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Epithelial restitution and cellular proliferation after gastric mucosal damage caused by hypertonic NaCl in rats*

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Summary. Hypertonic NaCl enhances gastric cancer in rats induced by N-nitroso compounds. This study was designed to examine the structural changes and alterations in mitotic activity occurring after mucosal exposure to hypertonic NaCl. Wistar rats were given one ml of 4.5 M NaCl by gastric tube and groups of 4–5 animals were sacrificed at different time intervals up to 120 h. An i.p. injection of thymidine was given 1 h before death. Samples of antral and corpus mucosa were prepared for microscopy and autoradiography. Hypertonic NaCl caused uniform destruction of surface mucous cells and pits in the corpus and antrum. Epithelial restitution with the formation of a thin epithelial layer occurred within one h of damage. The mucosa changed towards normal within 24–48 h. The distance between mucosal surface and the replicating cells decreased during the first 2 h. The proliferation zone remained in the middle of the glandular layer throughout the experiment. The proliferative activity increased during the first 24 h after mucosal damage. The number of labelled cells per unit area of mucosa was somewhat larger in the corpus than the antrum, but in the corpus the distance between proliferating cells and mucosal surface was double that of the antrum. Hypertonic NaCl causes a series of changes in the gastric mucosa. The increased mitotic activity can only partly explain the cocarcinogenic effect, since N-nitroso-induced adenocarcinomas occur predominantly in the antrum while the mitotic activity is maximal in the corpus.

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Introduction

Considerable evidence has been provided that gastric cancer may be related to the intake of salted food [4]. It has been shown experimentally that hypertonic NaCl acts as a cocarcinogen when given in combination with a carcinogen. Tatematsu et al. [14, 15] showed that saturated NaCl solution enhanced the carcinogenic effect of MNNG (N-methyl-N′-nitro-N-Nitrosoguanidine) in the rat stomach. Evidence has also been provided that the presence of an ulcer increases the carcinogenic effect of MNNG [13]. Capoferro and Torgersen [1] found that 2 M NaCl increased the uptake of tritiated 7.12-dimethylbenz(a) anthracene in the mucosa of pyloroligated rats. They also showed that hypertonic NaCl caused a marked decrease in the mucin content of the mucous surface cells.

It has been shown recently that damage to the gastric surface epithelium is followed by rapid epithelial restitution after removal of the injurious agent. Svanes et al. [10, 11] showed that exposure of the frog fundic mucosa mounted in Ussing chambers to 1 M NaCl for 10 min caused extensive damage to the surface epithelium. After washing and return to control solutions at pH 7.40, epithelial restitution occurred within 4–6 h. Similarly, Rutten and Ito [6] showed that guinea pig fundic mucosae mounted in chambers were extensively damaged when exposed to 1.25 M NaCl, and epithelial restitution occurred within 90 min after wash-out of hypertonic saline. Lacy and Ito [5] exposed in vivo rat gastric mucosa to 100% ethanol for 30–45 s which caused uniform damage confined mostly to the surface epithelium; thirty min later, more than 75% of the mucosal surface was restored.

The results referred to above suggests that the cocarcinogenic action of NaCl may be related to the effects of damage or repair in the gastric mucosa. It would therefore be important to establish the kind of changes which result from gastric mucosal exposure to concentrated NaCl solutions in the rat.

In the present study hypertonic NaCl was instilled into the rat stomach in volumes and concentrations that have been used in cancer research. The gastric mucosa was studied histologically at different time intervals after exposure to hypertonic NaCl, up to 120 h. The early restitution after mucosal damage caused by hypertonic NaCl is believed to be due to cellular migration [7]. However, the subsequent repair of the mucosa after damage, could be expected to be due to cellular proliferation. Autoradiography was therefore performed to obtain information about the location and activity of cellular proliferation after damage.

Materials and methods

Male Mol: WIST rats (Møllergård breeding laboratories, Ejby, Denmark), weighing 185–330 g, were used. During the last 24 h before an experiment food was withdrawn, access to water was free until one hour before the experiment.

One ml 4.5 M NaCl was instilled into the stomach with a gastric tube. Control animals received 1 ml 0.9% saline instead of hyperosmolar NaCl.

The rats were killed by means of a blow to the back of the cranium and subsequent severing of the carotid arteries.

The animals received an intraperitoneal injection of 1 μCi/g body weight of metyl (^{3}H)-Thymidine, (^{3}H-TDR spec. act. 24 Ci/mmol, Radiochemical Centre, Amersham, UK) one h before death. All animals had the injection at the same hour of the day.

Immediately after killing the animal, initial fixation of the mucosa was performed by instillation of 10 ml Bouin's solution into the in situ stomach for 10 min. The stomach was then removed, opened and inspected. Samples of the antrum, corpus and fundus were selected for histological examination and further fixed in Bouin's solution. Seven μm thick sections were prepared and stained with haematoxylin and eosin. The sections were coded so that the examiner was unaware of which experimental group they belonged to. Conventional light microscopy was used for histological examination. The lengths of mucosal surface of a tissue section showing normal, damaged and restituted surface epithelium (Figs. 1–4) were measured with an ocular micrometer.

The results were expressed as the percentages of mucosal surface showing normal, damaged, or restituted epithelium [5].

Tissue samples for autoradiography were fixed in Bouin's solution after removal of the stomach. After routine paraffin-wax embedding the samples were serially sectioned at 7 μm and prepared for autoradiographic processing in the standard way using Kodak autoradiographic 10 Fine Grain Stripping Plates. During the 7 days of exposure the slides were stored in a light proof box at a temperature of 0° C. After exposure the film strips were developed and fixed. Thereafter the slides were stained for 1.5 min in Harris's haematoxylin.

