N-Acetyl-Cysteine and Prostaglandin

Comparable Protection Against Experimental Ethanol Injury in the Stomach Independent of Mucus Thickness

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The role of barrier mucus in mediating the protective effects of 16,16 dimethyl PGE_2 (dm PGE_2) against ethanol-induced gastric injury, with and without concomitant treatment with N-acetyl-cysteine (NAC), a potent mucolytic agent, was evaluated. Fasted rats were orally administered either saline, 10 μg/kg dm PGE_2, 20% NAC, or 10 μg/kg dm PGE_2 plus 20% NAC. In the first study, the rats were killed 15 minutes later and their stomachs were removed and assayed for barrier mucus adherent to the gastric wall using the Alcian blue technique. In the second study, the rats were orally given 2 mL of absolute ethanol (EtOH) after receiving one of these pretreatment regimens, and 5 minutes later they were killed and their stomachs were evaluated histologically by light microscopy for the magnitude of EtOH injury. Although NAC significantly reduced the thickness of barrier mucus by 76% when compared with control animals, it did not adversely affect the ability of dm PGE_2 to spare the deep epithelium from injury by EtOH. In fact, NAC was as effective a protective agent as dm PGE_2. Neither agent prevented damage to the surface epithelium by EtOH, verifying previous studies regarding the protective effects of prostaglandins. These results indicate that both dm PGE_2 and NAC prevent EtOH-induced damage to the deeper layers of the gastric mucosa independent of mucus gel layer thickness, suggesting that other mechanisms than mucus are involved in mediating this protection.

CONSIDERABLE CONTROVERSY EXISTS regarding the role of mucus in the prevention of gastric mucosal injury.[1-7] Published reports indicate conflicting findings with respect to the output of gastric mucus in stomachs that have been exposed to damaging agents such as aspirin, ethanol, and hypertonic NaCl, as well as protective agents such as sulfhydryl drugs and prostaglandins (PGs).[3-8] Such studies demonstrate that the thickness of the mucus gel covering the epithelium can be influenced by the application of various damaging and protective agents.[3,5,7-9]; however, the actual importance of mucus in mediating the protective effect of an agent such as PG has yet to be established.

Current knowledge indicates that gastric mucus exists in two forms: one form adherent to the mucosal surface ("barrier mucus"), and a second free-flowing form within the luminal bathing solution ("free mucus").[2,5,8] Barrier mucus appears to be the more important form and has been proposed to be the component of mucus that is directly involved in mucosal defense.[2,5] Free mucus, on the other hand, seems to be derived from desquamated cells and/or secretory products of surface mucus cells with little or no protective properties. The current study investigated the role of barrier mucus in the prevention or attenuation of ethanol damage in the stomachs of rats as evaluated histologically when rats were pretreated with 16,16 dimethyl PGE_2 (dm PGE_2) alone or in combination with N-acetyl-cysteine (NAC), a potent mucolytic agent that reduces the thickness of barrier mucus .[3,9,10] Additionally, there is strong evidence that sulfhydryl compounds mediate the gastric protective effects of PGs and that ethanol damage to gastric mucosa is associated with decreased tissue levels of sulfhydryl compounds.[11,12] Since NAC is a sulfhydryl agent, the possibility that this compound itself protects against ethanol damage independent of mucus secretion was also investigated.

Methods and Materials

Female Sprague-Dawley rats, with an average weight of 200 g, were fasted overnight in cages with wire mesh

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bottoms to prevent coprophagia. On the day of experimentation, each rat was randomly assigned to one of four groups and received a 1.0-mL oral bolus by orogastric intubation of either physiologically normal saline, 10 μg/kg of dm PGE_2 in saline, 20% NAC in saline, or 10 μg/kg of dm PGE_2 in combination with 20% NAC. The concentration of dm PGE_2 used in this study was chosen because subcutaneous administration of this dose has been shown in studies in our laboratory to prevent deep mucosal injury induced by absolute ethanol,[13] suggesting that it should possess a similar action when given orally. A concentration of 20% NAC was used because it possesses effective mucolytic properties.[3,9,10] All solutions were buffered to pH 7.4; this pH was necessary to maintain NAC in solution.

