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The effects of 16,16-dimethyl prostaglandin E_2 + aspirin on the canine gastric mucosal barrier

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Summary. The canine gastric epithelium was exposed to solutions containing 20 mM aspirin and 20 mM aspirin + 30 μg/kg 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$) for periods of three and forty minutes. No macroscopic hemorrhagic lesions were seen. Light microscopically, surface lesions were reduced from 10 percent (aspirin alone) to 2.5% (aspirin + $dmPGE_2$). However, $dmPGE_2$ does not appear to attenuate aspirin induced tight junction alterations. Discontinuities in the apical occluding complexes, hyperplastic tight junctions and stand number variability were documented in freeze frature replicas of aspirin as well as aspirin + $dmPGE_2$ treated dog stomachs. The results of these experiments would seem to suggest that 30 μg/kg $dmPGE_2$ does not prevent aspirin induced damage to the tight junctions of the canine gastric epithelium or enhance their repair.

Key words: 16,16-dimethyl prostaglandin E_2 – Aspirin – Tight junctions – Stomach – Ultrastructure

Introduction

Occluding tight junctions between gastric epithelial cells prevent the paracellular passage of potentially damaging substances from the stomach lumen. As such, they represent a major morphological component of the gastric mucosal barrier. Previous thin section electromicroscopic studies on the effect of ulcerogens on gastric tight junctions have suggested that they remain morphologically intact following exposure to a variety of agents which result in severe disruption of the epithelium (Eastwood 1975; Eastwood 1985). No thorough freeze fracture studies of a similar nature have, to our knowledge, been performed. Prostaglandins have been shown to reduce the gastric injury caused by so called "barrier breakers" (i.e., aspirin, ethanol, bile salts) in man and a variety of laboratory animals (Robert 1976; Robert 1979; Konturek 1982; Miller 1983). One of the proposed mechanisms by which prostaglandins are thought to exert their protective effects is their ability to sustain transmucosal potential differences (Miller 1983). Since tight junctions are intimately involved in the generation and maintenance of transmucosal resistance, we have investigated, using the freeze fracture technique, the effects of aspirin and aspirin + $dmPGE_2$ on the tight junctions of the canine gastric epithelium. The results of this investigation which pertain to aspirin + $dmPGE_2$ treatment are described in this paper; data relating to the normal structure of the tight junctions of the canine gastric mucosa, and the effects of aspirin exposure have been reported in detail elsewhere (Meyer et al. 1984, Meyer et al. 1986).

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Materials and methods

Animals treatments (tap water, aspirin alone, and aspirin + $dmPGE_2$) were performed in a random manner. The duration of each treatment was either three for forty minutes. A total of eighteen mongrel dogs of both sexes ranging in weight from 14 kg to 20 kg were used in these experiments. Six dogs were placed in each treatment category, three of which were treated for three minutes, while the remaining three received forty minute exposures. The dogs, which had been fasted overnight, were anesthetized and a midline incision was made. The pylorus was tied and a gastric tube placed at the gastroesophageal junction. Test solutions were introduced and the gastric tube removed. The treatments consisted of 2 Bayer aspirin (Glenbrook Laboratories, New York, NY, USA) (650 mg) dissolved in 180 ml water (20 mM) and 2 Bayer aspirin + 30 μg $dmPGE_2$ (Upjohn Diagnostics, Kalamazoo, MI, USA) per kg of the dog's weight at a final pH of 3. Control animals were given 180 ml of tap water. After the proper treatment period the stomachs were rapidly removed and the dog sacrificed. The stomachs were filled with 180 ml of fixative containing 2% glutaraldehyde, 3% paraformaldehyde in 0.1 M cacodylic acid with 0.1 M sucrose at pH 7.2. After fixation each stomach was cut along

the greater curvature. None of the treatments resulted in grossly visible damage to the stomach. Pieces were sampled from three areas: the fundic region, the body of the stomach and the pyloric antrum (Meyer et al. 1986). The specimens were submerged in a 30% glycerol-fixative solution for at least 1 h, then frozen in Freon 22 cooled by liquid nitrogen. The samples were fractured and replicated −110° C in a Balzers 301 freeze-etch machine (Balzers High Vacuum Corporation, Santa Ana, CA, USA) equipped with a Pt gun and quartz-crystal monitor. Replicas were cleaned in dimethylformamide, then full strenght bleach with 0.2 N NaOH. The replicas were rinsed in double distilled water and examined in a JEOL 100B electron microscope (JEOL USA, Peabody, MA, USA) at 80 KV.

