Role of gastric blood flow, neutrophil infiltration, and mucosal cell proliferation in gastric adaptation to aspirin in the rat

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Abstract

Gastric mucosa exhibits the ability to adapt to ulcerogenic action of aspirin but the mechanism of this phenomenon is unknown. In this study, acute gastric lesions were produced by single or repeated doses of acidified aspirin in rats with intact or resected salivary glands and with intact or suppressed synthase of nitric oxide. A single oral dose of aspirin produced a dose dependent increase in gastric lesions accompanied by considerable blood neutrophilia and mucosal neutrophil infiltration, significant reduction in gastric blood flow, and almost complete suppression of biosynthesis of prostaglandins. After rechallenge with aspirin, the mucosal damage became smaller and progressively declined with repeated aspirin insults. Gastric adaptation to aspirin was accompanied by a significant rise in gastric blood flow, reduction in both blood neutrophilia and mucosal neutrophil infiltration, and a remarkable increase in mucosal cell regeneration and mucosal content of epidermal growth factor. Salivectomy, which reduced the mucosal content of epidermal growth factor, aggravated the initial mucosal damage induced by the first exposure to acidified aspirin but did not prevent the adaptation of this mucosa to repeated aspirin insults. Pretreatment with N^G-nitro-L-arginine (L-NNA), a specific inhibitor of nitric oxide synthase, eliminated the hyperaemic response to repeated aspirin but did not abolish the development of adaptation to aspirin showing that the maintenance of the gastric blood flow plays little part in this adaptation. In conclusion, the stomach adapts readily to repeated aspirin insults and this is accompanied by a considerable reduction in blood neutrophilia and the severity of neutrophil infiltration and by an extensive proliferation of mucosal cells possibly involving epidermal growth factor.

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Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, are very useful in treating painful inflammatory conditions but gastropathy associated with the use of these drugs is the single most commonly reported adverse reaction to this drug.[1]

The pathogenesis of NSAID gastropathy is poorly understood but it has been causally linked to the topical irritation of the mucosa as well as to the inhibition of prostaglandin biosynthesis.[2–5] Prostaglandins exhibit potent protective effects against various types of mucosal damage including that induced by NSAIDs[6] and the inhibition of endogenous prostaglandins considerably increases the susceptibility of gastrointestinal mucosa to injury.[7] Recently, vascular aetiology for NSAID gastropathy has been proposed[7] by the demonstrable ability of these drugs to cause vascular endothelial damage,[8–10] neutrophil activation within the mucosa,[11] and decrease in mucosal blood flow.[10–12]

It is of interest that with more prolonged administration of NSAID in rats[13] and in humans,[14 15] the tolerance or adaptation to ulcerogenic action of these drugs develops in the gastric mucosa. The mechanism of this phenomenon has not been clarified but increased epithelial cell proliferation was noted after two weeks of daily treatment with aspirin in rats.[16] In humans, the mucosal recovery during continued intake of indomethacin was accompanied by an increase in the mucosal blood flow.[17] Thus, increased cell proliferation and enhanced gastric blood flow were proposed to contribute to the development of gastric adaptation but neither the time course of this phenomenon nor its relation to mucosal blood flow and cell proliferation have been established.

In this study, we used as a model the formation of severe gastric erosions produced in fasted rats by intragastric administration of acidified aspirin. We investigated the time course and the duration of gastric adaptation to aspirin and its possible mechanisms, particularly the role of mucosal blood flow, proliferation of mucosal cells, and the participation of epidermal growth factor and nitric oxide biosynthesis.

Methods

ANIMALS, ASPIRIN TREATMENT, AND MEASUREMENTS

Female Wistar rats of 180–220 g body weight were used. The animals were fasted overnight in individual cages. Aspirin was dissolved in 1 ml of 0·2 N HCl and given intragastrically in various doses (12·5–200 mg/kg) but in most experiments a standard dose of 100 mg/kg was used. A dose of 100 mg/kg of aspirin was chosen because when dissolved in 0·2 N HCl and given orally it produced submaximal mucosal damage, which could be only slightly further enhanced by increasing the dose of

aspirin to 200 mg/kg. One hour later, the animals were lightly anaesthetised with ether, the stomach was exposed, and the gastric blood flow was measured in the oxyntic portion of the stomach using laser Doppler flowmetry (Laserflo BPM 403A, Vasamedics, St Paul, MN, USA) as described previously.[18] The stomachs were then removed, opened along the greater curvature, and the area of gastric lesions was measured by planimetry (Morphomat, Carl Zeiss, Berlin, Germany) on photographs of the stomach by someone unaware of treatment given. The erosions appeared as black spots because of blood oozing from the lesions. Erosions of at least 1 mm in diameter were considered and the average area of lesions per stomach was calculated for each group of rats. Two standardised longitudinal sections of the mucosa were taken from the gastric corpus, fixed in 10% buffered formalin, and paraffin wax sections were stained with haematoxylin and eosin. The sections of the mucosa were used for quantitative histological evaluation (morphometry) using Nikon microscope equipped with Microplan II. The disrupted surface of the mucosal strips denuded of epithelium, the deeper necrotic lesions penetrating the mucosa, and the strip length with regeneration of cells were measured and expressed as a per cent of total.