Fifteen minutes after the administration of these treatment regimens, the rats were killed by cervical dislocation and their stomachs were removed to measure mucus adherent to the gastric surface epithelium using Alcian blue, a cationic histologic dye that binds glycoproteins and soluble mucopolysaccharides into insoluble complexes without penetrating mucosal cells.[8,14] Dye quantitation was performed using the method described by Corne et al.[14] The stomachs were opened along the lesser curvature, the glandular portion excised and everted, and soaked for 2 hours in 10 mL of 0.1% Alcian blue dissolved in 0.16-mol/L sucrose and 0.05-mol/L sodium acetate, at pH 5.8. After three washes in 0.25-mol/L sucrose to remove the uncomplexed residue, the dye bound to the surface barrier mucus was eluted by immersion of the glandular stomach in 10 mL of 0.5-mol/L $MgCl_2$ for 2 hours. The resulting solutions were shaken with equal volumes of diethyl ether and the aqueous phase read on a Beckman spectrophotometer (Model 35, Beckman Instruments, Inc., Fullerton, CA) at 605 nm. The Alcian blue recovery recorded as optical density was converted to μg/mL of dye bound to the mucosal surface by comparison with a standard curve obtained from dilution of 0.1% Alcian blue solution. A previous study has shown that Alcian blue and mucus combine in constant proportions.[14]

In a second series of experiments, the rats were again randomly assigned to each of the previously described four groups and subjected to one of these treatment protocols. Fifteen minutes later, at the time the rats in the first series of experiments were killed and mucus determinations made, this second group of rats instead received an oral bolus of 2 mL of absolute ethanol. Five mintues after this damaging agent was given, they were killed by cervical dislocation. This time of killing the rats was chosen because previous studies have shown that 5 minutes after ethanol exposure widespread mucosal damage is present in the rat stomach, without evidence of healing.[13,15]

At the time the rats were killed in these experiments, their stomachs were quickly exposed through a midline laparotomy, ligated at the pylorus and the gastroesophageal junction, injected for fixation with 2 mL of half-strength Karnovsky's fixative[16] through a small puncture wound in the forestomach just distal to the proximal ligature, removed, and then placed in additional fixative for 24 hours. At the end of this time, the stomachs were opened along the lesser curvature, the mucosal surface was inspected for gross evidence of injury, and two samples were obtained and coded for light microscopic evaluation. The location of the samples taken was standardized; both were excised from the midline region along the greater curvature, one just below the limiting ridge of the forestomach, and the other just proximal to the antrum as previously described.[13] Sections were embedded in paraffin, stained with hematoxylin and eosin, mounted on glass slides, and evaluated for extent and depth of mucosal injury. Criteria for scoring the depth of injury have been described in detail previously.[13] Briefly, surface mucus cell damage and/or necrosis was indicated by cytoplasmic vacuolization or swelling, or nuclear pyknosis and/or swelling, with margination of nuclear chromatin. Lucent cytoplasm and pyknotic nuclei indicated parietal cell damage. Gland dilation, hyperemic vessels, and hemorrhage were indications of deeper glandular injury. Type 1 damage involved interfoveolar surface mucus cells only. If gastric pit surface mucus cells were also involved, the classification was Type 2. Injury extending from luminal cells through gastric pit cells and including as much as one third of the depth of the gastric glands was classified as Type 3. The most severe necrotic injury that extended deeper into the gastric glands below the upper third was designated Type 4. Scores for each type of damage from the two samples from each rat were averaged to give single values for that stomach, and results were expressed as per cent ± SEM for each of four categories of depth of cellular injury for all experimental groups.

For both mucus and gastric injury studies, statistical evaluation was performed using analysis of variance; $p < 0.05$ was considered significant.

Results

Results of the mucus assay are shown in Figure 1. By itself, dm PGE_2 did not stimulate an increase in the amount of mucus adherent to the gastric glandular surface 15 minutes after pretreatment, at the time when absolute ethanol was administered in the gastric injury study. Actually, a slight decrease in barrier mucus was noted in rats treated with dm PGE_2 when compared with control rats, although differences between these two groups were not statistically significant. Both groups treated with NAC, however, regardless of concomitant dm PGE_2 treatment, showed an average reduction of 76% in the bound layer when compared with control rats, which was significant.