The sections were coded so that the examiner was unaware of which experimental group they belonged to. Light microscopy was used for examination of labelling. Since (^{3}H)-Thymidine specifically labels the DNA during the S-phase of the cell cycle the silver grains were detected in the overlayed photographic emulsion over the cell nucleus. One section from each of the three different areas under investigation was examined. In each section 40 labelled cells were counted and their location expressed by μm distance to the mucosa surfaced and to glandular bottom (Fig. 6). The length of a section that contained 40 labelled cells was measured and interpreted as an expression of mitotic activity.

For morphological examination, the animals were distributed at random in sixteen experimental groups. One group served as a control without gastric mucosal damage. Groups of 4–5 animals were killed 5 min, 10 min, 20 min, 30 min, 45 min, 1 h, 2 h, 4 h, 12 h, 18 h, 24 h, 48 h, 72 h, 96 h and 120 h, respectively, after application of NaCl.

For autoradiography the same groups were used except the groups where exposure time was less than one hour.

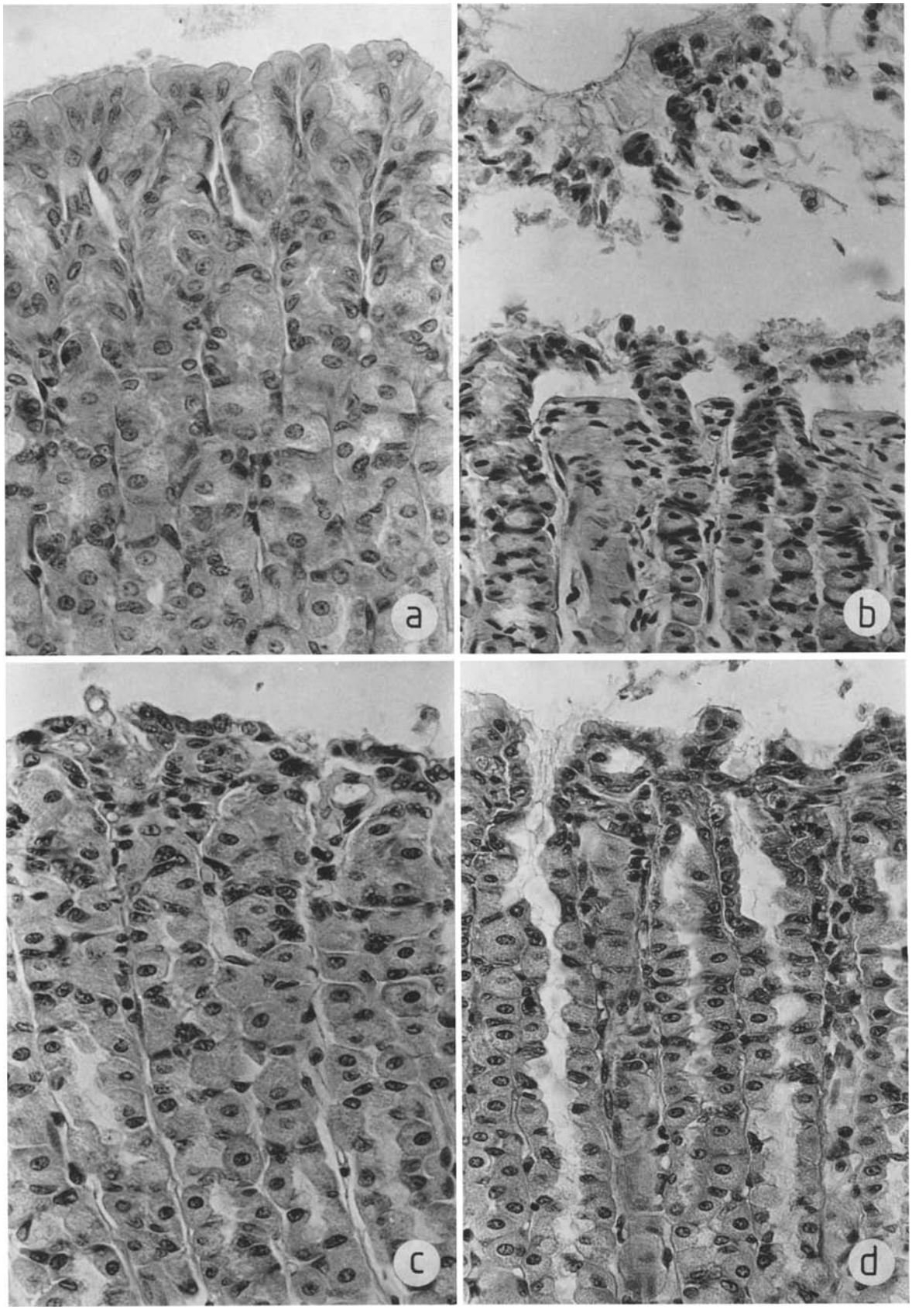
One way analysis of variance (ANOVA) was used on data from autoradiography measurements of distance to mucosal surface and glandular bottom (Fig. 6). Based on results obtained by microscopic examination the one hour data, 1+2 h, 12+18 h, and the 48–120 h data were selected as groups for analysis with Scheffe's test [16]. Regression analyses were used for the data in Fig. 7.

Results

Five minutes after installation of 1 ml of 4.5 M NaCl into the stomach about 90% of the corpus surface epithelium was damaged. The surface cells and most of the pit cells were damaged and lifted off from the basal lamina (Fig. 1 b). The parietal cells appeared shrunken with small hyperchromatic nuclei. Mucosal erosions with damage to the upper $^1/_4$–$^1/_2$ of the glandular layer were observed in about 7% of the surface lengths. Capillary stasis and some haemorrhages were present in the upper part of the mucosa.