All morphologic measurements were done by the same person (R.M.) on coded tissue samples and without knowledge of tissue source. For freeze fracture quantitation at least two freeze fracture runs were done producing a minimum of 8 replicas per stomach area; 24 replicas per dog. The replicas were examined and large fractured areas of membrane with a tight junction complex present (defined as an interface, IF) were photographed and printed at a final magnification of 40000. For each tight junction complex, the average depth and average number of horizontal strands were recorded. The average depth of the tight junction complex was determined by measuring the distance between the upper-most and lower-most strands (excluding aberrant loose strands) both at the center and one-fourth of the distance from each end of the exposed tight junction complex. The number of strands on the tight junction complex was counted by use of an overlay that consisted of a line parallel to the microvillar surface and lines perpendicular to this line every 0.5 μm. The intersection of these perpendicular lines with the tight junction strand was counted and averaged using a point-counting program in an image analysis system. The ladder-like extensions of tight junctions occurring at boundaries where three cells form junctions were excluded from these measurements. The total length of the occluding junctional complex parallel to the apical cell surface in a given micrograph was also measured with a Apple II computer equipped with a Hipad digitizing tablet. The data were analyzed by the student's *t*-test. A probability value of <0.05 was considered statistically significant.

Pieces of the stomachs fixed for freeze fracture were also examined by light microscopy. The samples were cut into 5 mm × 5 mm × 2 mm pieces. They were dehydrated in a graded series of alcohol and embedded in JB-4 plastic (Polysciences). Semithin section (0.2–0.5 μm) from 5–6 blocks for each area of each dog were cut and stained with hematoxylin and eosin or PAS (periodic acid-Schiff). Measurements of the length of the gastric muscosa and length of damage were made with a ocular micrometer, at 250 × on a Zeiss microscope (Carl Zeiss, Inc., Thornwood, NY, USA). At least 100 mm of surface epithelium was measured per dog.

Results

Light microscopy

The normal appearance of the surface epithelium of the canine gastric mucosa consists of a stratum of columnar cells with an overlying layer of extracellular mucous. No macroscopic hemorrhagic gastric lesions were seen when 20 mM aspirin and 20 mM aspirin + 30 μg/kg $dmPGE_2$ were administered to dogs for three and forty minute periods. Light microscopic damage in both groups was focal in nature and typically characterized by the mushroom-like swelling of small groups (3–10) of injured and necrotic surface epithelial cells (Fig. 1). Cells in the gastric pits and glands were not noticably impaired and complete erosion of the gastric mucosa was not observed. Quantitative measurement revealed that aspirin + $dmPGE_2$ damaged 2.53% of the surface epithelium examined (Table 1). This represented a significant reduction ($P<0.01$) in the amount of mucosal damage when compared with animals that were given aspirin alone (10.6%). There was no significant difference in the amount of mucosal damage observed when animals were exposed to aspirin + $dmPGE_2$ for three and forty minutes (Table 1).

Freeze fracture

The occluding junctions between surface mucous cells of the normal canine stomach are composed of five to eight strands which are interwoven into a honeycomb configuration (Fig. 2). Normal gastric gland cell tight junctions, in contrast, usually contain five to six strands arranged in a continuous parallel pattern. It was not possible to consistently distinguish between the various types of gastric gland cells in freeze fracture preparations. However, very little variation in tight junction strand number was observed in normal gastric glands (5.7 ± 0.1). Following administration of 20 mM aspirin and 20 mM aspirin + 30 μg/kg $dmPGE_2$ a number of abnormalities were seen: a) focal discontinuities in the occluding tight junction complex (Figs. 3, 4), b) variability in tight junction strand number, c) disorganization of strands in the tight junction complex, including tight junction hyperplasia (Figs. 5, 6). Exposure of the gastric mucosa to aspirin + $dmPGE_2$ for forty minutes did not significantly increase the number of tight junctional discontinuities between surface epithelial cells when compared to those treated for three minutes (Table 2). There was, however, an unexplained reduction in the number of tight junctional discontinuities between gland cells in specimens examined after the 40 min exposure period. Hereafter, in this section, the data of the two exposure periods to aspirin + $dmPGE_2$ will be expressed in a combined fashion (Table 3).

