Duodenogastric Reflux Increases the Penetration of N-³H-Methyl-N-nitro-N-nitrosoguanidine into the Antral Mucosa of Rats: A Possible Role for Mucosal Erosions and Increased Cell Proliferation in Gastric Carcinogenesis

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Duodenogastric reflux is a risk factor for gastric carcinogenesis, but the pathogenesis is not fully understood. We studied the risk of N-methyl-N-nitro-N-nitrosoguanidine (MNNG)-induced carcinogenesis in the antrum of rats with duodenogastric reflux. Duodenal fluid was directed into the stomach through the pylorus (pyloric reflux group) or through a gastrojejunostomy (jejunal reflux group). After twenty-four weeks, 5-bromo-2-deoxyuridine (BrdU) was injected intravenously and the stomach was exposed to N-³H-methyl-N-nitro-N-nitrosoguanidine (³H-MNNG). The antral mucosa was examined with immunohistochemistry and autoradiography for identification of proliferating cells (BrdU labelled) and cells at risk of MNNG-induced carcinogenesis (³H-MNNG and BrdU-labelled cells). Duodenogastric reflux increased the number of double-labelled cells in the antral mucosa from 4.8±0.6 per mm in the control group to 11.3±1.9 in the jejunal reflux group (*P*<0.05) and 12.7±0.9 in the pyloric reflux group (*P*<0.05). Mucosal erosions were observed in 15 of 28 animals with pyloric reflux and the number of double-labelled cells in the erosion area (4.3±0.7) was higher than in the same area of animals without erosion (1.4±0.5) (*P*<0.05). Duodenogastric reflux increased the cell proliferation and significantly changed the distance between the surface epithelial lining and the proliferating cells when compared to the controls. These results indicate that duodenogastric reflux increases the penetration of ³H-MNNG into the antrum mucosa of rats. Increased cell proliferation and erosions increase the number of cells at risk of an initiation process from a penetrating gastric carcinogen.

Key words: Methylnitronitrosoguanidine — Duodenogastric reflux — Stomach neoplasms — Gastric mucosa — Mucosa erosion

Duodenogastric reflux is one of several risk factors for gastric carcinogenesis.[1] Other risk factors are *Helicobacter pylori* (*Hp*) infection and smoking,[2, 3] which also increase duodenogastric reflux.[4, 5] Increased levels of bile acids have also been observed in stomachs with atrophic gastritis, intestinal metaplasia and dysplasia.[6, 7]

Experiments in rats confirm that duodenogastric reflux induces gastric cancer without addition of exogenous carcinogens.[8] However, increased gastric levels of *N*-nitroso compounds in patients with a resected stomach suggest that duodenogastric reflux favours a milieu for conversion of nitrite to possible carcinogenic substances.[9]

The effect of duodenogastric reflux on gastric carcinogenesis has been studied mostly in the corpus of animals with resected antrum.[10–12] The documentation for a role of duodenogastric reflux in carcinogenesis of the antrum is therefore scanty.[8, 13] Taurocholic acid supplied in the food to mimic duodenogastric reflux after a period of initiation with N-methyl-N-nitro-N-nitrosoguanidine (MNNG)

increases the tumour yield both in the antrum and in the corpus mucosa of rats.[14]

The mucosal changes by which duodenogastric reflux predisposes to cancer are not clear. In humans, duodenogastric reflux induces reactive gastritis and increases intestinal metaplasia in *Hp*-infected patients.[6] In animal experiments, duodenogastric reflux induces changes in the corpus mucosa that precede the cancer in time or are found adjacent to the cancer. These changes include loss of specialised cells, hyperplasia of several elements of the mucosa, adenocystic glandular proliferation, erosions, and ulcer.[15, 16] Moreover, duodenogastric reflux increases the cell proliferation, and elongates the cell cycle time and the S-phase in the corpus mucosa.[17] Similar results are not available for the antrum.

To initiate carcinogenesis, a gastric carcinogen must penetrate through the superficial gastric mucosa to reach the proliferation compartment and become incorporated into the DNA during cell replication.[18, 19] The proliferation compartment is identical with the regenerative neck area of the glands where early gastric cancers are found and from which adenocarcinoma of the stomach arises in

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humans.[20, 21] Bromodeoxyuridine (BrdU), which is incorporated into DNA during cell replication (S-phase), identifies proliferating cells.[22] MNNG can be labelled with tritium (^{3}H) on the methylating group and traced by means of autoradiography. The penetration of carcinogen is quantified in terms of the number of cells with BrdU-labelled nuclei (cells in S-phase) which have incorporated N-^{3}H-methyl-N-nitro-N-nitrosoguanidine (^{3}H-MNNG) into their DNA (double-labelled cells). Double-labelled cells are considered to be the cell population at risk of MNNG-induced carcinogenesis.[18] Thus, an increased number of double-labelled cells in the mucosa implies an increased risk of N-nitroso compound-initiated gastric carcinogenesis.

It is reasonable to assume that duodenal reflux to the antrum changes the morphology of the mucosa, alters the proliferation compartment, and influences the penetration of carcinogens from the gastric lumen to the proliferation compartment. The gastric adenocarcinomas appear after 32–40 weeks of exposure to duodenogastric reflux and rarely within 20–24 weeks of surgery.[15, 16] The present study was designed to test whether duodenal reflux to the antrum causes mucosal changes that alter the exposure of proliferating cells to a carcinogen before the occurrence of neoplasms in the mucosa.

MATERIALS AND METHODS

Animals Ninety-two Mol:WIST male rats weighing 254±14 g (mean±SD) were supplied by Møllergård Breeding (Ejby, Denmark). The animals arrived at the facility 10 days before the experiments and they were standardised on arrival according to the FELASA accreditation scheme (Federation of European Laboratory Animals Science Association, Utrecht, Netherlands). The rats were housed in groups of 4 in MAK IV cages on aspen bedding. The animal room was maintained at 22±0.5°C and 50±10% relative humidity. The light cycle was 12h/12h with simulated sunrise and sunset. Room air was changed 18 times/h. The rats had ad libitum access to water and were fed RM1 expanded diet (Special Diets Services, Witham, UK) ad libitum. The experimental animal board of the Norwegian Department of Agriculture approved the experiments.