Epithelial restitution occurred rapidly after the initial damage. Even at 10 min signs of early restitution were present in small areas of the mucosal surface. At 30 min after application of 4.5 M NaCl about 70% of the corpus mucosa had an epithelial lining which consisted of cuboidal and some flattened cells (Fig. 1 c). Two hours after damage most

Fig. 1 a–d. Changes in rat fundic mucosa observed 5–120 min after intragastric instillation of 1 ml 4.5 M NaCl solution. **a** photomicrograph of normal fundic mucosa; **b** 5 min after application of 4.5 M NaCl. The picture shows damage and detachment of the surface mucous cells and most of the pit cells. The parietal cells are small with small dark nuclei; **c** 30 min after exposure to 4.5 M NaCl. There is an incomplete epithelial lining consisting of cuboidal cells with scanty cytoplasm. Parietal cells are seen close to the surface epithelium; **d** fundic mucosa 2 h after damage, showing complete epithelial lining with irregular cuboidal cells, shallow pits and dilated glands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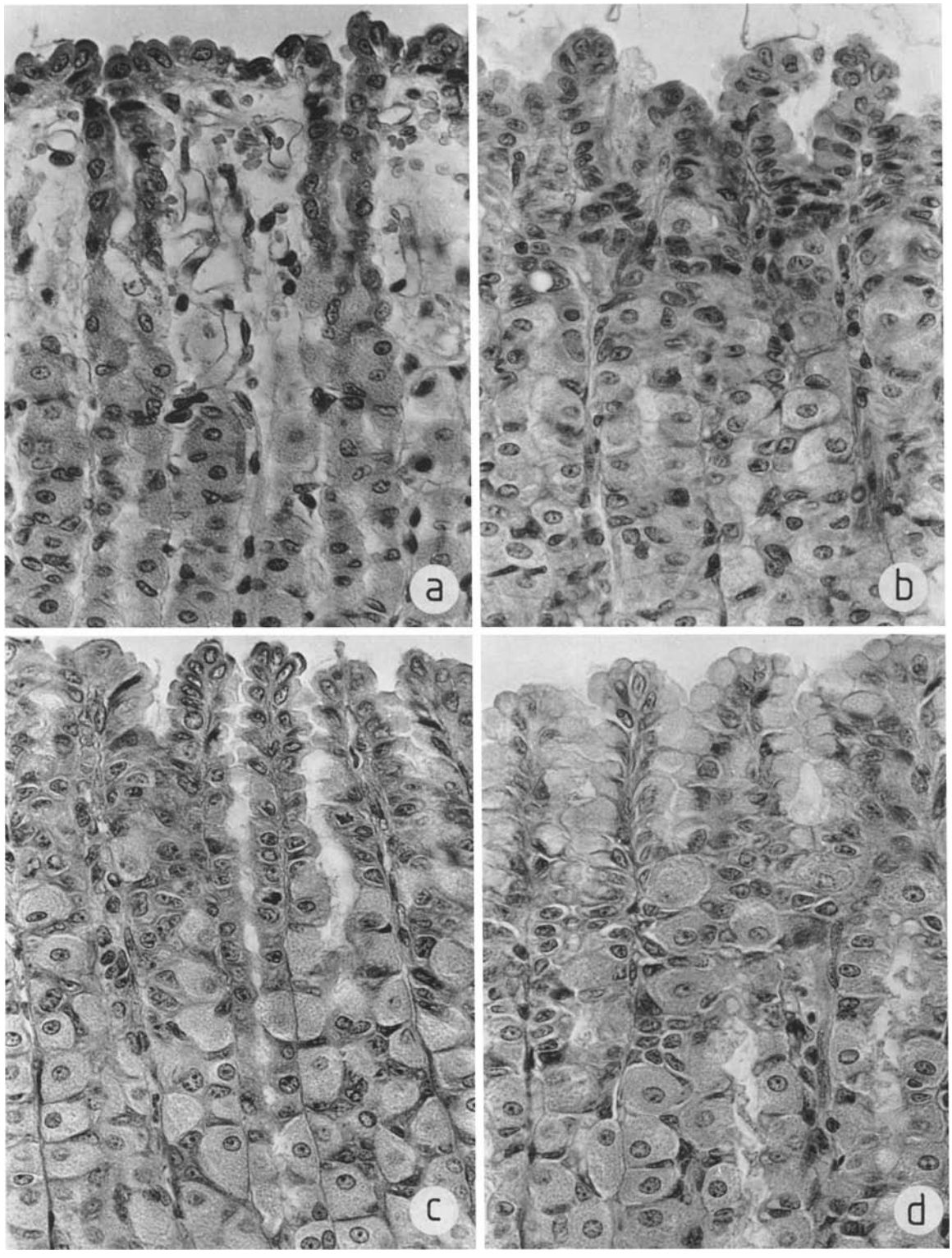

Fig. 2a–d. Changes in rat fundic mucosa observed 4–48 h after intragastric instillation of 1 ml 4.5 M NaCl. **a** Fundic mucosa 4 h after damage, showing epithelial lining and subepithelial edema and hemorrhage. **b**, **c**, **d** Rat fundic mucosa 12 h (**b**), 24 h (**c**) and 48 h (**d**) after exposure to 4.5 M NaCl, showing gradual normalization of surface epithelium, pits and glands