No discontinuities, interruptions in the occluding complexes, in the tight junction complexes of surface and glandular cells were seen in control animals (Fig. 7). There was no significant difference in the frequency of tight junction discontinuities between animals treated with aspirin and those treated with aspirin + $dmPGE_2$ (Fig. 7). There was,

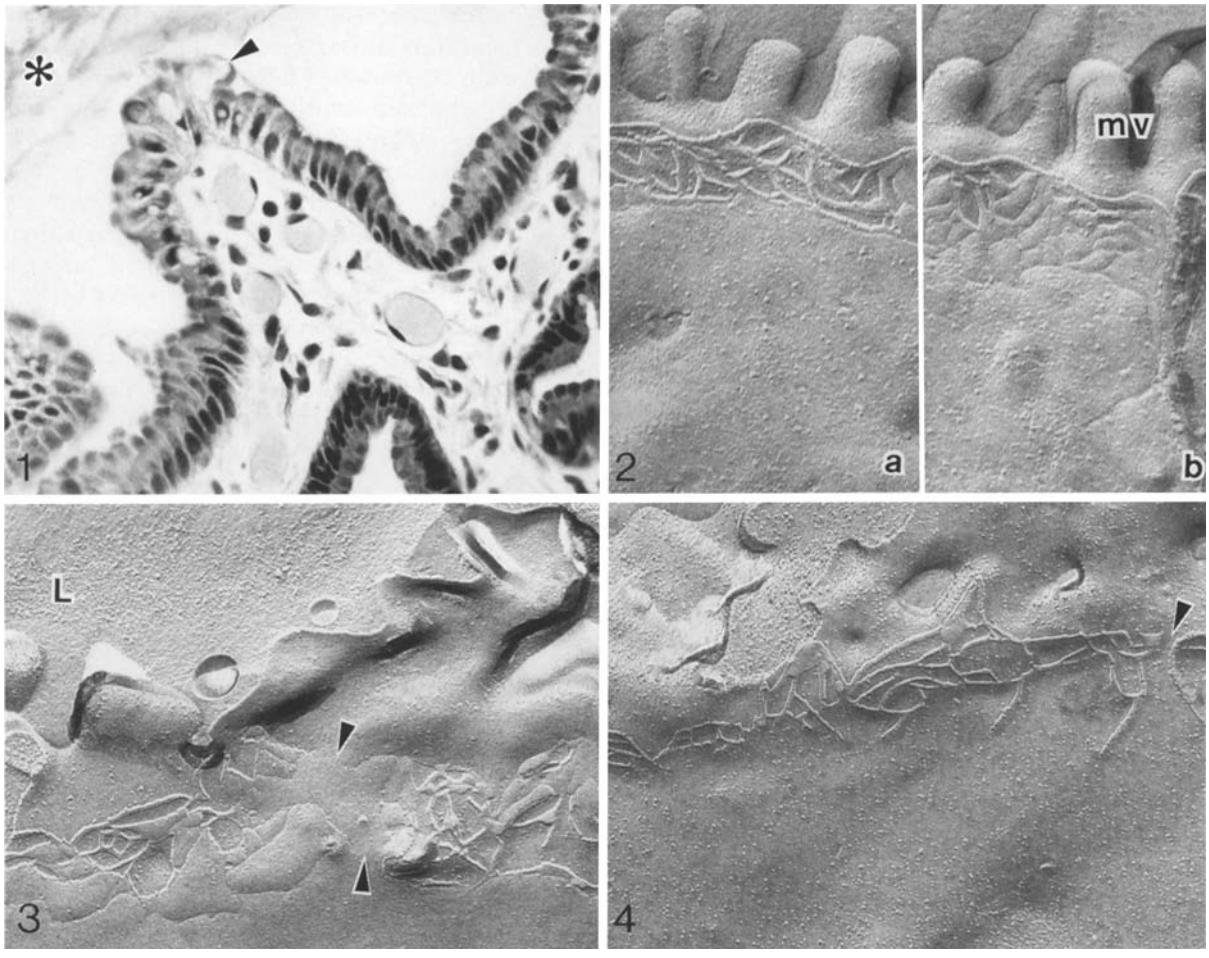

Fig. 1. A focal area of damaged surface epithelial cells (*arrowhead*) is illustrated with cells mushrooming into the gastric lumen (pale staining cells) flanked by undamaged cells. A layer of mucous (*asterisks*) covers the surface of the epithelium. Aspirin (*ASA*) + dmPGE$_2$ treated dog. Magnification × 100