In some experiments, the mucosa was scraped from the stomach, weighed, and used for the determination of DNA synthesis, DNA content, prostaglandin E_2 release, and epidermal growth factor content.

The rate of DNA synthesis was determined by incubating the tissues at 37°C for 30 minutes in medium containing 2 μCi of [^{3}H]thymidine (19·3 Ci/mmol, New England Nuclear, Boston, USA). Total DNA content in the gastric mucosa was also determined as described earlier.[19]

For ex vivo determination of prostaglandin E_2, the mucosa was processed, according to the method described previously.[3] Briefly, a portion of corpus mucosa (about 100 mg) was placed in an Eppendorf plastic tube containing 1·0 ml of 10 mM phosphate buffer (pH 7·4) and minced with fine scissors for 15 seconds. The samples were then incubated in a shaking bath (37°C) for 20 minutes after which they were centrifuged (30 seconds, 9000 *g*). The supernatant was frozen and subsequent determination of prostaglandin E_2 was performed by specific radioimmunoassays using prostaglandin E_2 kits (NEN Research Products, NEN Division, D-6072 Dreieich, Germany).

In rats with salivectomy and sham operation, the biopsy samples of oxyntic mucosa were taken for determination of mucosal content epidermal growth factor as described previously.[20 21] Briefly, the tissue samples were weighed and homogenised in ice cold 0·02 mol/l TRIS-HCl buffer and centrifuged, the supernatant being collected and frozen at −20°C until epidermal growth factor radioimmunoassay. The epidermal growth factor antiserum (gift of Dr H Gregory, ICI, Alderly Park, UK), raised in rabbits against human epidermal growth factor was used at a final dilution of 1:210 000 and this antiserum recognised equally rat and human epidermal growth factor. Iodinated ([13-^{125}I] iodotyrosyl) peptide and rat epidermal growth factor were calibration standards (Amersham, UK). The detection limit of the assay was 0·01 nmol/l. The interassay and intra-assay precisions were about 12 and 10%, respectively.

Cellular proliferation was also determined by immunocytochemical staining. Bromodeoxyuridine (BrdU) dissolved in saline (1 ml) was injected intraperitoneally in a dose of 200 mg/kg, 90 minutes before the animals were killed. Cells in DNA synthetic phase incorporate BrdU into their nuclei and this incorporation in proliferating cells can be detected in a single stranded DNA by anti-BrdU antibody, which was then visualised in the three stage immunoperoxidase technique in paraffin wax sections.[22] The number of labelled nuclei per microscopical field at 400× was determined.

DETERMINATION OF BLOOD NEUTROPHILS AND MUCOSAL NEUTROPHIL INFILTRATION

The number of circulating neutrophils was determined in intact and aspirin treated rats at the end of each experiment. For this purpose, rats were anaesthetised with ether, the abdomen was opened by midline incision, and about 2 ml of blood was withdrawn from the vena cava and added to the vials containing EDTA as an anticoagulant. Blood neutrophil counts were performed under ×100 objective of a light microscope in 80 fields of view using a Burker's chamber. Blood counts were assessed by an observer unaware of the treatment given.

Neutrophil infiltration into the gastric mucosa was determined by chemical staining for neutrophils using anti-neutrophil antibody (DAKO, Denmark) diluted 1:50. Reaction was completed with the ABC method using Extravidin kit (Sigma Chemical Co, St Louis, MO, USA). Density of neutrophil infiltration was assessed by counting the number of positive neutrophils per microscopical field at 400× magnification in non-necrotic mucosa of the oxyntic gland area.

DEMONSTRATION OF GASTRIC ADAPTATION

Several groups, each containing 8–10 rats, were used. One group of rats received acidified aspirin at a standard dose (100 mg/kg) 10 00 am and were killed three hours later. Another group also received acidified aspirin (100 mg/kg) at 10 00 am but were refed one hour later until 5 00 pm, fasted overnight, and then given acidified aspirin the next day at 10 00 am and killed one hour later. Other groups had the same schedule of daily treatment with acidified aspirin (100 mg/kg), refeeding and refasting for two, three, or four consecutive days after the first exposure to aspirin. Acidified aspirin was used also in a single oral dose of 12·5, 25, 50, or 200 mg/kg followed by four days of rechallenge with the same dose to determine the