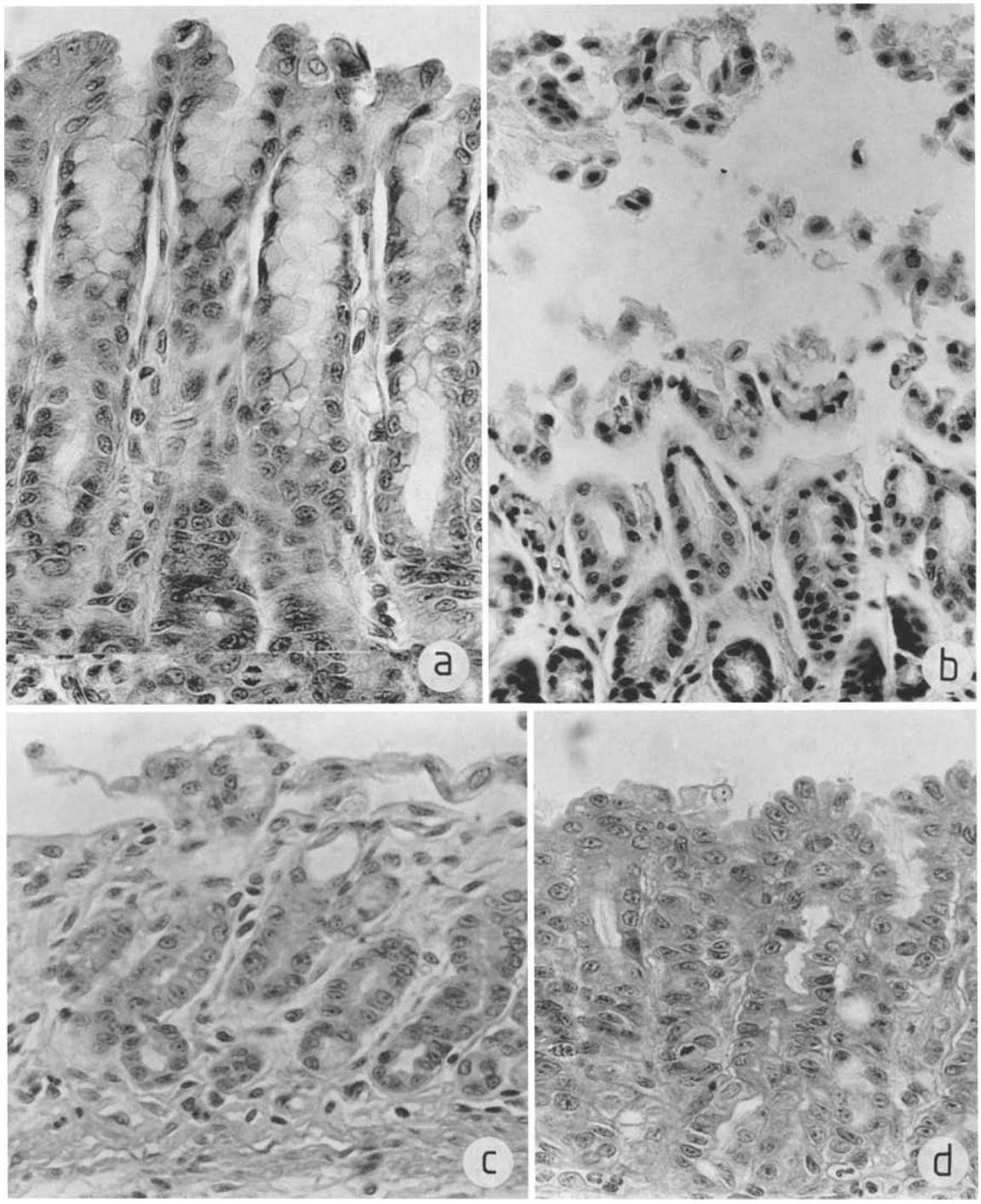

Fig. 3a–d. Changes in rat antral mucosa 5–120 min after instillation of 1 ml 4.5 M NaCl solution. (**a**) photomicrograph of normal rat antral mucosa; (**b**) antral mucosa 5 min after exposure to 4.5 M NaCl. The surface epithelium and parts of the pits are detached from the mucosa. The gland cells appear shrunken; (**c**) antral mucosa 30 min after application of 4.5 M NaCl, showing early, incomplete restitution of the surface epithelium; (**d**) antral mucosa 2 h after treatment with 4.5 M NaCl. The epithelial lining is complete with irregularly arranged surface cells. The glands are irregular with considerable variation in nuclear size and shape