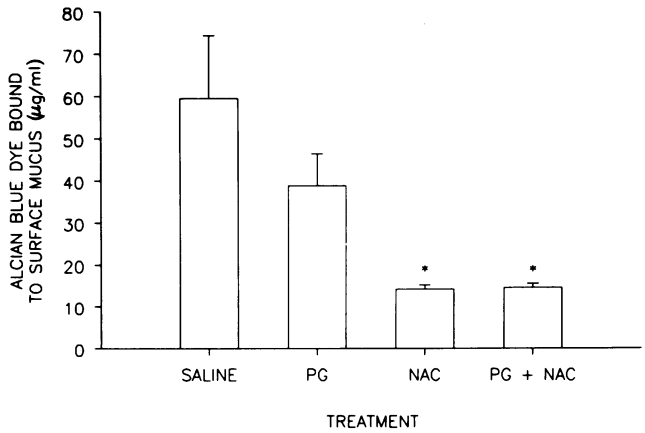

FIG. 1. Results of Alcian blue mucus assay expressed as μg/mL of dye bound to gastric barrier mucus layer 15 minutes after orally administering saline, PG (10 μg/kg dm PGE_2 treatment), NAC (20% NAC treatment), and PG + NAC (10 μg/kg dm PGE_2 plus 20% NAC treatment). N = 6 for each experimental group, *p < 0.025 compared with saline.

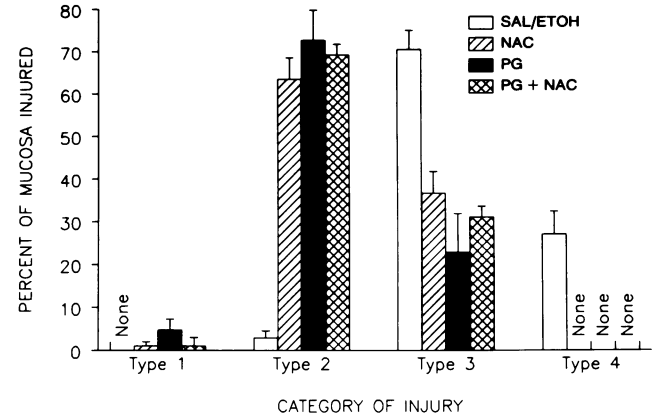

FIG. 2. Effect of prostaglandin and/or NAC pretreatment on the depth of gastric injury in rats killed 5 minutes after 2-mL oral EtOH administration. There was no Type 1 injury in rats treated with Sal/EtOH and no Type 4 injury in any of the other experimental groups. Sal/EtOH = oral saline followed by 100% ethanol, PG/EtOH = oral 10 μg/kg dm PGE_2 followed by 100% ethanol, NAC/EtOH = oral 20% NAC followed by 100% ethanol, PG + NAC/EtOH = oral 10 μg/kg dm PGE_2 plus 20% NAC followed by 100% ethanol. N = 6 for each experimental group.