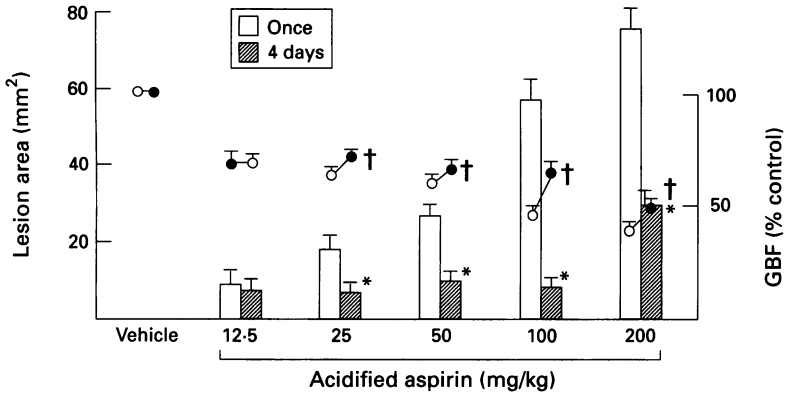

*Figure 1: Area of gastric lesions and gastric blood flow (GBF) in rats given orally acidified aspirin in various doses either once (once) or repeated for the next four days. Means (SEM) of 8–10 rats. *Shows significant decrease below the value obtained after acidified aspirin given once; †shows significant increase compared with the value obtained with acidified aspirin given once.*

threshold dose of this agent required to induce gastric adaptation.

In studies with the duration of adaptation acidified aspirin was given daily at a dose of 100 mg/kg intragastrically for four consecutive days. After this four day treatment with aspirin, the animals were divided into four groups and were refed and remained without treatment for the period of three, five, seven, or 10 days. After each of these time intervals, one group of rats was fasted overnight, and on the following morning, it received rechallenge with acidified aspirin and killed one hour later.

EFFECTS OF SALIVECTOMY

In studies with salivectomy, the sublingual-submandibular gland complexes were removed under pentobarbital anaesthesia (50 mg/kg intraperitoneally). In control rats, a sham operation with cutting of the skin on the neck was performed. Seven to 10 days after salivectomy the animals were subdivided into subgroups of 8–10 rats and had the same daily treatment with acidified aspirin, refeeding and refasting for one to four consecutive days. The animals were then anaesthetised to measure gastric blood flow, and killed to measure the area of gastric erosions. The samples of gastric mucosa were taken for the determination of the mucosal contents of epidermal growth factor as described.[21]

INHIBITION OF NITRIC OXIDE SYNTHASE

To test the possible implication of endogenous nitric oxide in gastric adaptation to aspirin, a potent and selective inhibitor of nitric oxide synthase, N^G-nitro-L-arginine (L-NNA).[23] This agent was given intravenously in a dose of 25 mg/kg either alone or in combination with L-arginine, a substrate of nitric oxide synthase (100 mg/kg intravenously), given 30 minutes before the first exposure to aspirin and before subsequent daily aspirin treatment for four days. Control rats received respective vehicles. Groups of rats were anaesthetised with ether for measurement of gastric blood flow and then killed for assessment of the area of gastric erosions one hour after acidified aspirin was given in tests without or with injection of L-NNA or L-arginine, or both.

GASTRIC ACID SECRETION AFTER FIRST EXPOSURE TO ASPIRIN AND AFTER REPEATED ADMINISTRATION OF ASPIRIN

The effects of single and repeated treatment with acidified aspirin on gastric acid secretion were studied on several groups of pylorus ligated rats. The acidified (0·2 N HCl) aspirin (100 mg/kg) or vehicle (0·2 N HCl) was introduced into the stomach in a volume of 1 ml and one hour later, the rats were anaesthetised with either and the pylorus was ligated. Two hours later the animals were killed, and the gastric content was collected for the measurements of gastric volume, pH and acid concentration, and output. Gastric secretion was examined one hour after first exposure to acidified aspirin or vehicle and after one to four days of repeated treatments with acidified aspirin or vehicle.

STATISTICS

The results are reported as means (SEM). Statistical significance was determined by analysis of variance and where appropriate by the unpaired Student's t test, a value of $p<0·05$ being considered significant.

Results

GASTRIC LESIONS INDUCED BY ACIDIFIED ASPIRIN AND GASTRIC ADAPTATION TO ASPIRIN

Acidified aspirin given orally for the first time (once) in various doses produced a dose dependent increase in gastric erosions reaching at a dose of 100 mg/kg the mean area of about 57 mm^2 (Fig 1). This dose was then used as a standard in most of the experiments. Doubling the dose of aspirin resulted in only a small further increase in lesion area, while the decrease of the dose of aspirin to 50, 25, and 12·5 mg caused a significantly smaller area of gastric damage averaging about 27·5, 18·2, and 9 mm^2, respectively. By contrast, the same doses of aspirin (12·5–200 mg/kg) given intragastrically without 0·2 N HCl produced only few erosions per stomach and these results have not been included for the sake of clarity.

After four days of treatment with acidified aspirin, the area of gastric lesions was significantly reduced at all doses of aspirin used except the smallest one (12·5 mg/kg), which after first treatment produced only few erosions and similar lesions were also seen when this dose was repeated daily for four days. After four days of treatment with acidified aspirin at a dose of 25 mg/kg or higher, the area of gastric lesions was significantly smaller than that seen after the first exposure to aspirin. This reduction was accompanied by a significant increase in gastric blood flow. The greatest reduction in lesions area was seen when aspirin was used at a dose of 100 mg/kg and the degree of gastric adaptation at that dose reached about 87%.