Fig. 2. Normal surface mucous cells are characterized by apical tight junction complexes consisting of a meshwork of interwoven strands (E fracture face; **a**) and grooves (P fracture face; **b**). Microvilli (*MV*) project in to the gastric lumen. Control (H_2O) treated dog. Magnification × 38700

Fig. 3. This freeze-fracture electron micrograph illustrates a surface epithelial cell tight junction complex discontinuity (*arrowhead*) and mushrooming of the apical cell surface into the stomach lumen (*L*). ASA + dmPGE$_2$ treated dog. Magnification × 37500

Fig. 4. A gastric gland tight junction complex discontinuity (*arrowhead*) is seen along with variability in complex strand number (1–5 strands). ASA + dmPGE$_2$ treated dog. Magnification × 37900

however, a significant difference in the number of discontinuities between surface epithelial cells and gland area cells in animals treated with aspirin and aspirin + dmPGE$_2$. In animals treated with aspirin + dmPGE$_2$ discontinuities (Figs. 3, 4) were encountered on 42% (48% aspirin alone) and 35% (20% aspirin alone) of surface epithelial and glandular cell plasma membrane interfaces respectively. In other words, when animals were treated with aspirin + dmPGE$_2$ a discontinuity was noted for every 10.5 μm of surface (7.9 μm aspirin alone) and every 17.5 μm (21 μm aspirin alone) gland epithelium tight junction complex examined.

There was no significant difference in the number of strands in the tight junctional complexes of animals treated with aspirin + dmPGE$_2$, those treated with aspirin alone, and control animals, however, considerable variability in strand numbers was seen. The mean number of strands in the tight junction complexes of aspirin + dmPGE$_2$ was 7.2 ± 0.3 for the surface epithelium (6.8 ± 0.9, aspirin; 5.8 ± 0.2, control), and 6.2 ± 0.3 for the glandular epithelium (6.2 ± 0.4, aspirin; 5.7 ± 0.1, control). Strand number measurements varied from 0–20 for aspirin + dmPGE$_2$ and aspirin treated animals compared to 4–9 for control

Table 1. Length of gastric mucosal sections examined for damage

Treatment	No of Animals	Total length of mucosa Measured (mm)	Total length Damaged (%)
Control (H_2O)[a]	9	347.9	0.56
Aspirin[b]	9	1244.7	10.6[c]
Aspirin + dmPGE$_2$	6	1268	2.53[d]

[a] Meyer et al. (1984)
[b] Meyer et al. (1986)
[c] With a student *t*-test – ASA compared to dmPGE$_2$ + ASA, $P = 0.01$
[d] Percent of mucosa damaged per experimental animal: 3 min (1.5, 2.7, 3.4); 40 min (0.25, 1.6, 3.4)

animals. Three percent of surface and glandular plasma membrane interfaces in aspirin + dmPGE$_2$ treated samples contained tight junction complexes in which the barrier was reduced to a single strand compared to six percent in aspirin treated animals.

Disorganization of tight junction complexes in treated (aspirin and aspirin + dmPGE$_2$) specimens represents a continuum of defects which range from focal strand disorientation to the uncontrolled proliferation of tight junction elements. Disorientation was characterized by deviations from either the parallel pattern exhibited by normal gland cells or the honeycomb configuration of normal surface epithelial cells. Another, more obvious, divergence from normal structure was the