Chemicals N-^{3}H-Methyl-N-nitro-N-nitrosoguanidine (^{3}H-MNNG) (specific activity 1.0 Ci/mmol) was supplied by Amersham, Buckinghamshire, UK. 5-Bromo-2-deoxyuridine (BrdU), minimum 99% crystalline, was purchased from Sigma Chemical Co., St. Louis, MO. Halothane ("Fluothane," Zeneca, Ltd., Macclesfield, Cheshire, UK), buprenorphin ("Temgesic," Reckitt & Coleman, Ltd., Hull, England, UK) and pentobarbital-Na (Pentobarbital Natrium) were purchased from NMD (Norwegian Medicinal Depot ASA, Oslo, Norway).

Animal preparation The surgical procedure was performed with the animal on a heated operating table and the snout in a ventilated mask for spontaneous breathing of 1–2% halothane in a 50:50 blend of oxygen and nitrous oxide. The corneal reflex, the pedal withdrawal response and the rate of the spontaneous ventilation were used to evaluate the depth of the anaesthesia. The abdomen was opened in the midline and the stomach and duodenum exposed, carefully avoiding damage to blood vessels and the vagus nerves. The surgical procedures are described below. All anastomoses were performed with an extramucosal running suture (6/0 "Vicryl," Ethicon, Norderstedt, Germany). The abdominal wall was closed in layers with a running suture (4/0 "Vicryl").

After surgery, the animals remained in an incubator at 37°C until they regained consciousness and mobility. For the following two days, each animal was housed alone and supplied ad libitum with carbohydrates and electrolytes (FK Salt balance, Lactamin, Stockholm, Sweden) dissolved in tap water. Pain relief was ensured by a subcutaneous injection of buprenorphin 0.05 mg·kg^{-1} every 12 h and on signs of discomfort. For the remaining experimental period, there were four animals in each cage. The animals received tap water and food pellets ad libitum. They were inspected daily and weighed every fourth week. Animals showing signs of discomfort, malnutrition or diseases were killed and those lost within the first two weeks of the operation were replaced. Animals that died later were excluded. Only those surviving the final experiment entered the evaluation.

After twenty-four weeks the carcinogen penetration experiment was performed. For the last 24 h, the animals received only tap water and were kept individually in wire-bottomed cages to avoid coprophagia. The anaesthesia was induced by inhalation of 5% halothane followed by an intraperitoneal injection of pentobarbital-Na (60 mg·kg^{-1}). Increments of pentobarbital were given intravenously upon return of the corneal reflex or pedal withdrawal response. The body temperature was kept at 38.0±0.1°C. Blood pressure and heart rate were monitored by inserting a polyethylene catheter (outer diameter 0.63 mm) through the femoral artery into the abdominal aorta. The catheter was connected to a SensoNor 840 pressure transducer (Horten, Norway) and a Gould WindoGraf 980 recorder (Cleveland, Ohio). Ringer acetate was infused (1 ml·100 g^{-1}·h^{-1}) via a catheter inserted into the right femoral vein. The stomach was exposed and a ligature around the distal oesophagus and duodenum prevented loss of fluid instilled into the stomach. In animals with a gastrojejunostomy, the jejunum was ligated close to the gastric wall. A polyethylene tube (outer diameter 0.35 cm and length 2.8 cm) inserted and fixed into the apical portion of the forestomach served as an entrance to the stomach. Food remnants were removed by flushing the stomach

with saline. After surgery, the animals were allowed to stabilize for 30 min. The stomach was rinsed with 2 ml of deionized water every 10 min during the experiment. BrdU (30 mg·kg^{-1}) was injected intravenously 1 h before euthanasia. 555 μCi ^{3}H-MNNG was dissolved in 2 ml of deionized water and the solution was shaken vigorously with a laboratory mixer before instillation into the stomach. Dilution of the carcinogen was avoided by rinsing and emptying the stomach with 2 ml of deionized water immediately before the ^{3}H-MNNG was instilled. The stomach was exposed to the ^{3}H-MNNG solution for 10 min and the animals were killed by severing the inferior caval vein. The stomach was removed, opened along the greater curvature and pinned flat onto a corkboard. The mucosa was inspected for tumours and any change of the mucosa was noted. The stomach was fixed in 70% ethanol for 48 h. Whole wall samples of the corpus were dehydrated and embedded in paraffin. Sections (4 μm thick) were prepared for immunohistochemistry and autoradiography or stained with hematoxylin and eosin for conventional light microscopy.

The abdominal cavity was searched for metastases, the liver was cut through and any noted change was the object of biopsy.

Immunohistochemistry and autoradiography Paraffin sections (4 μm thick) were mounted on glass slides coated with chrome alum-gelatin and deparaffinized. BrdU-labelled cells were stained by means of the avidin biotin peroxidase complex method. After immunohistochemistry, the sections were processed for autoradiography. Both methods have been described in detail.[23] Tissue sections devoid of ^{3}H-MNNG but processed identically served as negative controls. One experimental slide exposed to daylight before the four-week processing period served as a control for false negative result.[24] No background radiation, chemical reaction between diaminobenzidine and the autoradiography film, or fading of the latent image during the four weeks of exposure was detected. All slides were processed using the same batch of chemicals and slides lost or broken during the processing were not reprocessed alone to avoid biases from difference in chemicals.

