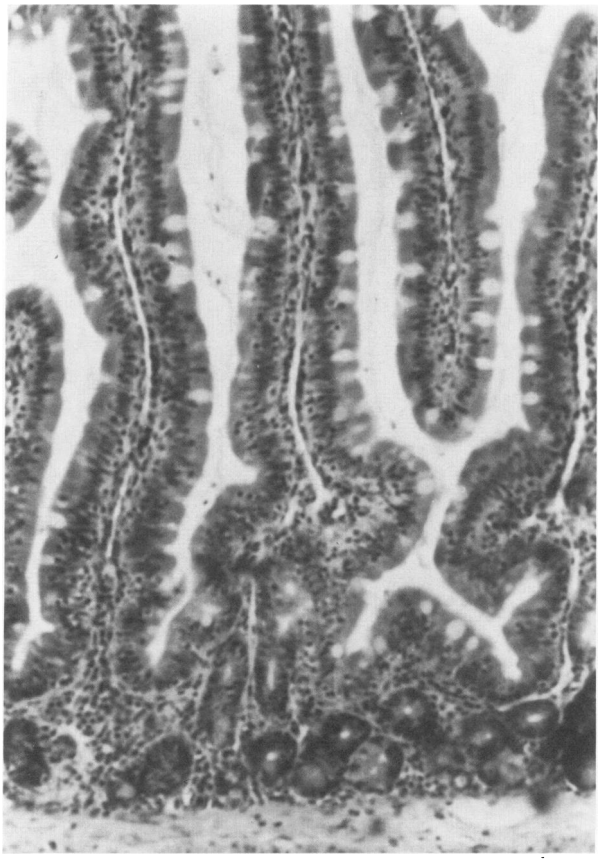

Figure 3—Light-microscopic appearance of a treated intestinal loop from a rabbit that received intravenous infusion of superoxide dismutase (4 mg/kg/hr). The superoxide dismutase infusion commenced 15 minutes after NEC induction and continued until sacrifice 3 hours later. Note the complete preservation of intestinal architecture.

throughout the intestine when compared with the 3-hour results, suggesting generalized intestinal leukocyte accumulation as the disease progresses.

Discussion

Superoxide anion and/or derivatives have been previously implicated in intestinal injury induced by ischemia and reperfusion.[11,12] This is based on the protective effects of superoxide dismutase and allopurinol, an inhibitor of xanthine oxidase. The present findings demonstrate that superoxide may also play a critical role in intestinal injury initiated by a luminal-based insult. There is a growing awareness that oxygen-derived radicals may be linked to a number of diseases of the circulation as well as inflammatory conditions.[14,15] Free radical production results in lipid peroxidation and damage to membranes. These deleterious effects may culminate in the loss of cellular integrity evident in NEC, in addition to the degradation of capillary basement membranes and the interstitial matrix associated with increased vascular permeability.[11]

A characteristic defense of the mature intestine in response to an insult is an increase in motility in order to expel noxious luminal material from a localized site. However, this response is compromised in premature infants, because they generally have poor gut motility. This phenomenon may explain the high degree of perforations in the clinical form of NEC as

Table 1—Effects of Superoxide Dismutase (SOD) on Intestinal Damage Induced by Experimental NEC

Intestinal loop	n	Score*	Lymphatic dilatation	Cellular infiltration	Edema
NEC					
Control	5	0.2	1/5	1/5	0/5
Interloop	5	0.2	2/5	1/5	1/5
Treated	5	2.2†‡	4/5	2/5	4/5
NEC + SOD (4 mg/kg/hr intravenously)§					
Control	9	0.2	1/9	0/9	0/9
Interloop	9	0.3	3/9	1/9	1/9
Treated	9	0.3	2/9	1/9	1/9
NEC + allopurinol (30 mg/kg by mouth)‖					
Control	7	0.7	4/7	2/7	0/7
Interloop	7	1.1	6/7	3/7	2/7
Treated	7	1.9	5/7	5/7	3/7
NEC + allopurinol (60 mg/kg by mouth)‖					
Control	12	0.3	3/12	2/12	0/12
Interloop	12	0.9	3/12	5/12	1/12
Treated	12	0.9	3/12	8/12	0/12

*Values given as mean, other variables described by their frequency.
†Significantly greater than untreated intestinal loops, $P < 0.001$ (ANOVA).
‡Significantly greater than group receiving SOD, $P < 0.001$ (ANOVA).
§Includes one experiment in which the SOD infusion commenced 1 hour, not 15 minutes, after NEC induction.
‖Administered for 2 days before the experiment.