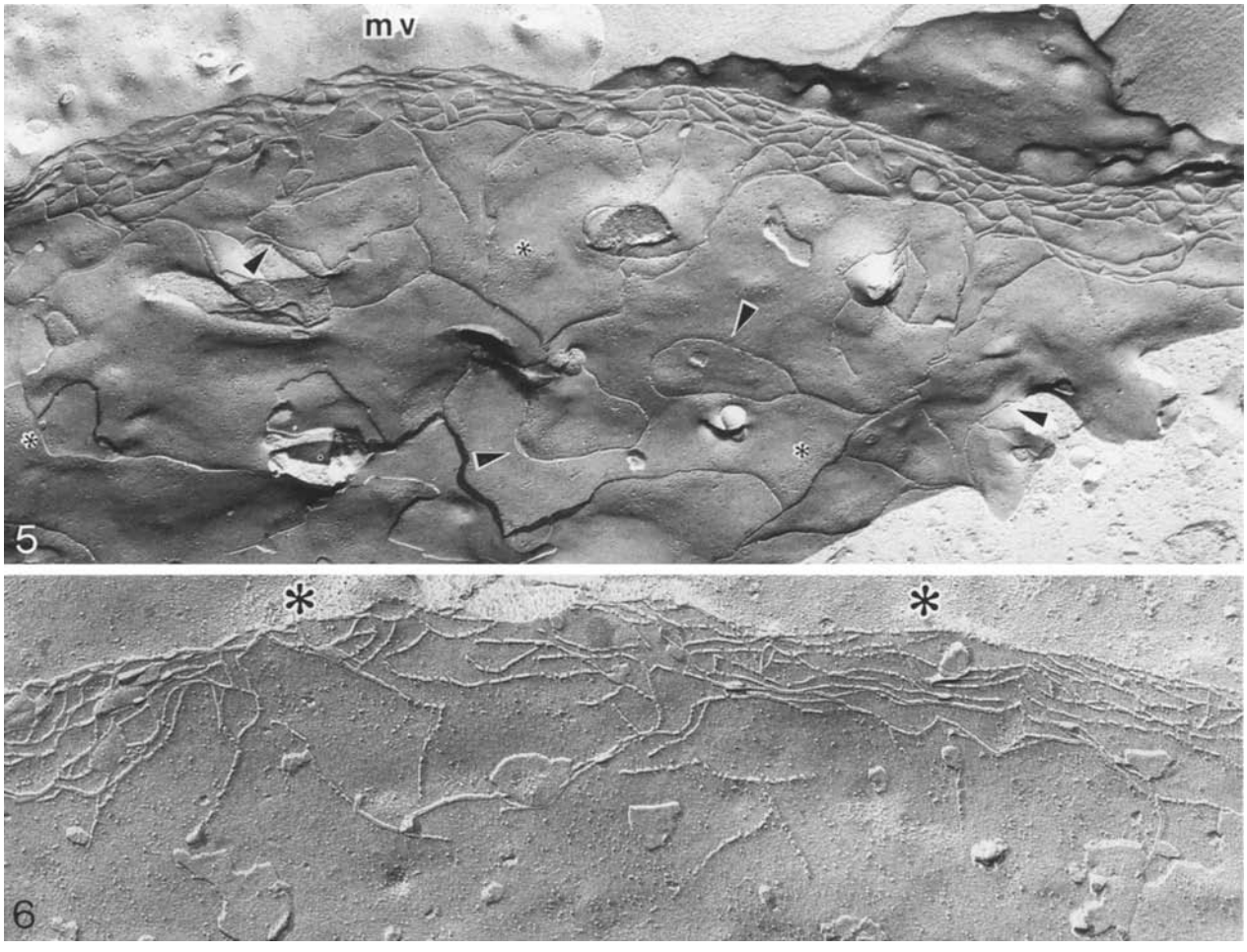

Fig. 5. Hyperplastic tight junctions (extensions of the apical tight junction strands radiating over the lateral plasma membrane (*arrowheads*)) is illustrated on a surface epithelial cell. A decrease in the number and length of microvilli (*MV*), swelling of apical plasma membrane, and clustering of intramembranous particles (*asterisks*) are also seen. ASA + dmPGE$_2$ treated dog. Magnification × 27500

Fig. 6. A surface epithelial tight junction complex with a wide variability in strand number (1–9) and an increase in the pariculate nature of many of the strands is seen. A loss of surface microvilli on the cell is also noted (*asterisks*). ASA + dmPGE$_2$ treated dog. Magnification × 33800

Table 2. Freeze-fracture quantitation of the tight junction complexes

	No. of interfaces[a]	Total Length of occluding junctions measured (μm)	No. of discontinuities in complex (% total IFs)	Average distance between discontinuities in the complex (μm)
3 min ASA + dmPGE$_2$[b]				
Gland area cells	346	2392	141 (35)	16.9
Surface cells	333	1906	220 (49)	8.9
40 min ASA + dmPGE$_2$				
Gland area cells	315	1706	93 (35)	118.3
Surface cells	343	1885	141 (26)	13.4