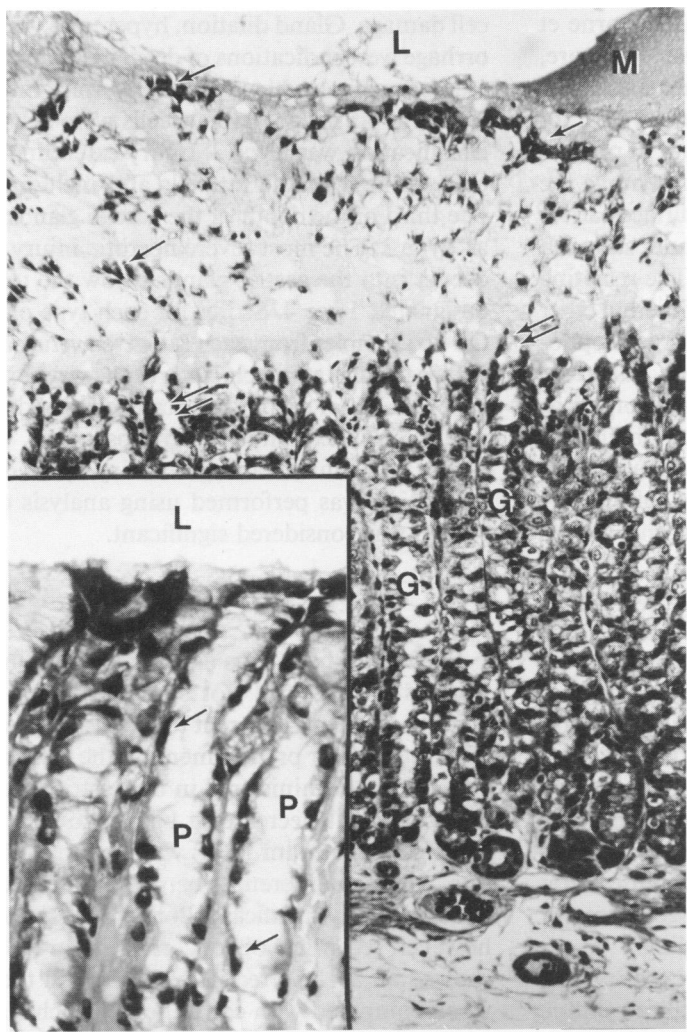

FIG. 3. Light micrograph showing Type 3 injury in rat gastric mucosa exposed to physiologic saline followed 15 minutes later by 100% ethanol. A sheet of mucus (M) and exfoliated pit and interfoveolar surface mucus cells (arrows) are shown within the gastric lumen (L). Collapsed remnants of gastric pits (double arrows) lead into dilated gastric glands (G). Paraffin-embedded, hematoxylin and eosin stain, (×230). (Inset) Light micrograph showing the mucosal surface from a region of necrotic (Type 4) damage. Cell nuclei are contracted and pyknotic and cell cytoplasm is shrunken (arrows). L = Gastric lumen, P = gastric pit, (×575).

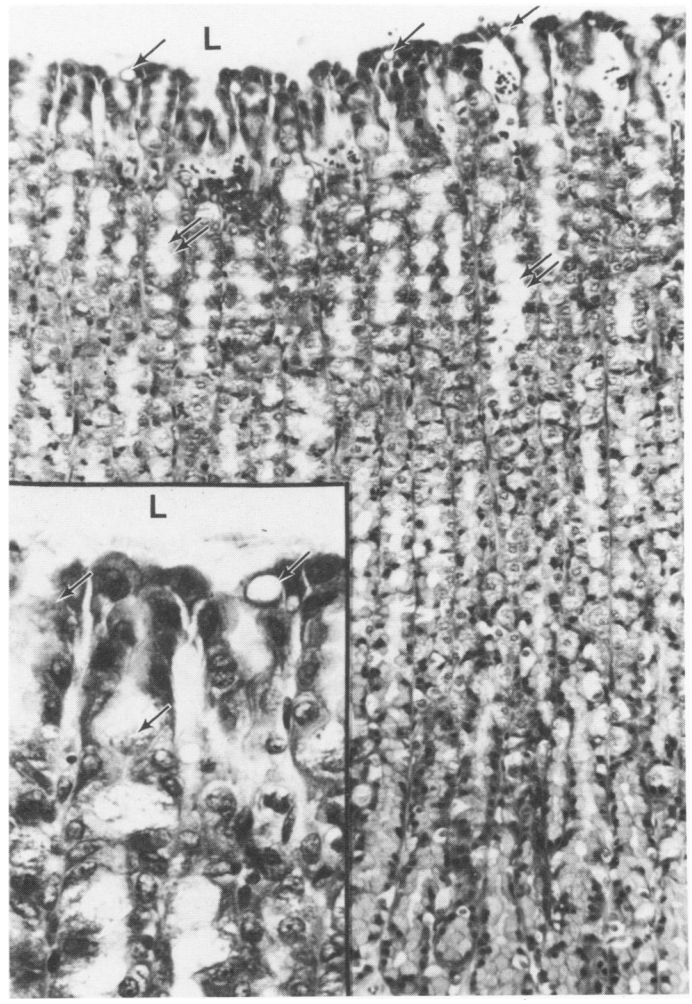

FIG. 4. Light micrograph showing gastric mucosa exposed to 10 μg/kg dm PGE_2 followed 15 minutes later by 100% ethanol. The mucosal histoarchitecture appears relatively undisturbed although the upper gland lumina are dilated (double arrows) and some cells contain large vacuoles (arrows). L = Gastric lumen. Paraffin-embedded, hematoxylin and eosin stain, (×230). (Inset) Higher magnification of a region showing cell injury in surface mucus cells and upper gland cells (arrows). L = Gastric lumen, (×575).