Microscopic examination Light microscopy and a structured examination scheme were used for the histological evaluation. With 400× magnification, the labelled cells were grouped into two categories. Cells with a brown deposit (DAB staining) over the whole nucleus had incorporated BrdU into their DNA and were classified as cells in the S-phase. BrdU-labelled cells with three or more silver grains (^{3}H-MNNG) over their nuclei were regarded as double-labelled cells and at risk of MNNG-induced carcinogenesis.[18, 25] An ocular micrometer, measuring 0.29 mm at 400× magnification was used as a unit and constituted one measuring field. BrdU and double-labelled cells were counted in 40 consecutive fields of the gastric mucosa from the pylorus towards the oesophagus along the lesser curvature. These fields were later grouped into five equal zones and numbered I through V from the pylorus and upwards along the lesser curvature.

The mucosa was divided into a superficial, a proliferation, and a deep compartment (Fig. 1). Two imaginary lines, made visible by the ocular micrometer, along the uppermost and lowermost BrdU-labelled cells in each field bordered the proliferation compartment. The superficial compartment was defined as the area between the surface epithelial lining and the proliferation compartment, and the deep compartment as the area between the muscularis mucosae and the proliferation compartment. The muscularis mucosa served as the reference point for the morphometric measurements, and when it could not be readily identified, the luminal side of the submucosa was used as the reference. The width of the different compartments was measured in all 40 fields according to Fig. 1.

Lesion was used as common term for ulcer and erosion. Whenever present, gastric mucosal lesions and their location were recorded. The term mucosal erosion was used when the surface mucous cell layer, the foveolar epithelium or the superficial part of the glands was injured or absent. When glands with the proliferating compartment were absent, the lesion extended through the muscularis mucosae, or the mucosa glands were replaced by a granulation tissue, the lesion was classified as an ulcer.
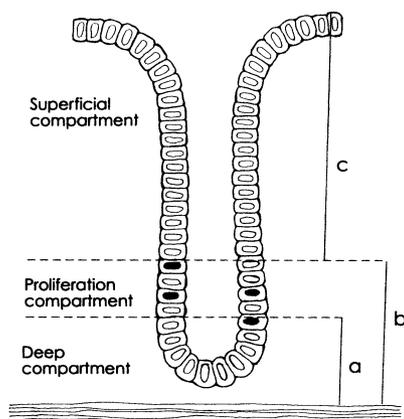

Fig. 1. Sketch illustrating the antrum mucosa. Base line is the muscularis mucosae. a, the deep compartment, measured as the distance from the muscularis mucosae to the top of the deepest BrdU-labelled cell in the proliferation compartment; b, the distance from the muscularis mucosae to the top of the uppermost BrdU-labelled cell in the proliferation compartment; c, the superficial compartment, measured as the distance from luminal border of the surface mucous cells to the top of the uppermost BrdU-labelled cell in the proliferation compartment. The width of the proliferation compartment is the difference between the lengths b and a.

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Experimental groups The animals were distributed at random into three groups. An 8 mm gastrotomy was fashioned along the greater curvature starting 2 mm distally to the border of the forestomach. Fig. 2 illustrates the surgical procedures. In the control group, the gastrotomy was closed as a sham operation. In the jejunal reflux group, the surgical procedure directed duodenal fluid through the gastrojejunostomy into the corpus area of the stomach, through the antrum and out through the pylorus. In the pyloric reflux group, the surgical procedure directed duodenal fluid through the pylorus into the antrum,

through the corpus, and out through the gastrojejunostomy. The jejunum was opened along the anti-mesenteric side to fit the gastrotomy.

Statistics The microscopy results were obtained with 400× magnification from 40 consecutive fields of the mucosa (each of 0.29 mm). The measured area was grouped into five equal zones each consisting of eight neighbouring fields and numbered I through V from the pylorus and upwards along the lesser curvature. For each measured variable, a mean value was calculated for every zone. The data analyses were performed with the SPSS ver. 9.0 statistical package. In two-way analysis of variance (ANOVA), the number of proliferating cells, the localization of the proliferating cells in the mucosa, and the number of double-labelled cells, were the dependent variables. The experimental groups (the surgical procedure) represented one grouping factor; the five microscopy zones represented the second. Otherwise, variables were compared by one-way analysis of variance with experimental groups as the grouping factor. Newman-Keuls multiple range tests were applied whenever justified by the
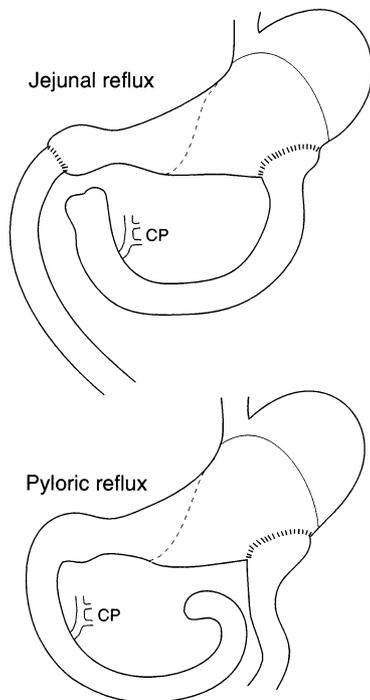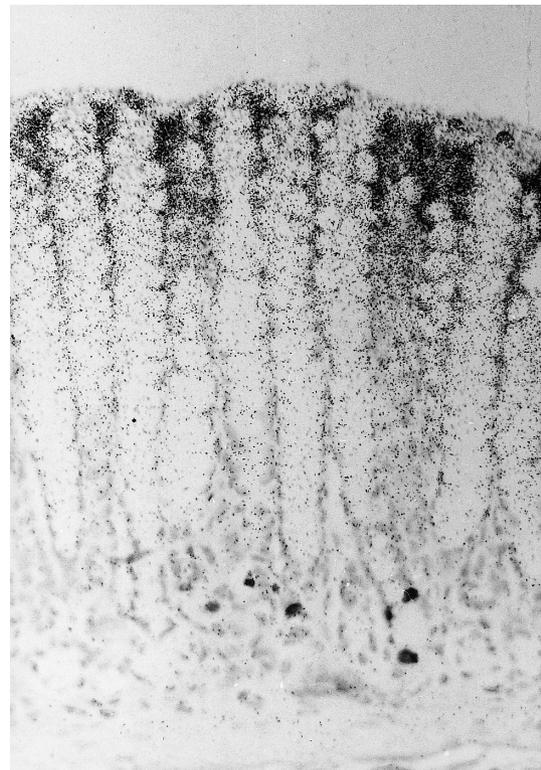

