Reversible Drug–Induced Oxyntic Atrophy in Rats

JAMES R. GOLDENRING,* GREGORY S. RAY,* ROBERT J. COFFEY, Jr.,‡ PAUL C. MEUNIER,§ PATRICK J. HALEY,§ T. BRADFORD BARNES,‖ and BRUCE D. CAR§

*Augusta Veterans Affairs Medical Center and Institute of Molecular Medicine and Genetics, Departments of Medicine, Surgery, and Cellular Biology and Anatomy, Medical College of Georgia, Augusta, Georgia; §DuPont Pharmaceutical Corp., Newark, Delaware; ‡Departments of Medicine and Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee; and ‖Cephalon, West Chester, Pennsylvania

Background & Aims: Oxyntic atrophy is the hallmark of chronic gastritis. Many studies have sought to develop animal models for oxyntic atrophy, but none of them are reversible. We now report that rats administered high doses of DMP 777 demonstrate reversible oxyntic atrophy. *Methods:* DMP 777 was administered to CD-1 rats by oral gavage (200 mg · kg^{-1} · day^{-1}). Serum gastrin level, in vivo acid secretion, and gastric histological changes were evaluated in DMP 777–dosed animals. Direct effects of DMP 777 on parietal cells were evaluated by assessment of aminopyrine accumulation into isolated rabbit parietal cells, as well as by assessment of DMP 777 effects on acridine orange fluorescence and H^+,K^+-adenosine triphosphatase (ATPase) activity in isolated tubulovesicles. *Results:* Oral dosing with DMP 777 caused a rapid increase in serum gastrin levels and severe hypochlorhydria. DMP 777 inhibited aminopyrine accumulation into rabbit parietal cells stimulated with either histamine or forskolin. DMP 777 reversed a stimulated proton gradient in isolated parietal cell tubulovesicles. Oral dosing with DMP 777 led to rapid loss of parietal cells from the gastric mucosa. In response to the acute loss of parietal cells, there was an increase in the activity of the progenitor zone along with rapid expansion of the foveolar cell compartment. DMP 777 treatment also led to the emergence of bromodeoxyuridine-labeled cells and cells positive for periodic acid–Schiff in the basal region of fundic glands. With extended dosing over 3–6 months, foveolar hyperplasia and oxyntic atrophy were sustained while chief cell, enterochromaffin-like cell, and somatostatin cell populations were decreased. No histological evidence of neoplastic transformation was observed with dosing up to 6 months. Withdrawal of the drug after 3 or 6 months of dosing led to complete restitution of the normal mucosal lineages within 3 months. *Conclusions:* DMP 777 acts as a protonophore with specificity for parietal cell acid-secretory membranes. DMP 777 in high doses leads to the specific loss of parietal cells. Foveolar hyperplasia, loss of normal gland lineages, and the emergence of basal mucous cells appear as sequelae of the absence of parietal cells. The results suggest that parietal cells are critical for the maintenance of the normal mucosal lineage repertoire.

The fundic gastric gland is assembled from a complex repertoire of cell types. Lineages in the gastric fundic mucosa arise from a progenitor zone that is located in the upper third of the gland. Progenitor cells give rise to second-order stem cells including preparietal, presurface, and preneck lineages.[1] Surface cells differentiate and migrate toward the lumen. These surface cells have a short half-life estimated at 3–5 days.[2] A small number of differentiating parietal cells migrate toward the lumen, whereas most parietal cells progress toward the base of the gland. Parietal cells have a long life of 50–60 days.[3] Differentiated mucous neck cells move toward the base of the glands and go through further differentiation into zymogenic cells.[3] Enteroendocrine cells (enterochromaffin-like [ECL] cells and somatostatin cells) also likely differentiate from the same progenitor zone.[4] The ontogeny of gastric mucosal lineages can be influenced by many endocrine and paracrine factors. Increases in transforming growth factor (TGF)-α levels in either patients with Ménétrier's disease[5,6] or mice overexpressing TGF-α under the metallothionein promoter (MT–TGF-α)[5,7–9] demonstrate a massive expansion of foveolar cells with a concomitant reduction in other gland lineages. Similarly, hypergastrinemia in patients with gastrinoma[10] and in rodents exposed to profound acid suppression[11] leads to expansion of parietal cell mass.