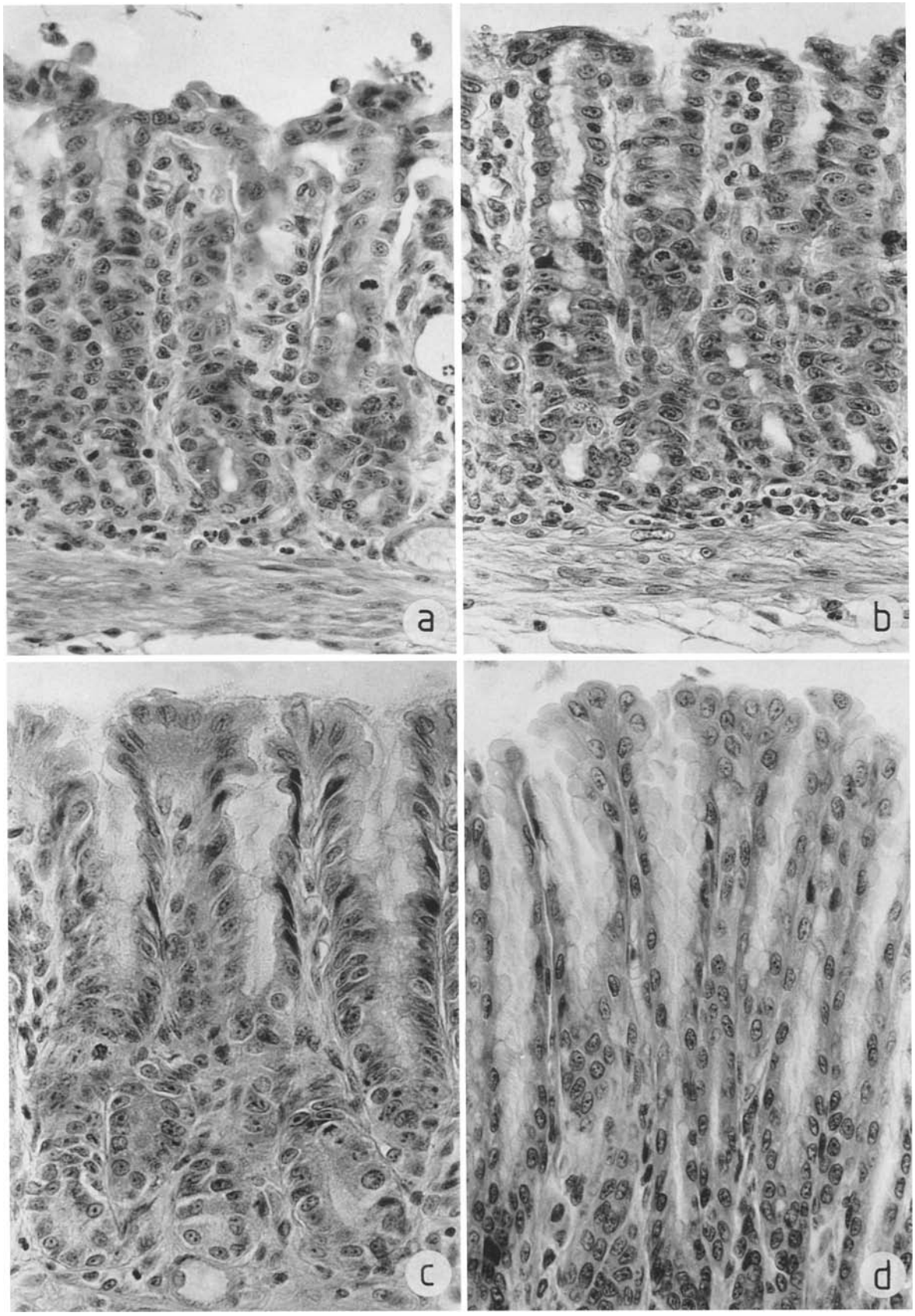

Fig. 4a–d. Structural changes in rat antral mucosa observed 4 h (**a**), 12 h (**b**) 24 h (**c**) and 48 h (**d**) after intragastric instillation of 1 ml 4.5 M NaCl. Between 4 and 48 h the surface epithelium, the pits and the glands gradually changes towards normal

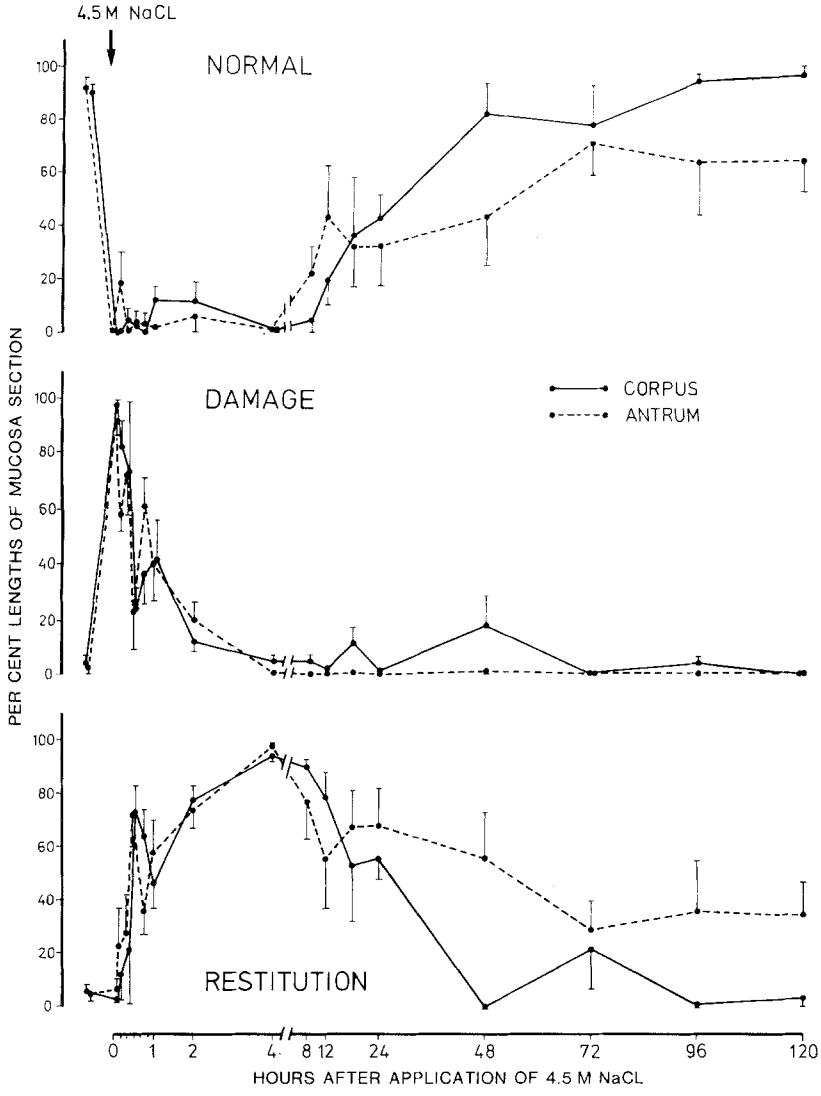

Fig. 5. Lengths of mucosal surface showing normal, damaged and restituted epithelium, were measured in sections of the corpus and antrum of rats. The figure shows the percentage distribution of normal, damaged and restored epithelium at different time intervals after mucosal exposure to 4.5 M NaCl