When the animals were resting for three or five days and then rechallenged with aspirin, the area of gastric lesions was not significantly different from that recorded after the first

TABLE I *Mean area of gastric lesions, prostaglandin E_2 generation, and gastric blood flow (GBF) (expressed as per cent of control value in the intact mucosa) after the first administration of acidified aspirin (day 0), after the rechallenge with aspirin for the next four days (day 4), and after rechallenge after 3, 5, 7, or 10 days of rest from the aspirin treatment*

	Lesion area (mm^2)	*Prostaglandin E_2 (%)*	*GBF (%)*
Day 0	54 (6)	12 (3)	45 (12)
Day 4	17 (3)*	10 (3)*	72 (14)*
After rest from aspirin treatment for:			
3 Days	12 (4)*	10 (2)*	74 (15)*
5 Days	22 (5)*	12 (3)*	62 (10)*
7 Days	38 (12)	29 (7)*	55 (10)
10 Days	48 (16)	34 (7)*	48 (12)

Data are mean (SEM) of 8–10 rats.
*Shows significant change compared with the values in the mucosa exposed only once to aspirin.

rechallenge. With further prolongation of the resting period to seven and 10 days, the area of lesions reached the value similar to that recorded after the first administration of aspirin. Gastric blood flow showed a similar value to that recorded when aspirin was applied once. Mucosal prostaglandin E_2 formation tended to increase reaching about 30% of the value in the intact mucosa (Table I).

Figure 2 shows the area of gastric damage by a single treatment (once) with a standard dose of acidified aspirin (100 mg/kg) and during repeated daily administration of this agent for the next four days. The large decrease (by about 80%) in the gastric damage was already seen the day after a single aspirin treatment when rechallenge (day 1) with acidified aspirin was made. After one to four days of aspirin treatment, no further significant change in the lesion area was seen so macroscopically full gastric adaptation to acidified aspirin was achieved.

Gastric blood flow, which in the intact stomach averaged about 55 ml/100 g^{-min} was significantly reduced by about 50% after first exposure to acidified aspirin (Fig 2). Gastric blood flow was significantly increased after the rechallenge with aspirin (day 1) and remained at the same value after four days of treatment with aspirin (day 4). Mucosal generation of prostaglandin E_2 in the intact mucosa averaged about 350 ng/g. A single treatment with acidified aspirin abolished by over 90% the

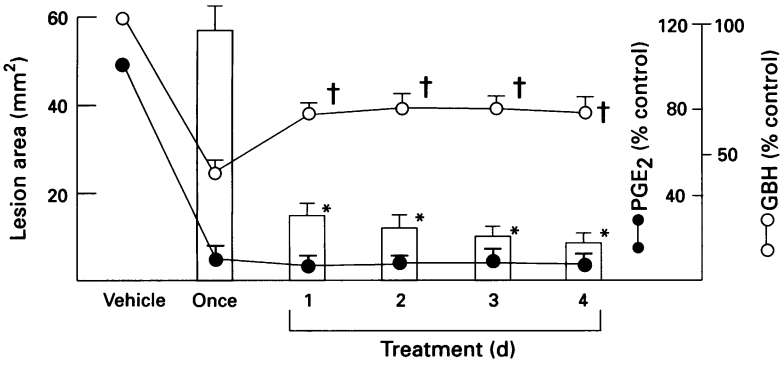

*Figure 2: Area of gastric lesions, gastric blood flow (GBF), and prostaglandin E_2 (PGE_2) generation in the gastric mucosa of rats given acidified aspirin for the first time (once) or given at the same dose for the next four days. Means (SEM) of 8–10 rats. *Shows significant decrease in the area of gastric lesions below the value recorded with aspirin given once; †shows significant increase above the value of GBF obtained with acidified aspirin given once.*

TABLE II *The infiltration by neutrophils of the oxyntic mucosa and the blood neutrophilia in intact rats and those treated with acidified aspirin (100 mg/kg) for the first time (day 0) or after one (day 1) and four day (day 4) rechallenge with acidified aspirin*

	Neutrophil infiltration (no/field)	*Total blood neutrophils ($\times 1000/mm^3$)*
Intact mucosa	17 (2)	3·4 (0·6)
Day 0	55 (5)*	8·5 (1·0)*
Day 1	25 (3)*†	6·0 (0·5)*†
Day 4	19 (3)†	3·9 (0·7)†

Data are mean (SEM) of 10 rats.
*Shows significant increase above the value recorded in the intact mucosa; †shows significant decrease below the values seen after first treatment with acidified aspirin.

generation of prostaglandin E_2 by the gastric mucosa (Fig 2). This suppression of prostaglandin E_2 biosynthesis persisted during continued four day treatment with acidified aspirin.

HISTOLOGICAL FINDINGS

In the intact gastric mucosa, the number of infiltrating neutrophils into the oxyntic mucosa averaged 17 per microscopic field and the blood neutrophil counts averaged 3·4×1000 per mm^2 (Table II).

Single exposure (day 0) to acidified aspirin at 100 mg/kg resulted in a considerable gastric hyperaemia, moderate neutrophil infiltration into the mucosa, submucosal oedema, and an appreciable increase of circulating neutrophils (Table II). Deep necrotic erosions occupied about 28% of the mucosal strip length. About 46% of the mucosal strip length was denuded of the surface epithelium and no mucosal regeneration was seen at this study time (Table III).