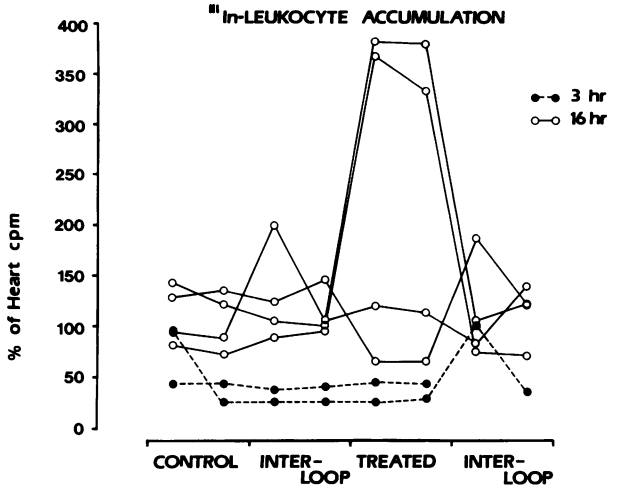

Figure 4—Accumulation of ^{111}In-labeled leukocytes in rabbit intestine 3 hours (●---●) and 16 hours (○——○) after induction of NEC in treated loops. Results from individual experiments are plotted and expressed as a percentage of radioactivity recovered in the heart (reference tissue).

opposed to the characteristics of adult inflammatory bowel disease.

It does not appear that invading leukocytes are required for the initiation of intestinal necrosis or the primary source of oxygen-derived free radicals, although they potentially may exacerbate intestinal injury at later stages. We have not measured intestinal blood flow during the induction of NEC. It is possible that leukocytes do not accumulate in treated loops by 3 hours, as a result of an impaired blood flow to this region, ie, reduced accessibility. On the other hand, blood flow should be enhanced due to metabolic hyperemia associated with digestion of casein in the test solution. Direct measurements of local blood flow are required for an assessment of the vascular contribution to this luminal-based injury.

The source of oxygen-derived radicals in NEC is uncertain. Xanthine oxidase, an enzyme arising from xanthine dehydrogenase, is thought to be the initial source of radicals in postischemic injury of the intestine.[11,12] The liver and intestine are particularly rich sources of xanthine oxidase.[16] However, allopurinol did not afford protection in this model of NEC, which suggests that free radical production induced in NEC must be derived from additional sources. We had suggested that circulating leukocytes do not play a primary role in the initiation of intestinal injury, but leukocytes resident in the bowel wall may be an important source of free radicals. In addition, a variety of mediators and chemotactic factors may be released by these cells which may contribute to the disease process.

Superoxide dismutase has a half-life of approximately 15 minutes and must be administered intravenously[11] which may limit its application in certain chronic disease states. These characteristics may not be limiting in the management of premature infants, where indwelling intravascular catheters are common and rapid termination of therapy upon untoward effects is advantageous. In general, agents that limit free radical formation or their effects may have widespread application in the premature infant, because several conditions may be related to oxygen toxicity, eg, bronchopulmonary dysplasia and retinopathy of prematurity. A promising feature of these results is that superoxide dismutase was effective when administered 15 minutes after NEC induction. In one experiment this protective effect extended to 1 hour after NEC initiation (data included in Table 1), whereas previous studies with superoxide dismutase in postischemic gastrointestinal injury were based on pretreatment with superoxide dismutase.[11,17] Because the diagnosis of NEC is difficult and often delayed therapy that can be introduced after the initial insult has the greatest clinical promise. Systemic administration of bovine superoxide dismutase has proven to be effective in reducing the severity of bronchopulmonary dysplasia associated with the management of respiratory distress syndrome of preterm infants.[18] Further, there were no local or systemic reactions associated with superoxide dismutase administration. The recent development of recombinant forms of human superoxide dismutase should also alleviate concerns over toxicity and immunologic problems associated with systemic administration of a protein.