Oxyntic atrophy is the hallmark of chronic gastritis. Loss of parietal cells is associated with chronic *Helicobacter pylori* infection[12,13] and Ménétrier's disease.[5,6,14] Attendant with loss of parietal cells, chronic gastritis is also characterized by foveolar hyperplasia, intestinal metaplasia, and an increased risk of gastric adenocarcinoma.[13] The association of *H. pylori* with chronic gastritis has led

Abbreviations used in this paper: BrdU, bromodeoxyuridine; ECL, enterochromaffin-like; HB-EGF, heparin-binding epidermal growth factor–like growth factor; MT, metallothionein; PAS, periodic acid–Schiff; pNPPase, *p*-nitrophenyl phosphatase; SPEM, spasmolytic polypeptide–expressing metaplasia; TGF, transforming growth factor.

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0016-5085/00/$10.00
doi:10.1053/gast.2000.7952

to the hypothesis that loss of parietal cells alters the orderly differentiation of gastric mucosal cell lineages.

Recently, it has become clear that, in addition to apical acid secretion, parietal cells also secrete many peptide growth factors including TGF-α, amphiregulin, and heparin-binding epidermal growth factor–like growth factor (HB-EGF).[5,15,16] The status of the parietal cell as a key source of critical mucosal growth factors has led to the hypothesis that parietal cells may coordinate many aspects of normal lineage differentiation in the gastric mucosa. Transgenic animal models have sought to address this hypothesis through the targeted ablation of parietal cells,[17,18] but the phenotypes tend to be irreversible. This limitation has hampered an analysis of the acute and chronic loss of parietal cells in phenotypically normal animals.

We now report a drug-induced, fully reversible oxyntic atrophy in rats. High doses of DMP 777 induce targeted death of gastric parietal cells in rats. These doses are associated with plasma drug concentrations approximately 700-fold greater than those associated with the highest human doses. Loss of parietal cells leads to rapid increase in gastrin levels and expansion of foveolar cell mass. Suspension of drug treatment for 3 months leads to complete reversal of all mucosal changes and restoration of normal gastric mucosa. These results represent the first reversible model of oxyntic atrophy and demonstrate that loss of parietal cells does not lead to irreversible changes in mucosal cell lineages.

Materials and Methods

Materials

CD-1 rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). [^{14}C]Aminopyrine was purchased form DuPont/NEN (Boston, MA). Polyclonal antibodies against H^+,K^+-adenosine triphosphatase (ATPase) were a gift from Dr. Adam Smolka (Medical University of South Carolina, Charleston, SC). Polyclonal antibodies against rat intrinsic factor were a gift of Dr. David Alpers (Washington University, St. Louis, MO). Murine monoclonal immunoglobulin M (IgM) antibody against human spasmolytic polypeptide was a gift of Dr. Nicholas Wright (Imperial Cancer Research Fund, London, England). Polyclonal antibodies against chromogranin A were a gift of Dr. Graham Dockray (University of Liverpool, England). Polyclonal antibodies against gastrin and somatostatin were purchased from Zymed (San Francisco, CA). Monoclonal antibromodeoxyuridine (BrdU) was purchased from Dako (Carpinteria, CA). Biotinylated secondary antibodies, streptavidin–alkaline phosphatase, and Vector Red substrate were purchased from either Vector Laboratories (Burlingame, CA) or Jackson Immunochemical (West Grove, PA). Rhodamine 123 was obtained from Molecular Probes (Eugene, OR).