Fig. 2. Sketch of the surgical procedures. CP, the common bile duct and the pancreatic ducts. An 8 mm gastrotomy was fashioned along the greater curvature starting 2 mm distally to the border of the forestomach. In the jejunal reflux group, a duodenal segment with the common bile duct and pancreatic ducts was isolated by dividing the duodenum proximally to the entrance of the bile and pancreatic ducts and the jejunum 5 cm distally to the duodeno-jejunal junction. The proximal part of this segment was closed blindly and the distal part was sutured end-to-side to the 8-mm gastrotomy in the corpus. The procedure directed the duodenal fluid first into the corpus of the stomach and then out through the antrum. In the pyloric reflux group, the jejunum was divided 2 cm distally to the duodeno-jejunal junction. The proximal end of the bowel was closed with a suture and the distal end was sutured end-to-side to the gastrotomy in the corpus. This procedure directed the duodenal fluid through the pylorus into the antrum, through the corpus, and out through the gastrojejunostomy.

Fig. 3. Photomicrograph from zone III in the antrum of a control rat (×320). Cells in S-phase are labelled with BrdU (black nuclei). Most of the ^{3}H-MNNG (black grains) is accumulated in the surface mucus cells and pit cells.

analysis of variance. Cross-tables with two-sided Pearson chi-square tests and Fisher's exact tests were used for comparison of categorical variables. A *P*-value less than 0.05 was regarded as statistically significant, except for the interaction effects in the two-way analyses of variance, where a *P*-value less than 0.1 was required. Unless otherwise indicated, data are presented with mean±SEM.

RESULTS

One animal with jejunal reflux died 21 weeks after the primary surgical procedure and one animal with pyloric reflux died during the second surgical procedure. Slides from eight of the animals were lost during the processing (2 of the control group, 4 of the jejunal reflux group, and 2 of the pyloric reflux group).

Double-labelled cells, cell proliferation and mucosa morphometry The duodenogastric reflux increased the number of double-labelled cells in the antrum mucosa
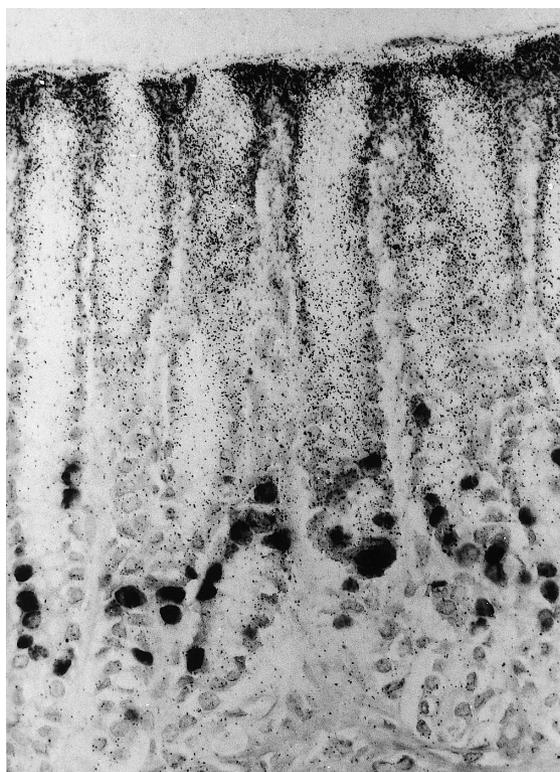

Fig. 4. Photomicrograph from zone III in the antrum of a rat with jejunal reflux (×320). Cells in S-phase are labelled with BrdU (black nuclei). Most of the ^{3}H-MNNG (black grains) is accumulated in the surface mucus cells and pit cells, but the proliferation compartment is expanded with numerous cells in the S-phase and many of them are located in areas heavily loaded with ^{3}H-MNNG.
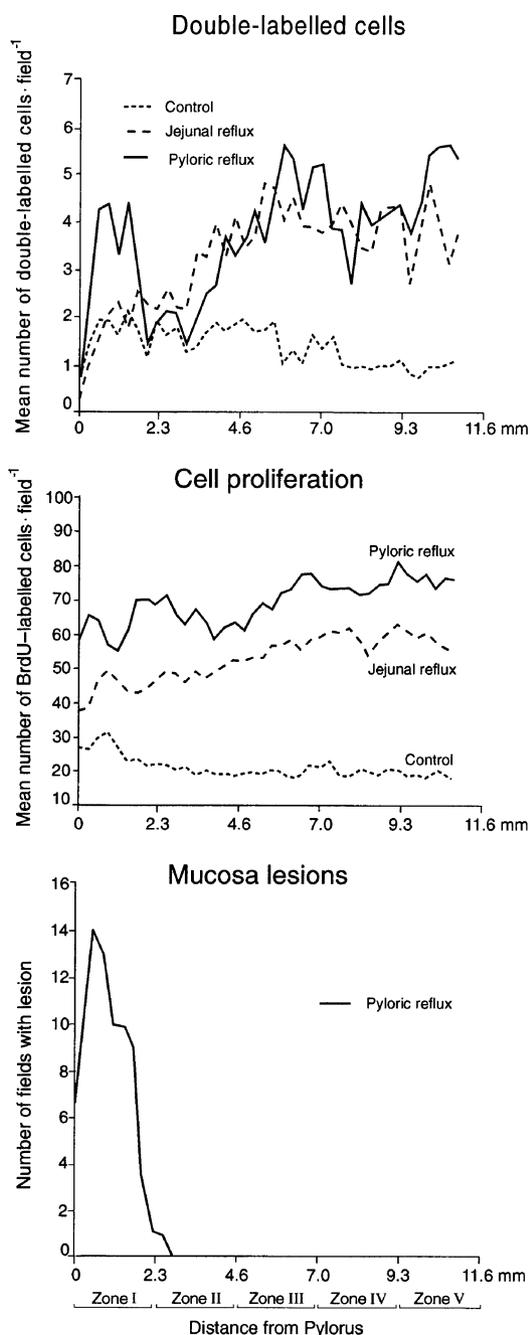