Results of light microscopic evaluation in the various experimental groups are shown in Figure 2. A detailed histologic description of rats exposed to absolute ethanol (EtOH) has been published previously by our laboratory.[13] The current study confirms both the quality and distribution of EtOH damage as detailed in our previous report.[13] Hemorrhagic macroscopic lesions were noted in all control rats pretreated with saline receiving ethanol in the current study. Microscopically, the deeper Types 3 and 4 damage predominated in this group, representing 70.2% and 27.0% of the mucosa, respectively, with no histologic evidence of normal epithelium 5 minutes after EtOH exposure (Fig. 3). Although only 0.2% of mucosa was normal in rats treated with dm PGE_2, the depth of injury was altered significantly, with 72.4% of the mucosa having Type 2 damage (Fig. 4). The amount of Type 3 damage was only 22.8%, and Type 4 damage was completely absent in this group. If the stomachs of rats were pretreated with 20% NAC before EtOH, the distribution of damaged cells was virtually identical to that seen with PG, the predominant injury again being Type 2 (63.3%), with some Type 3 (36.6%); normal cells and Type 4 damage were absent in this group (Fig. 5). NAC and PG in combination proved to be as protective but not more so than either agent given alone (Fig. 6). Macroscopically, the protection seen in these three groups (*i.e.*, PG alone, NAC alone, and PG plus NAC) was reflected by an absence of the gross lesions that were typical in the control rats pretreated with saline and exposed to EtOH.

Discussion

In a previous study from our laboratory,[13] we observed that dm PGE_2, when administered subcutaneously, significantly reduced the depth of gastric injury induced by absolute ethanol in the rat stomach when examined microscopically 5 minutes after exposure to this damaging agent. The current study demonstrates that oral administration of this PG analog is equally efficacious in preventing deep mucosal injury by ethanol. Although the surface epithelium was not spared from injury by dm

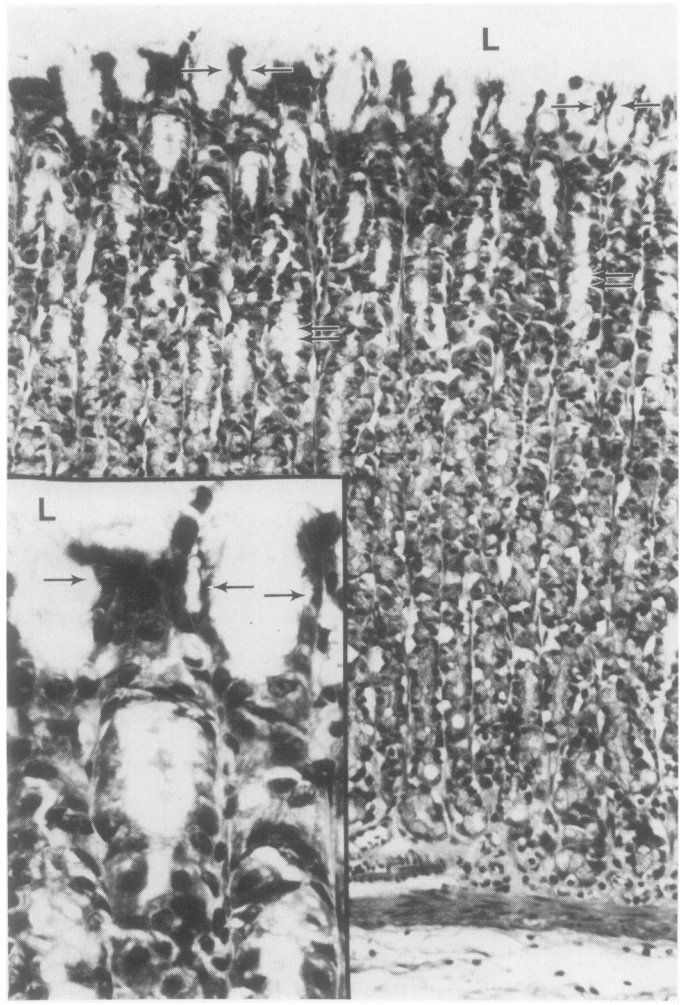

FIG. 5. Light micrograph showing gastric mucosa exposed to NAC followed 15 minutes later by 100% ethanol. The histoarchitecture of the gastric mucosa is minimally affected by the injurious agent. Injury to some surface mucus cells causes the zone between adjacent glands to assume a pinched appearance (arrows). Gastric glands are dilated (double arrows). L = Gastric lumen. Paraffin-embedded, hematoxylin and eosin stain, (×230). (Inset) Higher magnification of the gastric mucosa. Injured surface mucus cells contain pyknotic nuclei and frayed apical cell surfacers (arrows). L = Gastric lumen, (×575).