Dosing of DMP 777 in Rats

For all studies, DMP 777 ([*S*-(*R**,*S**)]-*N*-[1-(1,3-benzodioxol-5-yl)butyl]-3,3-diethyl-2-[4-[(4-methyl-1-piperazinyl)carbonyl]phenoxy]-4-oxo-1-azetidinecarboxamide) was formulated as a suspension in 0.5% methylcellulose and administered orally to rats as a gavage (5.0 mL/kg) once daily. Two hours before necropsy, BrdU (100 mg/kg) in saline was injected intraperitoneally. At necropsy, stomachs were excised, fixed in 4% paraformaldehyde, and embedded in paraffin.

Measurement of Gastrin

Gastrin analyses were performed by radioimmunoassay on EDTA-anticoagulated plasma by Anilytics (Gaithersburg, MD).

Immunohistochemistry

Replicate 5-μm sections were blocked with 1.5% normal goat serum in phosphate-buffered saline, pH 7.4, then incubated with primary antisera for 2 hours at room temperature. The primary antibody was detected with biotinylated secondary antibodies and biotin-streptavidin detection kit (Vector Laboratories). A red reaction product was developed with Vector Red alkaline phosphatase substrate (Vector Laboratories). The sections were counterstained with Mayer's hematoxylin and mounted. Sections were viewed and photographed on a Zeiss Axiophot bright-field microscope.

Measurement of In Vivo Gastric Acid Output

Sprague–Dawley male rats, 130–180 g, were fasted for 24 hours but had access to ad libitum water. Either vehicle or DMP 777 (50 or 200 mg/kg) in 0.5% methylcellulose (5.0 mL/kg) was administered orally by gavage 60 minutes before pyloric ligation. Each rat was anesthetized with methoxyflurane, and a midline incision was made. A silk ligature was placed around the pyloric sphincter, and the incision was closed with wound clips and sealed with collodion. Four hours after surgery, rats were killed with pentobarbital, abdomens were opened, the esophagus was clamped off, and the stomachs were removed. The gastric contents were emptied into a graduated centrifuge tube and centrifuged at 1000*g* for 10 minutes. For each sample, volume was recorded, acidity was determined by automatically titrating (Radiometer, Copenhagen, Denmark) a 1.0-mL aliquot to pH 7.0 with 0.1N NaOH, and total acid output (TAO) was calculated as TAO (μEq) = Volume (mL) × Acid Concentration (mEq/L). Results were determined as a mean ± SEM, and statistical significance was determined by analysis of variance followed by post hoc analysis Dunnett's test. In vivo gastric acid secretion assays were performed at Drug Research Laboratories (Buckingham, PA).

Isolation of Rabbit Parietal Cells

Rabbit parietal cells were isolated from the fundic mucosa following retrograde high-pressure perfusion of the celiac arterial distribution and pronase/collagenase digestion, as

described previously.[19] Gastric parietal cells isolated on Nycodenz (Accurate Chemical, Westbury, NY) gradients showed 85% ± 5% purity and >98% viability, as assessed by trypan blue exclusion.

Aminopyrine Accumulation

Aminopyrine accumulation into isolated rabbit parietal cells, as a semiquantitative assay of acid sequestration, was assayed using microtiter plate technology, as described previously,[19,20] with the exception that all incubations were carried out in a Boekel forced-air reciprocating incubator (Feasterville, PA).

Preparation of Gastric Tubulovesicles

Gastric tubulovesicles were prepared from resting rabbit gastric mucosa as described by Crothers et al.[21] Briefly, the gastric mucosa from New Zealand White rabbits was homogenized in 5 volumes of homogenization buffer (in mmol/L: 113 mannitol, 37 sucrose, 0.4 EDTA, 5 2-(*N*-morpholino)ethanesulfonic acid [pH 6.7], 5 benzamidine, and 0.1 4-(2-aminoethyl)benzenesulfonyl fluoride) plus a protease inhibitor cocktail (1.75 mg/mL aprotinin, 2.5 mg/mL soybean trypsin inhibitor, and 1 μg/mL chymostatin, pepstatin A, and leupeptin). The homogenate was centrifuged sequentially at 50*g*, 4000*g*, 14,000*g*, and 100,000*g*. The 100,000*g* pellet was resuspended in 10% sucrose buffer (5 mmol/L HEPES/NaOH at pH 7.4 plus 300 mmol/L sucrose) and fractionated over discontinuous sucrose gradients consisting of layers of 20%, 27%, and 33% sucrose. The vesicles partitioning at the 10%–20% sucrose interface were used as enriched tubulovesicles.