Fig. 5. Data from all animals. The figure shows the mean number of double-labelled cells, the mean cell proliferation (BrdU-labelled cells), and the location of mucosa lesions including their margins in the antrum of the stomach. Forty consecutive fields, each 0.29 mm long, were examined from the pylorus (zero) upwards along the lesser curvature of the antrum. The number and the location of fields with a lesion, including the margins of the lesions, are shown in the lower panel. The examined mucosa is divided into five equal zones (zones I–V) each of 2.3 mm. *N*=81 animals, 28 in the control group, 25 in the jejunal reflux group and 28 in the pyloric reflux groups.

Table I. Double-labelled Cells, Cell Proliferation, and Morphometric Findings in Different Zones of the Antrum Mucosa (Mean Number of Cells per Zone and Mean Distance per Zone)

| | | Zones | | | | | |
| | n | I | II | III | IV | V | ANOVA |
		(A)	(B)	(C)	(D)	(E)	
Double-labelled cells							
Control	28	1.6±0.3	1.7±0.3	1.6±0.2	1.2±0.2	0.9±0.1	P_g<0.001
Jejunal reflux	25	1.7±0.6	2.9±0.6	4.2±0.7[*, a]	3.9±0.6[*, a]	3.8±0.7[*, a]	P_z=0.001
Pyloric reflux	28	2.9±0.5	2.3±0.4	4.3±0.6[*, b]	4.1±0.4[*, b]	4.8±0.5[*, b]	P_i=0.006
Cell proliferation (BrdU labelled)							
Control	28	26.2±2.0	20.4±2.0	19.1±1.6	20.2±1.7	18.9±1.1	P_g<0.001
Jejunal reflux	25	43.5±4.0*	48.2±3.9*	54.6±3.4*	58.7±3.6[*, a]	58.7±3.8[*, a]	P_z=0.019
Pyloric reflux	28	62.5±5.0[*, †]	65.1±3.4[*, †]	68.7±4.0[*, †]	73.7±4.0[*, †]	76.3±4.6[*, †, a]	P_i=0.020
Superficial compartment (μm)							
Control	28	124.1±4.8	153.9±6.6[a]	150.4±4.6[a]	169.7±4.2[a]	178.5±3.7[a, b, c]	P_g<0.001
Jejunal reflux	25	117.3±7.9	153.9±6.2[a]	160.1±6.3[a]	168.7±6.2[a]	172.4±6.4[a]	P_z<0.001
Pyloric reflux	28	137.8±10.5	218.9±11.0[*, †, a, c, e]	186.7±10.0[*, †, a]	199.7±9.7[*, †, a]	190.9±7.3[a]	P_i=0.008
Proliferation compartment (μm)							
Control	28	41.2±2.5	40.3±2.6	37.8±2.3	39.5±1.9	40.5±1.7	P_g<0.001
Jejunal reflux	25	50.6±3.8	62.1±3.1*	68.7±3.9[*, a]	72.5±3.5[*, a]	73.2±4.0[*, a]	P_z=0.051
Pyloric reflux	28	125.9±7.8[*, †, b, c, d, e]	113.1±7.1[*, †, c, d, e]	82.4±3.6[*, †]	88.2±8.9[*, †]	83.3±4.2*	P_i<0.001
Deep compartment (μm)							
Control	28	33.6±1.3	33.9±1.3	33.6±1.3	36.9±1.3	36.8±1.2	P_g<0.001
Jejunal reflux	25	36.0±2.6	41.7±2.1	43.8±2.6*	38.2±1.8	38.8±1.9	P_z<0.001
Pyloric reflux	28	101.1±8.7[*, †, b, c, d, e]	64.7±3.0[*, †, c, d, e]	44.8±2.1*	38.9±1.5	40.4±1.9	P_i<0.001
Mucosa thickness (μm)							
Control	28	198.9±6.9	228.1±8.2	221.7±5.7	246.1±4.8[a]	255.8±4.5[a]	P_g<0.001
Jejunal reflux	25	203.9±12.5	257.8±8.2[*, a]	272.5±10.8[*, a]	279.3±7.9[*, a]	284.4±9.9[a]	P_z<0.001
Pyloric reflux	28	364.8±13.6[*, †, c, d, e]	396.7±17.3[*, †, a, c, d, e]	313.9±10.6[*, †]	326.9±15.8[*, †]	314.6±8.9[*, †]	P_i<0.001

Mean±SEM for each zone. n, number of animals; zones, see Fig. 5 for localization of the different zones; P_g, probability for group effects; P_z, probability for effects of different zones (I–V); P_i, probability for effects of interaction. Lower case letter denotes a significant difference from the zone with the corresponding capital letter within the same experimental group, P<0.05 by Newman-Keuls multiple range tests.
∗ P<0.05, different from control within the same zone by Newman-Keuls multiple range tests; † P<0.05, different from the other intervention group (group II versus group III) within the same zone by Newman-Keuls multiple range tests.

from 4.8±0.6 double-labelled cells·mm⁻¹ in the control group to 11.3±1.9 in the jejunal reflux group (P<0.05) and 12.7±0.9 in the pyloric reflux group (P<0.05). Figs. 3 and 4 show the microscopic picture of the antrum mucosa in the control group and in the jejunal reflux group. The distribution of double-labelled cells and cell proliferation along the lesser curve of the antrum is shown in Fig. 5. Results of the statistical analyses are given in Table I. In the control group, the mean number of double-labelled cells was similar throughout all zones. In the jejunal reflux group, the mean number of double-labelled cells increased significantly with the distance from the pylorus, and in

zones III–V the mean number of double-labelled cells was 3–4 times higher than in the control group (P<0.05). In zones III–V of the pyloric reflux group, the mean number of double-labelled cells was 3.5–5 times higher than in the control group (P<0.05). In zones III–V the number of double-labelled cells was similar in the pyloric and the jejunal reflux groups, whereas in zone I the number of double-labelled cells was approximately twice as high in the pyloric reflux group as in the jejunal reflux group and in the control group.