After the rechallenge with aspirin performed one day (day 1) after the first aspirin challenge, the persistence of acute gastritis, hyperaemia, and denudation of surface epithelium (44% of the mucosal strip length) were seen but the deep necrotic lesions were significantly diminished (to about 9% of the mucosal strip length). Also the severity of mucosal neutrophil infiltration and blood neutrophilia had declined. The regenerative changes were seen in about 2·5% of the mucosal strip length mostly as elongation of cristas, foveolas, and glandular neck areas lined with cuboidal, less differentiated regenerative epithelium. Submucosa became less oedematous and the inflammatory infiltrate was composed mostly of neutrophils.

TABLE III *Quantitative histology of gastric mucosa after first exposure (day 0) to acidified aspirin (100 mg/kg) and after rechallenge with aspirin after day 1 (day 1) and at day 4 (day 4)*

	Denuded surface	*Deep necrosis*	*Mucosal regeneration*
Day 0	46·2 (7·4)	27·8 (4·2)	0
Day 1	44·5 (6·0)	8·9 (2·3)*	2·5 (1·4)*
Day 4	5·2 (1·4)*†	1·2 (0·8)*†	45·5 (8·6)*†

Data are mean (SEM) of six examinations on six rats.
Results are expressed as per cent of the mucosal strip length.
*Shows significant change compared with first exposure;
†shows significant change compared with values obtained after four day rechallenge with aspirin compared with day 1 values.

TABLE IV *Bromodeoxyuridine uptake after first exposure to acidified aspirin (day 0) and after the one day (day 1) and four day (day 4) rechallenge with aspirin*

	Area with erosions	*Area without erosions*
Day 0	0	2·6 (0·9)
Day 1	3·1 (0·8)*	6·7 (1·6)*
Day 4	10·2 (1·8)*†	5·8 (0·7)*

Values represent number of labelled nuclei per microscopic field at 400× magnification. Data are mean (SEM) of six examinations on six rats.
*Shows significant increase above the value seen after the first exposure to aspirin; †shows significant increase above the values obtained after one day rechallenge with aspirin.

TABLE V *DNA synthesis and DNA content after first exposure to acidified aspirin (day 0), and after the one day (day 1) and four day (day 4) rechallenge with aspirin*

	DNA synthesis (dpm/μg DNA)	*Total DNA content (mg)*
Day 0	21 (3)	2·96 (0·11)
Day 1	36 (4)*	2·11 (0·16)
Day 4	66 (7)*†	2·44 (0·14)*

Values are means (SEM) of eight tests on eight rats. DNA synthesis is expressed in dpm/μg DNA and DNA content in mg. *Shows significant increase above the value seen after the first exposure to aspirin; †shows significant increase above the values obtained after one day rechallenge with aspirin.

TABLE VI *Gastric acid secretion in pylorus ligated rats without administration of aspirin and after single exposure to acidified aspirin or vehicle (0·2 N HCl) (day 0), after repeated treatment with acidified aspirin for one day (day 1) or four days (day 4)*

Test day	*Treatment*	*Volume (ml/h)*	*pH*	*Acid output (μmol/2 h)*
Day 0	Vehicle	3·0 (0·4)	1·4 (0·4)	250 (12)
	HCl – aspirin	7·3 (1·3)*	3·4 (0·8)*	180 (8)*
Day 1	Vehicle	4·0 (0·6)	1·0 (0·08)	421 (19)
	HCl – aspirin	6·6 (0·8)*	2·5 (0·5)*	210 (7)*
Day 4	Vehicle	3·2 (0·5)	1·2 (0·3)	258 (10)
	HCl – aspirin	4·0 (0·7)	2·4 (0·5)*	112 (4)*

Values are mean (SEM) of six to eight tests on six to eight rats. *Shows significant change compared with the values recorded in rats without administration of aspirin.

After four days (day 4) of repeated treatment with acidified aspirin, the mucosal infiltration with neutrophils and blood neutrophil counts was not significantly different from that seen in the intact mucosa. The gastritis was considerably reduced; only about 5% of the mucosa was denuded of surface epithelium and only 1·2% of the mucosal length was affected by deep necrotic lesions. In contrast, about 46% of the mucosa showed remarkable regenerative changes with elongation of neck and foveolar areas lined with cuboidal regenerative epithelium. In the denuded areas some damage to superficial lamina propria was still seen but even in these areas there was regeneration of the mucosa. In the submucosa, the inflammatory infiltrate was a mixture of polymorphonuclears and mononuclears (lymphocytes and macrophages).