H^+,K^+-ATPase Assays/pNPPase

H^+,K^+-ATPase activity was assessed in isolated tubulovesicles (1 μg protein) as the difference between ATPase activity in the absence or presence of 10 μmol/L nigericin using malachite green to detect free phosphate liberated, as described previously.[22] The *p*-nitrophenyl phosphatase (pNPPase) half-reaction of the H^+,K^+-ATPase was assayed in preparations of isolated tubulovesicles, as described previously.[23]

Acridine Orange Accumulation

Tubulovesicles were incubated at room temperature with 5 μmol/L acridine orange for 15 minutes to load vesicles.[24] Acid pumping was initiated with the addition of 0.3 mmol/L adenosine triphosphate and 2.0 μmol/L valinomycin. DMP 777 (10 μmol/L) or nigericin (10 μmol/L) was added 5 minutes after initiation of the proton gradient. Acid accumulation into vesicles was monitored by quenching of acridine orange absorbance (493 nm).

Rhodamine 123 Fluorescence in Mitochondria

To assess the integrity of the mitochondrial electrochemical gradient in parietal cells, isolated parietal cells were incubated with 10 μg/mL rhodamine 123 for 30 minutes at 37°C and then incubated for a further 10 minutes in the presence or absence of 10 μmol/L DMP 777. The presence of mitochondrial staining with rhodamine 123 was then evaluated in fluorescence microscopy (100 cells per condition in triplicate).

Electron Microscopy

For electron-microscopic examination, tissue was fixed by immersion overnight in 3% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L phosphate buffer, with postfixation in 1.0% OsO_4 with 1.5% potassium ferricyanide. Subsequently, tissue was dehydrated in a graded ethanol series, followed by propylene oxide and embedding in Epon 812. Thin sections (50–60 nm) were mounted on formvar-coated copper grids and stained with uranyl acetate and lead citrate.

Results

DMP 777 Causes a Rapid Increase in Serum Gastrin Levels

DMP 777 is a cell-permeant inhibitor of neutrophil elastase that can be administered orally (Figure 1). Short-term administration of DMP 777 orally at doses of either 50 or 200 mg/kg caused a rapid increase in serum gastrin levels that was statistically significant after 60 minutes (Table 1) and was sustained at even higher levels after 4 hours. This increase in gastrin was correlated with higher plasma concentrations of DMP 777 measured at 4 hours (data not shown).

DMP 777 Profoundly Inhibits Gastric Acid Secretion In Vivo

In light of the rapid increase in serum gastrin secretion after oral dosing of DMP 777, we evaluated the effects of DMP 777 on gastric acid secretion in pylorus-ligated rats. Administration of a single oral dose of 200 mg/kg DMP 777 caused a profound decrease in acid output over 4 hours (Table 2). The results showed that DMP 777 elicits profound hypochlorhydria.

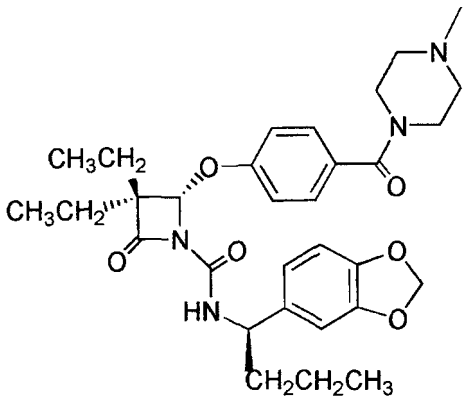


Figure 1. Chemical structure of DMP 777.