The cell proliferation in the jejunal reflux group and in the pyloric reflux group was significantly increased

throughout all zones when compared to the control group (Fig. 5, Table I) and in all zones the cell proliferation was significantly higher in animals with pyloric reflux than in animals with jejunal reflux. Whereas the cell proliferation was similar in all zones of the control group, the cell proliferation increased significantly with the distance from the pylorus in the two intervention groups.

The mucosa thickness (Table I) was significantly higher in the jejunal reflux group and in the pyloric reflux group than in the control group, and the mucosa was significantly higher in the pyloric reflux group than in the jejunal reflux group. In the control group and in the jejunal reflux group the mucosal thickness increased significantly with the distance from the pylorus, whereas in the pyloric reflux group the mucosa was highest close to the pylorus (zones I–II).

The width of the superficial compartment was similar in the jejunal reflux and the control groups, and in both groups the width increased significantly with the distance from the pylorus. Pyloric reflux expanded the superficial compartment significantly in most zones when compared to jejunal reflux and the control animals, and the expansion of the superficial compartment was most distinct in zone II.

Both pyloric and jejunal reflux expanded the proliferation compartment significantly when compared to the control group, and the proliferation compartment was signif-
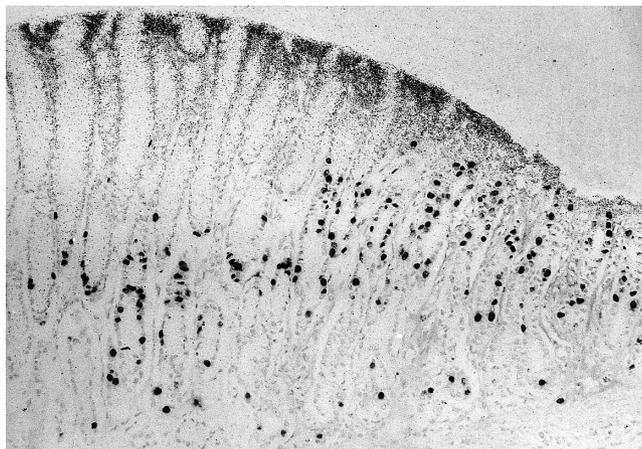
icantly wider in the pyloric than in the jejunal reflux group. In the control group, the width of the proliferation compartment was similar in all zones. In the jejunal reflux group, the width of the proliferation compartment increased with the distance from the pylorus, whereas in the pyloric reflux group, the proliferation compartment decreased with distance from the pylorus.

The deep compartment was significantly wider in animals with pyloric reflux than in animals with jejunal reflux or in controls, but the width of the deep compartment and the differences between the groups declined with the distance from the pylorus.

Animals with a mucosa lesion A mucosa lesion was observed in 15 of 28 (54%) animals with pyloric reflux but not in animals with jejunal reflux or in controls ($P<0.001$). The pyloric reflux group was therefore considered separately. Most of the lesions were erosions where the superficial compartment was partly or completely detached. In the centre of two lesions, the glands with the proliferation layer were lost, and data from the ulcer base were therefore excluded from the analyses. The mucosal erosions were 0.5 ± 0.1 mm long and located in zone I, the pre-pyloric area.

Fig. 6 shows the microscopic picture of an erosion and neighbouring mucosa. Table II and Fig. 7 compare results obtained in animals with and without mucosal erosions in the pyloric reflux group. In zone I (the erosion area) the mean number of double-labelled cells and the cell proliferation were significantly higher, and the proliferation compartment was significantly wider in animals with a mucosa erosion than in animals without erosion. Moreover, the thickness of the mucosa and the superficial compartment were significantly reduced in animals with erosion when compared to animals without erosion.

In zone II, the area adjacent to the erosions, the mean number of double-labelled cells tended towards being lower (though not significantly) in animals with erosion than in animals without erosion. In animals with erosion, however, the number of double-labelled cells was significantly lower in zone II than in the rest of the mucosa. The superficial compartment and the proliferation compartment in zone II were significantly wider, and the mucosa was significantly thicker in animals with erosion than in those without.

Both in animals with and without mucosa erosion, the deep compartment was significantly wider in zone I and II than in the rest of the mucosa. Except for zone I, the cell proliferation was similar throughout all zones. In zones IV–V all the examined variables were similar in animals with or without mucosa erosion.

Mucosa malignancy No malignant tumours or carcinoma of the mucosa was observed in either of the groups. Low-grade dysplasia was found in the pre-pyloric area in three (10%) animals with pyloric reflux.

Fig. 6. Margin and neighbouring mucosa of erosion in antrum of a rat with pyloric reflux (photomicrograph, ×125). Cells in S-phase are labelled with BrdU (black nuclei). ^{3}H-MNNG (black grains) is present at the surface of the mucosa, in the pits and the upper part of the glands. The proliferation compartment is expanded with numerous cells in the S-phase and in the margin, many of them being located close to the mucosa surface. Note the different distance from the mucosa surface to the cells in S-phase within the erosion (zone I) and the neighbouring mucosa (zone II).