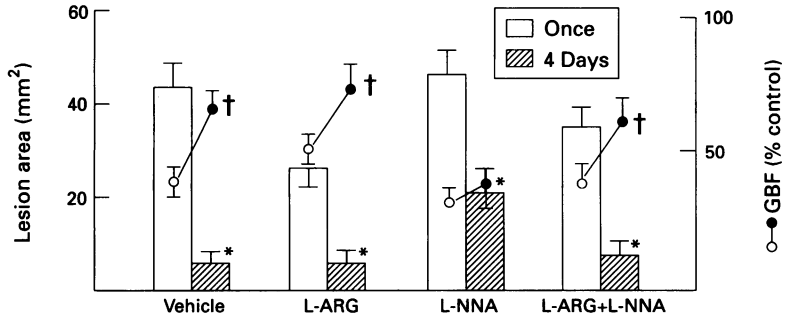

*Figure 3: Area of gastric lesions and gastric blood flow (GBF) in rats pretreated with vehicle, L-arginine, L-NNA or their combination and then exposed to acidified aspirin for the first time or challenged with aspirin for the next four days. Means (SEM) six to eight rats. *Shows significant decrease in the area of gastric lesions below the value obtained after acidified aspirin given for the first time (once); †shows significant increase in GBF above the value obtained with acidified aspirin given once.*

BROMODEOXYURIDINE UPTAKE AND DNA SYNTHESIS DURING TREATMENT WITH ACIDIFIED ASPIRIN

In the intact stomach, the bromodeoxyuridine (BrdU) uptake in the oxyntic mucosa was seen only in the neck area (4–5 nuclei per microscopic field at 400× magnification). After the first exposure (day 0) to acidified aspirin, the BrdU uptake by glandular epithelium in the area of necrotic lesions was virtually absent but after the first rechallenge with aspirin (day 1), the BrdU uptake was significantly increased (to 3·1 (0·8) of labelled nuclei per microscopic field) and after four days of treatment with aspirin this uptake was still remarkably increased (to 10·2 (1·8) of labelled nuclei per microscopic field). In non-necrotic oxyntic mucosa, the number of labelled nuclei after the first aspirin treatment was low and showed significant increase at day 1 and 4 of rechallenge with aspirin though this increase was less noticeable than in necrotic areas (Table IV).

DNA synthesis, as measured by the incorporation of labelled thymidine into DNA, averaged about 41 (5) dpm/μg DNA and total DNA content averaged about 2·6 (0·4) mg in the intact mucosa. After first exposure to aspirin, both the DNA synthesis and DNA content were significantly reduced by about 50% and 30%, respectively (Table V). With the rechallenge with aspirin at day 1 after the first exposure to aspirin, the DNA synthesis was still below the value in the intact mucosa but after four days of treatment with aspirin it was increased by about 52% above the value in the intact mucosa or about 210% compared with the value in the mucosa after first exposure to aspirin. DNA content did not significantly change at day 1 after the first exposure to aspirin but after four days of treatment with aspirin it was significantly increased by about 19% above the value obtained at day 0 (Table V).

GASTRIC ACID SECRETION AFTER FIRST EXPOSURE TO ASPIRIN AND DURING ADAPTATION TO ASPIRIN

After first exposure to acidified aspirin (100 mg/kg), the volume of gastric juice in pylorus ligated rats was almost doubled but the concentration and output of HCl were significantly reduced and pH increased compared with the values obtained with administration of vehicle (0·2 N HCl) (Table VI). A similar increase in the volume of gastric juice with the significant reduction in acid output and the rise in pH was seen after repeated treatment with aspirin at day 1 and 4.

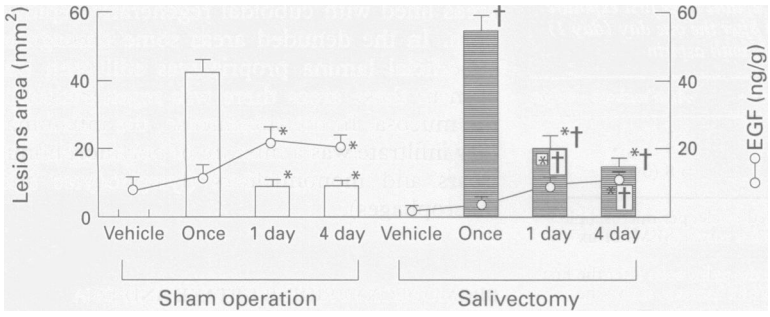

*Figure 4: Area of gastric lesions and the mucosal content of immunoreactive epidermal growth factor (EGF) in sham operated or with salivectomised rats given acidified aspirin for the first time (once) or after one or four days rechallenge with acidified aspirin. Means (SEM) of six to eight rats. *Shows significant difference compared with the value obtained with acidified aspirin given once; †shows significant decrease compared with the values obtained in sham operated rats.*

EFFECTS OF INHIBITION OF NITRIC OXIDE SYNTHASE, AND SALIVECTOMY ON GASTRIC ADAPTATION TO ASPIRIN

In rats with inhibited nitric oxide synthase by intravenous L-NNA, gastric lesions induced by first exposure to acidified aspirin were of similar area to those seen in vehicle treated rats and with subsequent daily rechallenge with aspirin, a significant decrease in lesion area was seen. This decrease in lesion area was significantly smaller in L-NNA than in vehicle treated rats (Fig 3). Gastric blood flow that was significantly reduced by the first exposure to aspirin, failed to show any significant increase after a subsequent four days' treatment with aspirin in L-NNA treated rats.