of the mucosa had a continuous epithelial lining with irregularly arranged cells and shallow pits (Fig. 1d). With increasing time after damage the surface epithelium became more compact and the cells more regularly arranged. The pits became deeper and more regularly shaped (Fig. 2a, b). At 24 h the mucosa did not differ much from normal. However, the nuclei of the surface cells were darker and the cytoplasm more scanty than normally (Fig. 2c). After 48 h it became difficult to decide whether the tissue was from a repaired mucosa or a control (Fig. 2d). As shown in Fig. 3b the hyperosmolar NaCl solution caused similar changes in the antrum and the corpus. The surface mucous cells and most of the pit cells were destroyed and detached from the mucosal surface. Restitution of the surface epithelium and the pits showed a similar time course as in the corpus (Figs. 3–4). In contrast with the corpus, the antral glands became very irregular with cells and nuclei showing great variation in shape and size and many mitoses.

The lengths of gastric mucosal surface showing normal, damaged or restituted epithelium were measured in sections from the corpus and antrum of 88 rats. A summary of the results is given in Fig. 5. About 95% of the surface epithelium was damaged after the application of 4.5 M NaCl. About 75% of the epithelial lining was restituted 2 h after the damage, and the epithelial lining was complete at 4 h. After 48 h it became difficult to decide whether a tissue represented late recovery or a control. This explains the observed fall in fraction of restored epithelium and the increased fraction of normal surface epithelium observed 48 h or later after mucosal damage. As shown in Fig. 5 the extent of damage and restitution and the changes over time were very similar in the corpus and antrum of the rat stomach.

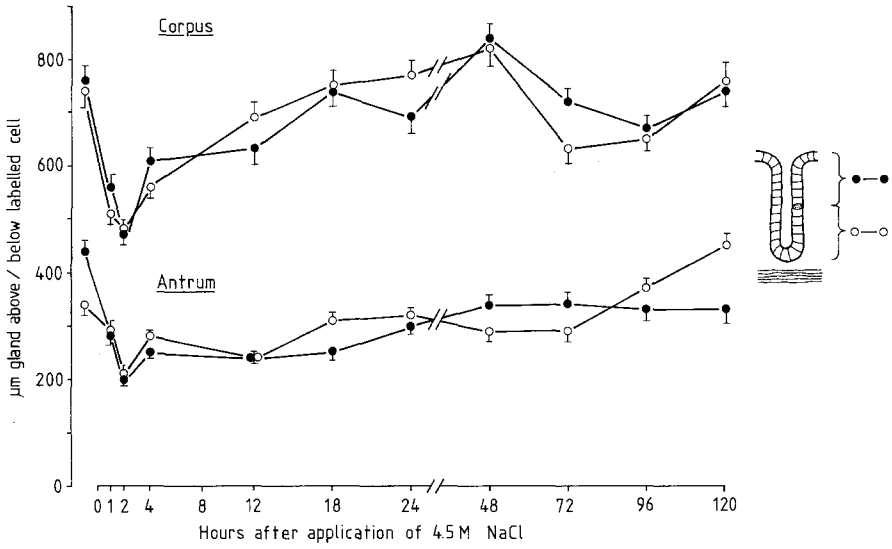

Fig. 6. Location of labelled cells in terms of µm distance to mucosal surface and glandular bottom in the corpus and antrum of rats at different time intervals after mucosal exposure to 4.5 M NaCl. In the corpus each point represents the mean value of 240 cells in 3 rats, in the antrum 120 cells in 3 rats

Table 1. One-way analysis of variance with Scheffe's test of corpus data in Fig. 6

	Distance to surface	Distance to bottom
DF	10;2629	10;2629
MS	188.9;12.9	228;12.9
F	14.6	17.7
p	<0.0005	<0.0005
Contrasts:		
h_0 vs h_1+h_2	7.53	7.27
h_0 vs $h_{12}+h_{18}$	2.35	0.48
h_0 vs h_{48-120}	0.71	0.65
h_1+h_2 vs $h_{12}+h_{18}$	6.30	8.30
h_1+h_2 vs h_{48-120}	9.65	10.30
$h_{12}+h_{18}$ vs h_{48-120}	2.40	0.65

Contrasts of Scheffe. The critical s-value for $p<0.05$ for each comparison is 4.28. h_0: Time for mucosal exposure to 4.5 M NaCl. h_1, h_2, —: One hour, two hours etc. after application of hypertonic NaCl

Table 2. One-way analysis of variance with Scheffe's test of antrum data in Fig. 6

	Distance to surface	Distance to bottom
DF	10;1309	10;1309
MS	38.85;2.76	35.93;2.76
F	14.09	13.0
p	<0.0005	<0.0005
Contrasts:		
h_0 vs h_1+h_2	6.18	2.68
h_0 vs $h_{12}+h_{18}$	5.96	2.80
h_0 vs h_{48-120}	3.50	0.32
h_1+h_2 vs $h_{12}+h_{18}$	0.26	0.17
h_1+h_2 vs h_{48-120}	4.10	4.20
$h_{12}+h_{18}$ vs h_{48-120}	3.78	4.40

Contrasts of Scheffe. The critical s-value for $p<0.05$ for each comparison is 4.28. h_0: Time for mucosal exposure to 4.5 M NaCl. h_1, h_2, —: One hour, two hours etc. after application of hypertonic NaCl

In autoradiographs the distance between labelled cells and mucosal surface or the distance between the labelled cells and the bottom of the glands were measured. The results obtained on the corpus/fundus mucosa are illustrated in Fig. 6. Statistical analysis of the data are recorded in Table 1. The distance between the labelled cells and the surface of the glands showed a significant decrease 1–2 h after application of hyperosmolar sodium cloride. The initial decrease was followed by an increase up to control levels between 12 and 18 h after the damage. No significant changes occurred between 18 and 120 h.