Thus, we propose that superoxide plays an early and critical role in the etiology of NEC, and that therapeutic strategies like the use of superoxide dismutase may greatly alter the outcome of this devastating neonatal disease.

References

1. Kanto WP Jr, Wilson R, Breart GL, Zierler S, Purohit DM, Peckham GJ, Ellison RC: Perinatal events and necrotizing enterocolitis in premature infants. Am J Dis Child 1987, 141:167–169
2. Wilson R, Kanto WPJ, McCarthy BJ, Burton T, Lewis P, Terry J, Feldman RA: Epidemiologic characteristics of necrotizing enterocolitis: A population based study. Am J Epidemiol 1981, 114:880–887
3. Ryder RW, Shelton JD, Guinan ME: Necrotizing enterocolitis: A prospective multicenter investigation. Am J Epidemiol 1980, 112:113–117
4. Brown EG, Sweet AY: Neonatal necrotizing enterocolitis. Pediatr Clin North Am 1982, 29:1149–1170
5. Santulli TV, Schallinger JN, Heird WC, Gongaware RD, Wigger J, Barlow B, Blanc WA, Berdon WE: Acute necrotizing enterocolitis in infancy: A review of 64 cases. Pediatrics 1975, 53:376–387
6. Kleigman RM, Fanaroff AA: Necrotizing enterocolitis. N Engl J Med 1984, 310:1093–1103

7. Frantz ID, L'Heurex PL, Engel RR, Hunt CE: Necrotizing enterocolitis. J Pediatr 1975, 86:259–263
8. Virnig NL, Reynolds JW: Epidemiological aspects of neonatal necrotizing enterocolitis. Am J Dis Child 1974, 128:186–190
9. Clark DA, Thompson JE, Schneider AJ, Weiner L, McMillan JA, Rokahr J: Necrotizing enterocolitis: intraluminal biochemistry and bacteria and an animal model. Pediatr Res 1985, 19:919–921
10. Clark DA, Oliphant M, Thompson JE, Gordon L, Lougnot K: Necrotizing enterocolitis: A radiographic and pathologic model (Abstr). Pediatr Res 1984, 18:315A
11. Granger DN, Rutili G, McCord JM: Superoxide radicals in feline intestinal ischemia. Gastroenterology 1981, 21:22–29
12. Grisham MB, Hernandez LA, Granger DN: Xanthine oxidase and neutrophil infiltration in intestinal ischemia. Am J Physiol 1986, 251:G567–G574
13. Kasai GJ, Burrows W: The titration of cholera toxin and antitoxin in the rabbit ileal loop. J Infect Dis 1966, 116:606–608
14. McCord JM: Superoxide radical: A likely link between reperfusion injury and inflammation. Adv Free Radical Biol Med 1986, 2:325–345
15. Weiss SJ: Oxygen, ischemia and inflammation. Acta Physiol Scand 1986, Suppl 548:9–37
16. Parks DA, Granger DN: Xanthine oxidase: biochemistry, distribution and physiology. Acta Physiol Scand 1986, Suppl 548:87–99
17. Adkinson D, Hollwarth ME, Benoit JN, Parks DA, McCord JM, Granger DN: Role of free radicals in ischemia-reperfusion injury to the liver. Acta Physiol Scand 1986, Suppl 548:101–107
18. Rosenfeld W, Evans H, Concepcion L, Jhaveri R, Schaeffer H, Friedman A: Prevention of bronchopulmonary dysplasia by administration of bovine superoxide dismutase in preterm infants with respiratory distress syndrome. J Pediatr 1984, 105:781–785

Acknowledgments

The authors wish to thank Mrs. Pamela Blank and Mrs. Carol Cristiano for assistance in manuscript preparation.