Addition of L-arginine to L-NNA failed to affect the area of gastric lesions but reversed, in part, the fall in gastric blood flow caused by L-NNA. L-arginine alone tended to decrease though not significantly the area of gastric lesions but failed to affect the gastric blood flow in response to first exposure to acidified aspirin and to four days' treatment with this agent.

Figure 4 shows that in salivectomised rats, the first exposure to acidified aspirin and rechallenge with aspirin resulted in a significantly larger area of gastric lesions than in sham operated rats. After one to four days' treatment with aspirin, the area of gastric lesions was reduced both in salivectomised and in sham operated rats though this reduction in salivectomised rats was significantly smaller compared with sham operated animals. With repeated administration of aspirin, there was a significant increase in the content of epidermal growth factor in the gastric mucosa. Salivectomised rats showed smaller contents of mucosal epidermal growth factor but this was also significantly increased after one to four days repeated treatment with aspirin.

Discussion

This study confirms that gastric mucosa shows a remarkable ability to adapt to the ulcerogenic action of aspirin and that this adaptation is accompanied by a noticeable decrease in neutrophil infiltration into the mucosa and in blood neutrophilia, an increased gastric blood flow (hyperaemia), and mucosal cell proliferation.

This study confirms that the appearance of widespread mucosal damage by aspirin is closely correlated with the considerable infiltration of neutrophils into the mucosa and the increase in the number of circulating neutrophils.[7 11] Similar association of antral ulcerations with neutrophil infiltration to the mucosa, enhanced leukotriene B_4 release, and blood neutrophilia were recently seen in indomethacin treated rats.[24] These results suggest that indomethacin induced ulcerations in gastric antrum depend on neutrophil infiltration and activity. As shown by Wallace *et al*,[7 11] the vascular endothelial injury precedes the development of haemorrhagic lesions caused by NSAIDs and such changes in the endothelial integrity were not seen in neutropenic rats. This strongly suggests that neutrophil endothelium plays a crucial part in the pathogenesis of acute mucosal lesions induced by NSAIDs. Our results show that aspirin induced acute gastric lesions in the oxyntic mucosa may also have a dependence upon neutrophil infiltration for their pathogenesis. The activation of neutrophils and their interaction with the endothelium resulted in about 50% reduction in the mucosal blood flow seen in rats upon the first exposure to acidified aspirin. This reduction in gastric blood flow could contribute to the formation of acute gastric damage.

The important finding of this study is the adaptation of the gastric mucosa to repeated insults of acidified aspirin, which is accompanied by the attenuation of both the number of circulating neutrophils and the severity of neutrophil infiltration into the gastric mucosa. The reduction in mucosal neutrophil infiltration and the fall in blood neutrophilia were already seen after the first rechallenge with aspirin and it was accompanied by the significant increase in the gastric blood flow. It is not clear what the mechanism of gastric hyperaemia[25] is in response to repeated exposures to aspirin and whether this hyperaemia plays any part in gastric adaptation to aspirin.

As prostaglandins have been implicated in adaptive cytoprotection[26 27] and this phenomenon was also accompanied by mucosal hyperaemia,[27 28] the appealing hypothesis was that gastric hyperaemia accompanying the adaptation to repeated exposures to aspirin might also be mediated by endogenous prostaglandins. This was not the case, however, as it was found in this study that the mucosal prostaglandins was suppressed (by over 90%) by the first exposure to aspirin and remained suppressed during repeated doses of this drug when the adaptation to aspirin developed. Thus, the increased generation of endogenous prostaglandin did not occur and prostaglandin is probably not involved in the mediation of gastric adaptation and accompanying gastric hyperaemia.

An alternative explanation of increased blood flow occurring during the rechallenge with aspirin and mucosal adaptation is the activation of nitric oxide synthase and release of nitric oxide. This mediator, which is formed

in the vascular endothelial cells from L-arginine through the action of nitric oxide synthase,[29] has been shown to contribute to local vasodilation in the gastric mucosa exposed to topical irritation.[30] We tested, therefore, whether the blockade of nitric oxide synthase[24] using L-NNA affects the enhancement of mucosal blood flow during gastric adaptation. Our previous study[18] showed that the pretreatment with L-NNA by itself reduced basal gastric blood flow by about 30%. In this report, the pretreatment with L-NNA reduced significantly the gastric blood flow after the first exposure to aspirin and abolished gastric hyperaemia after four days aspirin treatment but failed to eliminate the ability of the mucosa to adapt to aspirin. These results show that nitric oxide plays a crucial part in hyperaemia accompanying gastric adaptation to aspirin but is not essential for the development of gastric adaptation itself to aspirin. This does not exclude some influence of endogenous nitric oxide on the adaptation process. In fact, after the inhibition of nitric oxide synthase with L-NNA the ability of the mucosa to adapt was somewhat attenuated and the addition of L-arginine (a substrate of nitric oxide synthase) to L-NNA not only restored the mucosal hyperaemic response to rechallenge with aspirin, but enhanced the adaptation to that seen in vehicle treated rats with intact nitric oxide biosynthesis. These results show that increased blood flow in the stomach is mediated by nitric oxide but this has only a small influence on the development of gastric adaptation to aspirin.