[a] Interface (IF) is defined as 58 μm^2 of fractured membrane surface, containing tight junctional elements, that has an indication of another cell present
[b] Three animals per exposure period

Table 3. Freeze-fracture quantitation of the tight junction complexes

	No. of interfaces[a]	Total Length of occluding junctions measured (μm)	No. of discontinuities in complex (% total IFs)	Average distance between discontinuities in the complex (μm)
Control (H_2O)[b]				
Gland area celles	304	1431	0	
Surface cells	344	1729	0	
Aspirin[c]				
Gland area cells	957	5637	269 (20)	21
Surface cells	1075	5254	668 (48)	7.9
Aspirin + dmPGE$_2$ (times combined)				
Gland area cells	661	4098	234 (31)	17.5
Surface cells	676	3791	361 (42)	10.5

[a] Interface (IF) is defined as 58 μm^2 of fractured membrane surface, containing tight junctional elements, that has an indication of another cell present
[b] Meyer et al. (1984)
[c] Meyer et al. (1986)

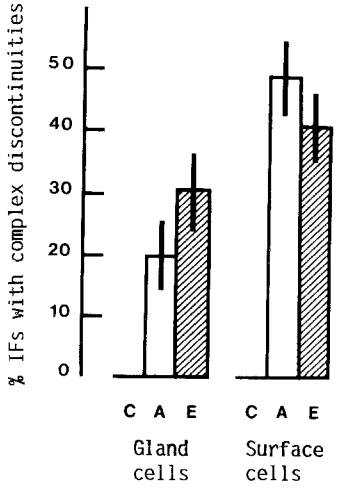

Fig. 7

presence of free-ending basal extensions of tight junction strands. Finally, tight junction hyperplasia, which appeared to be the result of unrestrained and unoriented tight junction growth, was perhaps the most unusual abnormality seen in this study (Fig. 5). There was no significant difference between aspirin and aspirin + dmPGE$_2$ treated animals with regard to the presence of hyperplastic tight junctions. In aspirin + dmPGE$_2$ samples hyperplastic tight junctions were seen on 25% (29%, aspirin alone) of surface epithelial and 12% (12%, aspirin alone) of glandular epithelial plasma membrane interfaces.

Necrotic cells, those which would be expected to exhibit extensive clustering of intermembranous particles, were very rarely observed in freeze fracture preparations. This leads us to believe that these cells must have been perferentially cross fractured.

Discussion

Morphologically aspirin results in two types of gastric epithelial damage: (1) lethal injury, which is characterized by the obvious signs of cell necrosis, is an irreversible process, and can be adequately documented by light microscopy, and 2) sublethal injury, which involves alterations in the structure of the tight junctions between gastric epithelial cells, is potenitally reversible, and can only be demonstrated using the freeze fracture technique. The primary purpose of this study was to examine the effect of $dmPGE_2$ on sublethal, aspirin induced injury to the canine gastric mucoa. Our experiments have shown that simultaneous oral administration of non-antisecretory doses of $dmPGE_2$ reduces light microscopic, aspirin induced damage to the surface epithelium of the canine gastric mucosa. At the ultrastructural level $dmPGE_2$ does not appear to prevent alterations of the tight junctions complexes of the gastric surface and glandular epithelium caused by aspirin exposure or enhance their repair. As was mentioned in the introduction, earlier thin section electronmicroscopic studies reported that gastric tight junctions remain intact after treatment with, among other substances, aspirin. Our results illustrate the necessity of using the freeze fracture technique when attempting to characterize the status of tight junctions following experimental manipulation.

Many PG's, and their synthetic analogs, have been shown to protect the gastric mucosa of numerous species from the action of noxious agents administered under a variety of experimental conditions. For example, PG's have been used in a wide range of concentrations, applied before or along with necrotizing agents, given orally, parenterally, and subcutaneously, with and without ligation of the pylorus. Additionally, injurious substances, with differing potencies, have also been employed at various concentrations. Diverse criteria have also been used to assess gastric damage, these include gross observation, light and electronmicroscopic examination and physiological measurement. However, despite these somewhat dissimilar approaches, the vast majority of these studies, including the present report, have reached the same conclusion; PG's decrease the severity of experimentally induced gastric injury. Thus, our experimental design has produced results that were similar, at the light microscopic level, to most other studies. However, further investigation using ultrastructural techniques revealed that tight junction abnormalities were almost identical to those seen when the canine stomach was treated with aspirin alone.