The distance between the labelled cells and the bottom of the glands showed a similar significant initial decrease followed by an increase to control level between 12 and 18 h. It should also be noted that the distance between the labelled cells and the surface and the distance to the bottom showed very similar values which means that most of the labelled cells were located in the middle of the mucosa during the whole experiment.

As shown in Fig. 6 and Table 2, the distance between labelled cells and mucosal surface in the antrum decreased significantly during the first 2 h after application of hyperosmolar sodium chloride.

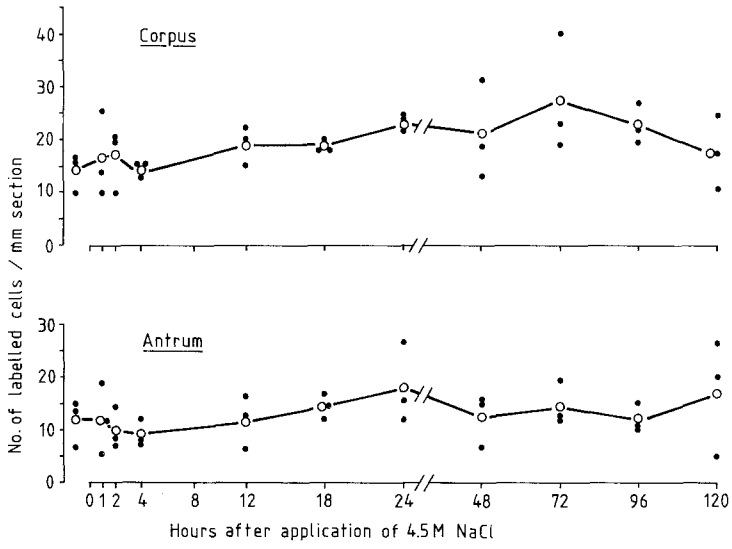

Fig. 7. Number of labelled cells per mm section in the corpus and antrum of rats, at different time intervals after mucosal exposure to 4.5 M NaCl. The corpus values represent mean values of counts obtained in the fundus and the corpus. The closed circles represent individual values for each animal and the open circles mean values

After the initial decrease, the values remained fairly constant up to 18 h after damage, after which the values increased towards control values between 48 and 120 h. The distance between labelled cells and gland bottom showed a similar curve as the distance to the surface. However, the statistical analysis showed no significant changes except for an increase towards the end of the experiment.

Regression analysis of the data in Fig. 7 showed that the concentration of labelled cells increased significantly during the first 24 h after application of 4.5 NaCl, both in the antrum ($y = 10.2 + 0.266\,x$, DF = 19, slope $t = 2.35$, $p < 0.05$) and the corpus ($y = 14.8 + 0.306\,x$, DF = 19, slope $t = 3.13$, $p < 0.01$). Between 24 h and 120 h the number of labelled cells/mm did not change significantly.

Discussion

In the present study intragastric instillation of hypertonic NaCl caused destruction and detachment of the mucosal surface epithelium in most of the corpus/fundus and antrum. After the mucosal damage, epithelial recovery occurred rapidly, so that most of the surface had an epithelial lining within 30–60 min. This corresponds very well to observations made on the in vitro frog and guinea pig gastric mucosa [6, 10, 11] and to results obtained in vivo on rats and cats [3, 5]. This early rapid epithelial repair is considered to be due to migration of cells form the bottom of the pits or the upper part of the glands, and not to cell proliferation [7].

After the initial epithelial restitution the mucosa gradually changed towards normal within 24–48 h after the damage. This change included a marked increase in the number of surface mucous cells which probably is the results of increased cellular proliferation although part of it might be due to decreased detachment of cells from the mucosal surface.

The distance between the labelled cells and the mucosal surface was found to be decreased 1–2 h after the damage, which is obviously due to detachment of surface epithelium and pits cells. This distance increased to predamage levels within 24 h which corresponds to the repair and normalization of the superficial part of the mucosa. The distance between the labelled cells and the bottom of the glands also decreased initially, probably as a result of dehydration and shrinkage of the cells, caused by the hypertonic NaCl. It is never the less surprising that the proliferative zone remained in the middle part of the glandular layer throughout the experiment.

Since the proliferative zone is located in the middle part of the glandular layer, and very few replicating cells are present in the superficial part of the mucosa, a labelling index in terms of the number of labelled cells per total number of the cells counted would not give satisfactory results under the present experimental conditions, since detachment of the upper part of the mucosa would lead to increased labelling index even if the mitotic activity remained constant.

After application of 4.5 M NaCl the surface epithelium and pits were destroyed while the glands remained essentially intact. This means that

the number of glands remained unchanged after the mucosal damage. The number of labelled cells per mm length of section therefore gives an expression of the number of labelled cells per gland independent on the total number of gland cells. It also reflects the potential number of proliferating cells per unit mucosal area that may be exposed to a carcinogen. The value will be influenced by interstitial oedema and cellular dehydration, which makes the results less reliable during the first hours after damage. In our study the number of labelled cells per mm section increased between 4 and 24 h after application of 4.5 M NaCl, which agrees well with the observation of Charnley et al. [2] who found that the percent of S-phase cells in the rat antral mucosa, as measured with flow cytometry, was increased by about 70% at 24 h after mucosal exposure to 3 M or 6 M NaCl.