Table II. Animals in the Pyloric Reflux Group with or without a Mucosa Erosion. Double-labelled Cells, Cell Proliferation, and Morphometric Findings in Different Zones of the Antrum Mucosa (Mean Number of Cells per Zone and Mean Distance per Zone)

	n	Zones					ANOVA
		I (Erosion area)	II	III	IV	V	
		(A)	(B)	(C)	(D)	(E)	
Double-labelled cells							
With erosion	15	4.3±0.7*	1.5±0.3[a, c, d, e]	4.3±0.9	4.4±0.6	5.2±0.8	P_z=0.001
Without erosion	13	1.4±0.5[c, d, e]	3.1±0.7	4.4±0.7	3.8±0.6	4.3±0.6	P_e=0.205
							P_i=0.019
Cell proliferation (BrdU labelled)							
With erosion	15	75.8±6.3*	67.6±4.8	64.9±5.8	69.5±5.0	75.8±6.1	P_z=0.069
Without erosion	13	47.1±5.7[c, d, e]	62.1±4.9	73.0±5.5	78.4±6.5	76.8±7.3	P_e=0.379
							P_i=0.010
Superficial compartment (μm)							
With erosion	15	95.7±7.8[*, b, c, d, e]	236.0±15.4[*, e]	203.4±16.6*	198.9±13.6	190.2±9.8	P_z<0.001
Without erosion	13	186.3±9.0	199.2±14.1	167.5±7.7	200.6±14.4	191.6±11.3	P_e=0.596
							P_i<0.001
Proliferation compartment (μm)							
With erosion	15	146.9±8.0[*, c, d, e]	130.3±10.5[*, c, d, e]	83.8±4.3	77.6±4.7	81.6±4.4	P_z<0.001
Without erosion	13	101.6±10.7	93.3±5.7	80.8±6.1	100.5±18.2	85.4±7.7	P_e=0.036
							P_i<0.001
Deep compartment (μm)							
With erosion	15	97.3±13.8[b, c, d, e]	68.4±3.9[c, d, e]	44.6±2.3	39.3±1.8	42.6±3.1	P_z<0.001
Without erosion	13	105.5±10.3[b, c, d, e]	60.4±4.6[c, d, e]	44.9±3.9	37.8±2.5	37.8±1.7	P_e=0.747
							P_i=0.746
Mucosa thickness (μm)							
With erosion	15	339.9±18.7*	434.6±25.0[*, a, c, d, e]	331.8±16.4	316.4±12.0	314.3±9.5	P_z<0.001
Without erosion	13	393.4±17.4[c, e]	352.9±17.5	293.2±10.7	338.9±31.6	314.9±16.2	P_e=0.454
							P_i=0.004

Mean±SEM for each zone. n, number of animals; zones, see Fig. 7 for localization of the different zones; P_z, probability for effects of different zones (I–V); P_e, probability for effect of erosion; P_i, probability for effects of interaction. Lower case letter denotes a significant difference from the zone with the corresponding capital letter within the same experimental group, P<0.05 by Newman-Keuls multiple range tests. *P<0.05, different from animals without erosion within the same zone by Newman-Keuls multiple range tests.

DISCUSSION

Several important results in terms of gastric carcinogenesis were identified in the present study. Duodenogastric reflux increased the number of double-labelled cells in the antrum mucosa, cells that are considered to be at risk of MNNG-induced carcinogenesis (Fig. 5, Table I).[18, 25] The increased number of double-labelled cells appeared at the lesser curvature, the most frequent location of gastric cancer in animal models.[26] The amount and distribution of double-labelled cells were similar in the jejunal and pyloric reflux groups except for zone I, in which there was mucosa erosion in the pyloric reflux group (Fig. 5). The increased number of double-labelled cells was related to increased cell proliferation and expansion of the proliferation compartment, which implies an increase in the number of cells at risk of becoming initiated for carcinogenesis. Mucosal erosion was associated with an increased number

of double-labelled cells in zone I. An expanded superficial compartment adjacent to the erosions (zone II) reduced the number of double-labelled cells, probably by increasing the distance the carcinogen has to penetrate before reaching the proliferation compartment. Low-grade dysplasia was found in the antrum mucosa of 10% of the animals with pyloric reflux, but as expected, no cancer was discovered as early as 24 weeks after the surgical procedure.[15, 16] We assume that the morphologic changes observed in this study precede the development of cancer and explain, at least in part, the carcinogenic effect of duodenogastric reflux in the antrum.[13, 14]

It is generally believed that increased cell proliferation represents an increased risk for carcinogenesis.[27] We have shown that increased cell proliferation and an expanded proliferation compartment are associated with an increased number of double-labelled cells. A possible mechanism for the carcinogenesis in duodenogastric reflux is therefore
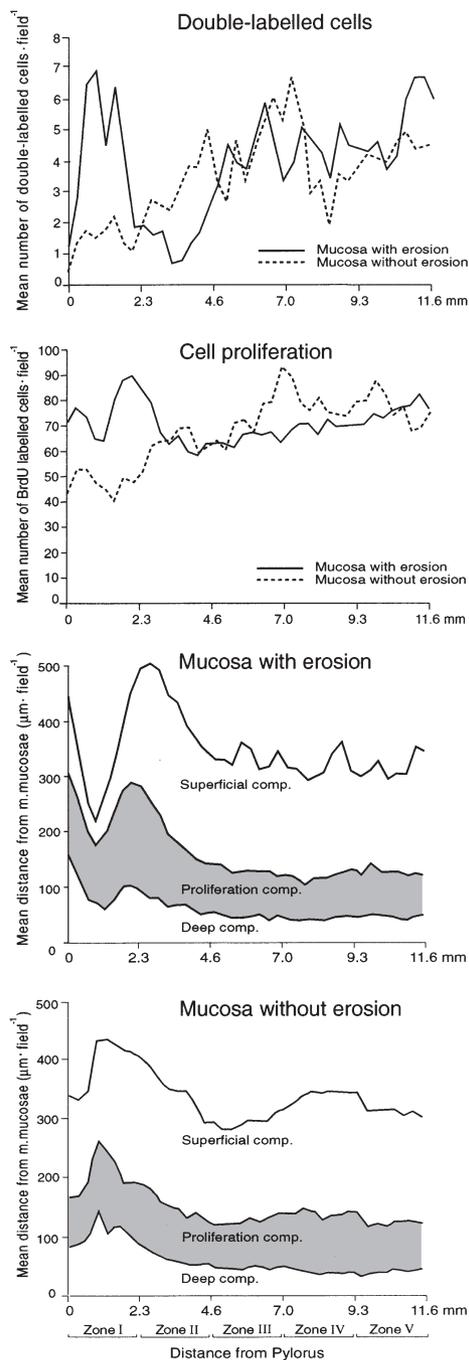
Fig. 7. Animals with pyloric reflux. The figure shows the mean number of double-labelled cells and the mean cell proliferation (BrdU-labelled cells) in animals with pyloric reflux, 15 animals with a mucosa erosion and 13 animals without mucosa erosion. The two lower panels show the morphometry of the antrum mucosa of animals with or without mucosa erosion (see Fig. 1). Forty consecutive fields, each 0.29 mm long, were examined from the pylorus (zero) upwards along the lesser curvature of the antrum. For the statistical analyses, the examined mucosa is divided into five equal zone areas (zones I–V).