PGE_2 in either study, confirming the work of other investigators,[15,17,18] the ability of dm PGE_2 in both studies to greatly reduce the depth of injury and virtually eliminate the formation of necrotic lesions commonly seen in alcohol injury reaffirms the remarkable protective properties of the PGs.

Although mucus production has been a popular hypothesis to explain the mechanism by which PGs mediate their protective effects, this study confirms other reports indicating that the thickness of barrier mucus, that component of mucus believed to be important in gastric mucosal defense, either is not altered by PGs, or any observed alterations do not coincide with the presence or absence of mucosal injury. Bolton and colleagues,[8] for example, did not measure any increase in gastric barrier mucus in rats after either oral or intravenously administered natural PGE_2 or the prostaglandin analogs 15-methyl PGE_2 and dm PGE_2. Similarly, Robert and associates[7] noted no enhancement of mucus gel thickness with oral administration of natural PGE_2 or synthetic dm PGE_2 in the rat even though both of these agents prevented macroscopic ethanol injury. Although McQueen et al.[5] found that intragastric dm PGE_2 induced an increase in mucus gel thickness as measured micrometrically by inverse microscopic viewing before exposure to various damaging agents, they concluded that the physical dimensions of the pH gradient provided by this mucus gel and the relatively unimpeded permeability to agents such as aspirin, ethanol, and hydrogen ion were insufficient deterrents in preventing damage to underying cells, despite the fact that the mucus layer appeared to remain intact. Further, LaMont et al.[4] observed protection against alcohol-induced damage in the rat with both a high and low dose of oral natural $PGF_{2\beta}$, but could demonstrate an increase in barrier mucus only with the higher dose. In the same study, these investigators showed that doses of the sulfhydryl agent cysteamine, which afforded protection against ethanol damage, stimulated the release of mucin glycoproteins, but that this protection could be reversed by the thiol blocker N-ethylmaleimide without a con-