The epithelial cell proliferation in response to aspirin given for four weeks in rats, as measured by [^{3}H]-thymidine uptake, was previously reported in rat fundic mucosa by Eastwood and Quimby.[16] Baumgartner *et al*,[31] who studied the effect of longterm parenteral administration of indomethacin in rats, also reported an increased DNA synthesis both in the fundic and antral mucosa. Levi *et al*[32] reported that NSAIDs stimulate the mitotic activity of mucosal cells in arthritis patients and misoprostol does not reverse this effect. It was suggested that the increase in mitotic activity in gastric glands seen after two weeks of NSAID treatment could be one of the mechanisms underlying gastric adaptation to NSAIDs[32] but no attempts were made to measure the proliferation before the start and after the treatment with NSAIDs in the same subjects. Thus, neither the time course of changes in mucosal proliferation during gastric adaptation to NSAIDs or the mechanism triggering this proliferation has been elucidated.

Our study shows that the exposure of gastric mucosa to acidified aspirin leads to a widespread damage of surface epithelium and deep necrotic lesions. Already after the first rechallenge with aspirin, when deep necrotic lesions were significantly reduced, the first sign of mucosal regeneration developed in the mucosa. This process was remarkably impressive after four days of repeated treatment with aspirin as evidenced by the elongation of gland necks and foveolar areas lined with cuboidal regenerative epithelium. Both the bromodeoxyuridine uptake in vivo and the [^{3}H]-thymidine incorporation to DNA in vitro, which were diminished after the first exposure to aspirin, showed a severalfold increase after repeated treatment with aspirin. Furthermore, repeated exposures to aspirin caused a significant increase in mucosal content of DNA showing that the mucosal growth is greatly stimulated during the process of adaptation.

The mechanism of the remarkable regenerative changes in the gastric mucosa in response to continued administration of aspirin is not clear but it could be a compensatory response to the widespread injury of the surface by the first exposure to acidified aspirin. As shown by Lacy,[34] the denudation of the surface epithelium after injury is rapidly repaired by the process of the foveolar and neck cell migration and this is later followed by increased cell proliferation in the regenerative zone. As the cell cycle of gastric epithelium is 16–22 hours, it means that in our experiments with aspirin rechallenge (one to four days after the first aspirin treatment), the cells reach the surface epithelium by migration from a massively proliferating regeneration zone. Lacy[34] showed that the surface epithelium in chronically rechallenged rats was more resistant to injury but chronic injury failed to change rapid epithelial restitution. Our study shows an increased regeneration of the gastric mucosa rechallenged by aspirin. The mechanism of the stimulation of cell proliferation is not explained but the fact that the mucosal content of immunoreactive epidermal growth factor was also significantly increased in the adapting mucosa suggests that epidermal growth factor may be implicated in this mucosal regeneration. As the removal of salivary glands, the main source of endogenous epidermal growth factor, did not eliminate gastric adaptation to aspirin, it may be concluded that salivary epidermal growth factor is not crucial for gastric adaptation. On the other hand, the increased content of epidermal growth factor in the mucosa adapting to aspirin suggests that this growth factor may originate mainly from the local production. Wright *et al*[35] reported that ulcerations in the human gastrointestinal tract may induce the development of novel cell lineages (from stem cells) that secrete locally epidermal growth factor. It is probable that gastric mucosa responds to aspirin induced damage by excessive local epidermal growth factor production to initiate the mucosal repair such as mitogenesis and cell proliferation. Tarnawski *et al*[36] showed that such an increase of epidermal growth factor receptors occurs in the area of gastric ulcer healing in rats. Epidermal growth factor could participate in the proliferation of mucosal cells due to overexpression of receptors of this peptide in the mucosa and also in the inhibition of gastric acid output seen throughout the period of gastric adaptation to aspirin. It is not excluded that the mucosal damage by aspirin also increases local production of transforming growth factor α, which stimulates mucosal cell

proliferation and inhibits gastric acid secretion in the same way as epidermal growth factor receptor. This possibility is supported by recent findings[37] that acute mucosal damage causes almost immediate increase in the content of transforming growth factor in gastric mucosa. Transforming growth factor α, like epidermal growth factor, could participate in the repair of acute mucosal damage as described previously[38] and in gastric adaptation to repeated mucosal challenge by aspirin as well as accompanying the inhibition of gastric acid secretion seen in this study.

It is of interest that the volume of gastric juice collected from animals pretreated by acidified aspirin was almost doubled while acid concentration and output were significantly reduced. The mechanism of the increase in gastric residual volume after mucosal injury and adaptation is not clear. Further studies are needed to find out if the increased gastric volume flow combined with the reduction in gastric acidity in the stomach adapting to aspirin results from the increased back diffusion of acid and increased plasma leakage or if it represents another protective mechanism enhancing water flow and limiting the extent of mucosal damage by diluting irritants[28] such as acidified aspirin.'

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