Table 1. Mean Serum Gastrin Levels After DMP 777 Administration

DMP 777 dosage	Control (n = 5)	15 mg/kg (n = 6)	30 mg/kg (n = 6)	50 mg/kg (n = 6)	200 mg/kg (n = 6)
Before dosing	27 ± 8.7				
1 h after dose	33.8 ± 20.5	28.0 ± 5.0	74.6 ± 47.9	95.0 ± 44.9	95.5 ± 53.6
4 h after dose	36.6 ± 7.1	40.1 ± 6.5	69.7 ± 60.9	113.1 ± 75.6	209.5 ± 75.8

NOTE. Results are pg/mL (mean ± SD).

DMP 777 Acts Directly on Parietal Cells

To identify whether DMP 777 effects were caused by direct action on parietal cells, we investigated the effects of DMP 777 on accumulation of the weak-base [^{14}C]aminopyrine into isolated rabbit parietal cells. Figure 2 shows that DMP 777 elicited a dose-dependent inhibition of aminopyrine accumulation stimulated by histamine with an IC_{50} of 1 μmol/L. DMP 777 also elicited a similar inhibition of aminopyrine accumulation stimulated by forskolin. DMP 777 also inhibited aminopyrine accumulation stimulated by 8-Br–cyclic AMP and inhibited basal uptake down to the levels observed in the presence of 50 mmol/L KSCN (750 ± 23 cpm) with a similar IC_{50} (data not shown). These results suggested that the effects of DMP 777 on acid secretion were likely a result of direct effects on parietal cells at a level beyond the stimulation of the histamine receptor.

To evaluate the structure-function basis of DMP 777 action on parietal cells, a series of DMP 777 analogues with varying potency as neutrophil elastase inhibitors, from inactive to potent, were tested as inhibitors of aminopyrine accumulation. Table 3 shows that, with the exception of nonlipophilic amidated derivatives, all showed activity as inhibitors of aminopyrine accumulation and their potency did not correlate with their potency as neutrophil elastase inhibitors. These results indicate that the acid-inhibitory effects of DMP 777 and its analogues are caused by a general amphiphilic chemical backbone structure of the basic series of analogues.

Given the profound nature of the effects of DMP 777 on second messenger–stimulated acid secretion, we sought to determine whether DMP 777 might inhibit directly the action of H^+,K^+-ATPase. We therefore tested the effects of DMP 777 on the activity of the H^+,K^+-ATPase as well as pNPPase half-reaction in isolated tubulovesicles from rabbit parietal cells. Figure 3 shows that 1–10 μmol/L DMP 777 had no effect on H^+,K^+-ATPase activity. Similarly, DMP 777 also did not affect the pNPPase half-reaction (data not shown). In contrast, omeprazole profoundly inhibited the activity of H^+,K^+-ATPase (data not shown).

Because we could not detect an alteration of the enzymatic activity of H^+,K^+-ATPase, we then evaluated the ability of DMP 777 to dissipate an established proton gradient in isolated tubulovesicles in the presence of the kalophore valinomycin. Figure 4 shows that isolated tubulovesicles could establish a proton gradient in response to the addition of ATP, as noted by the quenching of the fluorescence of acridine orange. Addition of 10 μmol/L DMP 777 to vesicles after the establishment of the gradient rapidly dissipated the proton gradient (Figure 4). The ability of DMP 777 to dissipate the gradient was similar to that observed for the nonselective protonophore nigericin (Figure 4). Pretreatment of vesicles with 10 μmol/L DMP 777 blocked the establishment of the gradient (data not shown). In contrast with these studies, DMP 777 had no effect on the maintenance of

Table 2. Effects of DMP 777 on Acid Secretion in Pylorus-Ligated Rats

	pH	Titratable acid (*mEq/L*)	Total acid output (*μEq/4 h*)
Vehicle	1.37 ± 0.03	106.0 ± 3.5	517.0 ± 73.5
DMP 777 (50 mg/mL)	1.77 ± 0.09	64.4 ± 6.4[a]	337.1 ± 43.5
DMP 777 (200 mg/mL)	6.03 ± 0.25	9.9 ± 1.5[a]	71.2 ± 11.6[a]

NOTE. Results are mean ± SEM; n = 10 for all groups.
[a]$P < 0.05$ compared with vehicle.