the increased number of cells at risk of repeated hits by the carcinogen over a long period of time (multi-hit process) due to the chronic stimulation of cell proliferation.[28] Moreover, the increased cell proliferation may expand clones of single hit cells and thereby increase the probability for a second or several new hits in the same cell.[28] A total of four to six genetic events are regarded as necessary for tumour development and carcinogenesis.[29] Persistently increased cell proliferation due to duodenogastric reflux could also promote the progression from initiated cells to overt cancer development.[19]

A changed width of the superficial compartment could be expected to influence the number of double-labelled cells by changing the distance the carcinogen has to penetrate before reaching the proliferating cells. In animals with pyloric reflux, the cell proliferation was significantly higher than in those with jejunal reflux, whereas the number of double-labelled cells was similar (Table I, Fig. 5). However, the superficial compartment was wider in animals with pyloric reflux than in those with jejunal reflux, which may explain why the two intervention groups had about the same number of double-labelled cells in zones II–V. It should also be noted that in zone II of animals with pyloric reflux the superficial compartment was thicker and the number of double-labelled cells lower than in the other zones. These observations indicate that the expanded superficial compartment observed in animals with pyloric reflux provides some protection against carcinogen penetration.

Pre-pyloric erosions were found in about half of the animals with pyloric reflux. Animals with jejunal reflux did not have erosions, which is probably due to dilution of the duodenal fluids during passage from the gastrojejunostomy via the corpus to the antrum. In the pyloric reflux group the number of double-labelled cells in zone I was much higher in animals with a pre-pyloric erosion than in those without erosion (Fig. 7). This is in accordance with a former study where we demonstrated that superficial injury of mucosa induced by salt facilitates the penetration of MNNG into the proliferating layer of the gastric mucosa.[25] Thus, the present findings suggest a role for antrum mucosal erosions in gastric carcinogenesis by being an area of reduced resistance to penetrating carcinogens. Erosions have also been observed overlying microscopic or early gastric cancers in man.[21] Epidemiological studies have provided evidence for increased risk of gastric cancer in humans with a history of gastric ulcer, but have not been able to establish a significant relationship between pre-pyloric ulcers or erosions and an increased risk of gastric cancer.[30, 31]

It is likely that the increased cell proliferation and the expanded proliferation compartment of the gastric mucosa, which increase the number of cells at risk of being hit by a carcinogen, are the most important factors contributing to

the increased number of double-labelled cells in this study. In addition, the thickness of the superficial compartment appears to play a role in controlling the penetration of carcinogen, and erosion of the superficial compartment increases the exposure of the proliferating cells to the carcinogen. However, increased tissue permeability to the carcinogen is also a potential explanation of the increased number of double-labelled cells. Duodenogastric reflux increases the pH of the fluids in the antrum,[32] and MNNG is absorbed from the intestine by passive diffusion, with a maximum at pH 6. The diffusion is greatly facilitated by the bile salt taurocholate[33] and conjugated bile salts disrupt the gastric mucosa barrier by dissolving phospholipids and cholesterol from the mucosa.[34] The bile salts increase the back-diffusion of H^+ and eventually cause mucosal injury.[35] It is, however, difficult to evaluate changes in permeability in the present model because of the multiple morphologic changes occurring simultaneously.

It is possible that the associations between increased cell proliferation, mucosal lesions and a high number of double-labelled cells in the antral mucosa of this study represent basic mechanisms in gastric carcinogenesis of the antral mucosa. *Hp*, which is considered a risk factor for gastric cancer in both humans and animals,[3, 36, 37] increases the cell proliferation in the antral mucosa and induces gastritis and mucosal lesions.[38, 39] Moreover, the cell proliferation is significantly higher in mucosa with chronic atrophic gastritis and intestinal metaplasia than in the normal gastric mucosa.[40] It is therefore possible that one of the carcinogenic effects of *Hp* infection and mucosal changes such as chronic atrophic gastritis and intestinal metaplasia is mediated by an increased number of proliferating cells at risk of being exposed to a penetrating carcinogen.

Increased duodenogastric reflux causes mucosal changes that result in increased exposure of proliferating cells to carcinogens (increased number of double-labelled cells). This is mainly due to increased cell proliferation and expansion of the proliferation compartment in the mucosa. The superficial compartment between the surface epithelial lining and the proliferation compartment provides some protection against carcinogens penetrating from the gastric lumen into the mucosa. Mucosal erosion in the antrum probably represents an increased carcinogenic risk by increasing cell proliferation and reducing the thickness of the mucosa above the proliferation compartment.

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