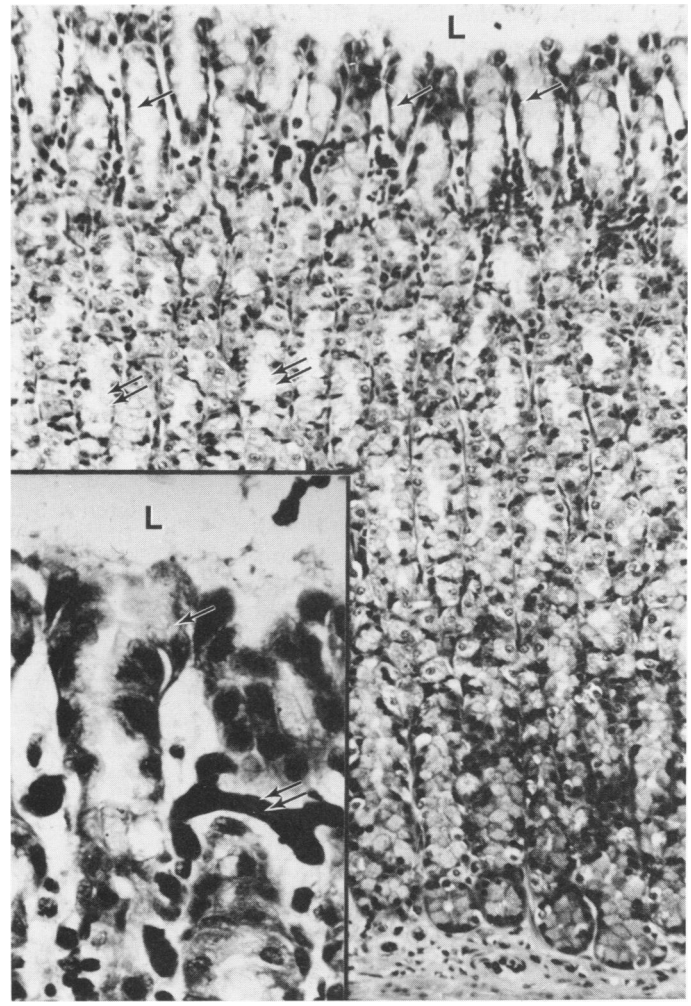

FIG. 6. Light micrograph showing gastric mucosa exposed to dm PGE_2 and NAC followed 15 minutes later by 100% ethanol. Compare with mucosa treated with saline/ethanol in Figure 3. Although the general histoarchitecture of the mucosa is relatively undisturbed, pit surface mucus cells are injured (arrows) and gland lumina (double arrows) are dilated. L = Gastric lumen. Paraffin-embedded, hematoxylin and eosin stain, (×230). (Inset) Higher magnification of a different region of the gastric mucosa. Mucus is seen at the apical zone of some surface mucus cells (arrow). Vasocongestion is also visible in this region (double arrows). L = Gastric lumen, (×575).

comitant decrease in mucus production. These studies raise serious questions concerning the proposed role of barrier mucus as an important mechanism by which mucosal defense is maintained and/or mediated.

In an earlier study, Parke[19] showed that sulfhydryl compounds reduce the disulfide bridges of the insoluble gel form of gastric mucus that is normally adherent to the epithelial surface, thereby converting it into a water-soluble liquid state that is shed into the gastric lumen as free mucus. The most efficient of these mucolytic agents is NAC,[10] making it particularly well suited to define the role of barrier mucus in mediating the defense afforded by various gastroprotective drugs such as PGs. In addition, since NAC belongs to the sulfhydryl family of compounds that have protective effects themselves,[11,12] mucus would not be the likely mediator of defense if NAC was shown to be protective at the same time that it decreased the thickness of barrier mucus. For these reasons, NAC was used as a mucolytic agent in the current study.

Our findings clearly demonstrate that NAC greatly reduces the thickness of barrier mucus at a time when its maintenance should be vital if it does indeed play a major role in mucosal protection. The fact that the protective effects of dm PGE_2 on preventing deep mucosal injury were unaltered despite concomitant treatment with NAC seriously questions an important role for barrier mucus in this protection. Of equal significance was the observation that NAC by itself could provide a degree of protection against alcohol injury that was not significantly different from that observed with dm PGE_2. Interestingly, NAC has also been shown to be protective against aspirin damage independent of mucus thickness.[3] Using a pylorus-ligated rat model, Bottcher et al.[3] instilled aspirin and different concentrations of NAC simultaneously into the stomach, and after 2 hours saw a dose-related lesion reduction by NAC accompanied by a reduction of gel mucus thickness by the higher doses. In fact, there was an observed increase in barrier mucus in aspirin-damaged stomachs of control rats, suggesting more of a correlation between mucus thickness and damage than with the pres-

ervation of mucosal integrity in rats treated with NAC. This study, as well as our demonstration of the protective effects of both dm PGE_2 and NAC against ethanol damage, argue strongly against any primary beneficial effects of mucus in mediating protection.

Since a number of investigators have shown that mucus release often follows exposure to a damaging agent, a more likely role for mucus might be as a protective covering to inhibit further deterioration of focally injured areas. In rats with visible erosions induced by ethanol, McQueen et al.[5] described replacement of the normal gel by a mixture of damaged tissue and mucus at the lesion site, and suggested that the mucus layer acted as a "protective environment" to restrict contact with luminal acid and pepsin and thereby delay further damage during the repair period. Morris et al.[6] made a thorough study of the morphologic features of the extracellular mucus coat in the rat under control conditions and after exposure to aspirin and isobutyric acid. Five minutes after contact with these damaging agents, they detected a massive release of glycoprotein from damaged and exfoliated surface epithelial cells as well as accelerated mucus exocytosis by intact foveolar and isthmic cells. This gelatinous mass mixed with cell remnants was maintained over sites of erosion by an overlying fibrous mucus network while the lesion was being resurfaced, and then dispersed as the reepithelialization process was completed,.