Our choice of dogs as experimental animals in this investigation was based on the fact that PGE's are the predominant prostaglandin found in the canine gastric mucosa. This situation is very similar to that which exists in the human stomach. In the rat gastric mucosa PGI_2 is the principal prostaglandin and therefore studies in this species may yield results that are not relevant to humans (Rainsford et al. 1980). Aspirin was selected as a gastric irritant because PGE has been shown to protect the surface epithelial cells of the stomach against the damaging effects of this agent (Ohno et al. 1985). In contrast, while low PGE doses will reduce macroscopic mucosal lesions caused by ethanol (Ohno et al. 1985; Lacy and Ito 1982), a hundred fold greater dose is required to decrease surface epithelial cell loss (Whittle et al. 1985). Our decision to use 30 μg/kg $dmPGE_2$ was based on a previous SEM study in which this nonantisecretory dose was shown to protect the gastric mucosa from aspirin induced injury (Ohno et al. 1985). This same study also demonstrated that $dmPGE_2$, by itself, in doses of 3–100 μg/kg caused no morphologic changes (Whittle et al. 1985). Additionally, Weinstein et al. (1986) using the freeze fracture technique, have established that a synthetic analog of PGE, (misoprostol) in doses of 50–100 μg/kg does not alter gastric epithelial tight junction structure (Weinstein et al. 1986).

Prevention of gastric mucosal barrier disruption is among the many mechanisms by which PG's are thought to exert their protective effect. Support for this theory comes mainly from physiological experiments which show that PG's forestall the typical aspirin associated drop in transmucosal potential difference (Miller 1983). However, such results are difficult to interpret in light of our observations which demonstrate that $dmPGE_2$ reduces, but does not eliminate aspirin induced light microscopic lesions and has little or no attenuating effects on the disruption of gastric tight junction complexes. Thus, it may be that other factors stimulated by $dmPGE_2$ may be acting to increase the resistance of the gastric mucosal barrier (Miller 1983; Rowe et al. 1985; Hawkey and Rampton 1985).

An explanation for the maintenance of the integrity of the gastric mucosal barrier by PG's following aspirin treatment may involve changes in the mucous component of the surface epithelium. Mucous has traditionally been regarded as a substance which prevents mechanical cell injury by acting as a lubricant. As a result, tight junction complexes, which have become more or less synonymous with barrier systems, have often been given

primary attention when questions of altered permeability are investigated. In view of the results of this experiment and other evidence, the role of mucous in cytoprotection should perhaps be re-evaluated. Recent investigations have shown that gastric mucous in combination with bicarbonate secretion may provide a barrier to the movement of hydrogen ions from the gastric lumen to the surface epithelium (Allen and Garner 1980; Allen 1981; Thomson 1981). Prostaglandins have been demonstrated to elicit a rapid incraese in mucous and nonparietal cell alkaline (e.g. bicarbonate) secretion (Miller 1983). Furthermore, this response occurs within a time frame that could deter the destructive action of damaging substances such as aspirin. Based upon the results of the experiments cited above, and others (Morris and Harding 1984) the potentially cytoprotective capacity of mucous in our experiments might be envisioned as follows: 1) blatant gaps in the mucosa caused by cell necrosis would be occluded by mucous released from the damaged surface epithelial cells; a highly localized "mixing barrier" would thus be formed during which re-epithelialization and re-establishment of the mucosal barrier could occur (Morris and Harding 1984); 2) a general increase in mucous thickness stimulated by PG's would prohibit the paracellular passage of hydrogen ions through discontinuites in the tight junctions between surface epithelial cells which have sustained sublethal injury and allow time for their repair.

In summary, we have established the fact that gastric tight junction abnormalities caused by aspirin persist even in the presence of exogenously applied dmPGE$_2$. In addition, light microscopic necrotic lesions, though reduced, were still noted. Thus, two important cellular components of the gastric mucosal barrier, namely an intact epithelium and occluding tight junction complexes, remain impaired. Therefore, it would seem to be necessary to propose another factor, including perhaps extracellular mucous, in order to explain the ability of PG's to sustain transmucosal potential differences following aspirin exposure.

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