It has been shown that concentrated sodium chloride instilled into the stomach of rats 24 h before the administration of MNNG increases the carcinogenic effect of MNNG [8, 9]. In our experiment concentrated NaCl was administered in the same way as in the cancer experiments referred to. At 24 h after application of 4.5 M NaCl the mucosal structure did not differ much from normal, however, the mitotic activity was increased. Proliferating cells are known to have increased suceptibility to carcinogens, and therefore the increased number of proliferating cells present at 24 h after application of hypertonic NaCl might explain the cocarcinogenic effect of hypertonic NaCl. However, in the present experiment the number of labelled cells per mm section was found to be just as large, or larger, in the corpus than the antrum. On the other hand, NaCl/MNNG induced tumours occur more frequently in the antrum than the corpus [9, 12], which means that increased mitotic activity, although important, cannot be the only reason to the cocarcinogenic action of hypertonic NaCl.

The chance that a MNNG molecule in the gastric lumen will reach a target cell in the middle of the mucosa, will depend on the number of proliferating cells per unit area of mucosa, but also on other factors such as concentration of MNNG in the gastric lumen, exposure time and permeability of the tissue between the mucosal surface and the replicating cells. The antral mucosa is much thinner than the corpus mucosa. In the antrum the distance between the mucosal surface and the proliferating zone was about half that of the corpus (Fig. 6), and this would be expected to influence the transport of a carcinogen from the gastric lumen to the proliferating cells.

After ingestion of concentrated NaCl in humans, as well as in experimental models where MNNG is given with the drinking water [14, 15] the mucosa may be exposed to carcinogens at various phases of damage and repair. The present study disclosed the morphological changes that occur after mucosal exposure to concentrated NaCl. However, except for the increased cell proliferation, we don't know if these changes are related to the cocarcinogenic action of NaCl.

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References

1. Capoferro R, Torgersen O (1974) The effect of hypertonic saline on the uptake of tritiated 7,12-dimethylbenz(a) anthracene by the gastric mucosa. Scand J Gastroent 9:343–349
2. Charnley G, Tannenbaum SR (1985) Flow cytometric analysis of the effect of sodium chloride on gastric cancer risk in the rat. Cancer Res 45:5608–5616
3. Dzienis H, Grønbeck JE, Varhaug JE, Lekven J, Svanes K (1987) Regional blood flow and acid secretion associated with damage and restitution of the gastric surface epithelium in cats. Eur Surg Res 19:98–112
4. Joossens JW, Geboers J (1983) Epidemiology of gastric cancer: A clue to etiology. In: Sherlock P, Morson BC, Barbara L, Veronesi U (eds) Precancerous lesions of the gastrointestinal tract. Raven Press, New York, pp 97–113
5. Lacy ER, Ito S (1984) Ethanol-induced insult to the superficial rat gastric epithelium: A study of damage and rapid repair. In: Allen A, Flemstrøm G, Garner A, Silen W, Turnberg LA (eds) Mechanisms of mucosal protection in the upper gastrointestinal tract. Raven Press, New York, pp 49–56
6. Rutten MJ, Ito S (1983) Morphology and electrophysiology of guinea pig gastric mucosal repair in vitro. Am J Physiol 244(7):G171–G182
7. Silen W, Ito S (1985) Mechanisms for rapid re-epithelialization of the gastric mucosal surface. Ann Rev Physiol 47:217–229
8. Shirai T, Imaida K, Fukushima S, Hasegawa R, Tatematsu M, Ito N (1982) Effects of NaCl, Tween 60 and a low dose of N-ethyl-N-nitro-N-nitrosoguanidine on gastric carcinogenesis of rat given a single dose of N-methyl-N-nitro-N-nitrosoguanidine. Carcinongenesis 3:1419–1422
9. Shirai T, Fukushima S, Ohshima M, Masuda A, Ito N (1984) Effects of butylated hydroxytoluene, and NaCl on gastric carcinogenesis initiated with N-Methyl-N-nitro-N-nitrosoguanidine in F344 rats. JNCI 72:1189–1198
10. Svanes K, Ito S, Takeuchi K, Silen W (1982) Restitution of the surface epithelium of the in vitro frog gastric mucosa after damage with hyperosmolar sodium chloride. Morphological and physiological characteristics. Gastroenterology 82:1409–1426
11. Svanes K, Takeuchi K, Ito S, Silen W (1983) Effect of luminal pH and nutrient bicarbonate concentration on restitution after gastric surface cell injury. Surgery 94:494–500
12. Takahashi M, Fukushima S, Sato H (1973) Carcinogenic effect of N-methyl-N-nitro-N-nitrosoguanidine with various kinds of surfactant in the glandular stomach of rats. Gann 64:211–218

13. Takahashi M, Shirai T, Fukushima S, Ito N, Kokubo T, Furukawa F, Kurata Y (1981) Ulcer formation and associated tumor production in multiple sites within the stomach and duodenum of rats treated with N-methyl-N-nitro-N-nitrosoguanidine. JNCI 67:473–479
14. Tatematsu M, Takahashi M, Fukushima S, Hananouchi M, Shirai T (1975) Effects in rats of sodium cloride on experimental gastric cancers induced by N-methyl-N-nitro-N-nitrosoguanidine or 4-nitroquinoline-1-oxide. JNCI 55:101–106
15. Tatematsu M, Takahashi M, Hananouchi M, Shirai T, Hirose M, Fukushima S, Ito N (1976) Protective effect of mucin on experimental gastric cancer induced by N-methyl-N-nitro-N-nitrosoguanidine plus sodium chloride in rats. Gann 67:223–229
16. Zar JH (1984) Biostatistical analysis, Sec. Ed. Prentice-Hall, Inc, Englewood Cliffs, New Jersey, pp 196–198

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