Current evidence indicates, then, that gastric mucus is not of primary importance as one of the mechanisms whereby protective agents mediate their preservation of gastric mucosa against deep necrotic injury after exposure to a damaging agent such EtOH. This protection of deeper cell layers from alcohol injury by dm PGE_2[13,15,17] has been confirmed in the current study and it has been shown also that NAC is equally as effective as dm PGE_2 in this experimental situation. Although it is not known if these two drugs have a common mechanism of action, decreased levels of endogenous sulfhydryls have been associated with tissue damage by various chemical agents.[11,12] Pretreatment with dm PGE_2 prevents this depletion, as well as increases levels of tissue sulfhydryls.[11,12] In conclusion, maintenance of a mucus gel barrier is probably not a primary mechanism of protection, although it may be an important means of delaying further damage, providing time and a proper environment for healing of an injury that has already ocurred.

References

1. Miller TA. The protective effects of prostaglandins against gastric mucosal damage: current knowledge and proposed mechanisms. Am J Physiol 1983; 245:G601–623.
2. Allen A, Garner A. Progress report: mucus and bicarbonate secretion in the stomach and their possible role in mucosal protection. Gut 1980; 21:249–262.
3. Bottcher W, Golanska EM, Kauffman GL Jr. N-acetyl-cysteine affords gastric mucosal protection while reducing gel mucus thickness. Surg Forum 1982; 33:164–166.
4. Lamont JT, Ventola AS, Maull EA, Szabo S. Cysteamine and prostaglandin $F_{2\beta}$ stimulate rat gastric mucin release. Gastroenterology 1983; 84:306–313.
5. McQueen S, Hutton D, Allen A, Garner A. Gastric and duodenal surface mucus gel thickness in rat: effects of prostaglandins and damaging agents. Am J Physiol 1983; 8:G388–393.
6. Morris GP, Harding RK, Wallace JL. A functional model for extracellular gastric mucus in the rat. Virchows Arch (Cell Pathol) 1984; 46:239–251.
7. Robert A, Bottcher W, Golanska E, Kauffman GL Jr. Lack of correlation between mucus gel thickness and gastric cytoprotection in rats. Gastroenterology 1985; 86:670–674.
8. Bolton JP, Palmer D, Cohen MM. Stimulation of mucus and nonparietal cell secretion by the E_2 prostaglandins. Dig Dis 1978; 23: 359–364.
9. Bickel M, Kauffman GL Jr. Gastric gel mucus thickness: effect of distention, 16,16 dimethyl prostaglandin E_2, and carbenoxolone. Gastroenterology 1981; 80:770–775.
10. Sheffner AL. The reduction in vitro in viscosity of mucoprotein solutions by a new mucolytic agent, N-acetyl-L-cysteine. Ann NY Acad Sci 163; 106:298–310.
11. Szabo S, Trier JS, Frankel PW. Sulfhydryl compounds may mediate gastric cytoprotection. Science 1981; 214:200–202.
12. Miller TA, Li D, Kuo Y-J, et al. Nonprotein sulfhydryl compounds in canine gastric mucosa: effects of PGE_2 and ethanol. Am J Physiol 1985; 12:G137–144.
13. Schmidt KL, Henagan JM, Smith GS, et al. Prostaglandin cytoprotection against ethanol-induced gastric damage in the rat: a histologic and cytologic study of the surface epithelium. Gastroenterology 1985; 88:649–659.
14. Corne SJ, Morrissey SM, Woods RJ. A method for the quantitative estimation of gastric barrier mucus. J Physiol 1974; 242:116–117.
15. Guth PH, Paulsen G, Nagata H. Histologic and microcirculatory changes in alcohol-induced gastric lesions in the rat: effect of prostaglandin cytoprotection. Gastroenterology 1984; 87:1083–1090.
16. Karnovsky MJ. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 1965; 27: 137A–138A.
17. Lacy ER, Ito S. Microscopic analysis of ethanol damage to rat gastric mucosa after treatment with a prostaglandin. Gastroenterology 1983; 83:619–625.
18. Wallace JL, Morris GP, Krausse EJ, Greaves SE. Reduction by cytoprotective agents of ethanol-induced damage to the rat gastric mucosa: a correlated morphologic and physiologic study. Can J Physiol Pharmacol 1982; 60:1686–1699.
19. Parke DV. Pharmacology of mucus. Br Med Bull 1978; 34:89–94.