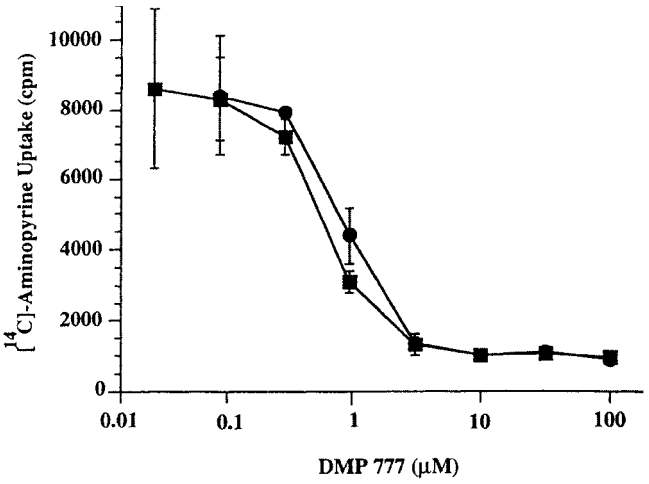


Figure 2. DMP 777 inhibits aminopyrine accumulation into rabbit parietal cells. Rabbit parietal cells were stimulated with either 100 μmol/L histamine (●) or 10 μmol/L forskolin (■) for 30 minutes. Basal (unstimulated) aminopyrine accumulation was 1450 ± 38 cpm. Each determination is the mean (±SEM) of quadruplicate samples. The data are representative of 4 identical experiments. DMP 777 inhibited aminopyrine accumulation in response to both histamine and forskolin with an IC_{50} of 1 μmol/L.

Table 3. IC_{50}s of Neutrophil Elastase Inhibitors

Compound	Elastase K_i ($mol/L^{-1} \cdot s^{-1}$)	Molecular weight (*daltons*)	Molecular form	Aminopyrine K_i ($\mu mol/L$)
L-694,458 (DMP 777)	3,800,000	699	Basic	0.5
L-680,831	400,000	453	Acid	NA[a]
L-680,859	1,200,000	482	Acid	NA
L-697,239	2,200,000	521	Neutral amide	NA
L-743,571	3,700,000	551	Neutral amide	NA
L-693,910	1,700,000	534	Free amine (basic)	0.6
L-740,449	4,500,000	590	Malate salt (basic)	0.9
L-696,509	2,000,000	698	Malate salt (basic)	0.5
L-687,847	1,900,000	671	Malate salt (basic)	0.5
L-707,538	220,000	622	Malate salt (basic)	1.1
L-734,635	Inactive[b]	623	Malate salt (basic)	0.7
L-739,401	425,000	652	Malate salt (basic)	1.6
L-709,516	200,000	532	Free amine (basic)	0.3
L-698,770	200,000	695	Citrate salt (basic)	0.7
L-709,932	150,000	606	Malate salt (basic)	0.4
L-739,406	530,000	636	Malate salt (basic)	0.8
L-702,737	500,000	733	Citrate salt (basic)	0.9

[a]Not applicable: IC_{50}s for these compounds were not calculated because they were unable to inhibit [^{14}C]aminopyrine uptake.
[b]Inactive enantiomer of L-707,538.

proton gradients in mitochondria, as assessed by fluorescence of rhodamine 123 (data not shown). These studies indicated that DMP 777 inhibits parietal cell acid secretion by acting as a protonophore with some specificity for the acid-secretory membranes.

DMP 777 Elicits Oxyntic Atrophy

During initial 3-month toxicity studies in rats and monkeys, DMP 777 at doses of 200 and 135 mg · $kg^{-1} \cdot day^{-1}$, respectively, elicited a marked change in the gastric mucosa with loss of parietal cells. Foveolar hyperplasia was also observed in rats. These doses are associated with plasma drug concentrations hundreds of fold greater than those observed with clinically used doses of DMP 777 (data not shown). No other significant sequelae of DMP 777 dosing were noted. To study these gastric mucosal changes in more detail, rats were dosed

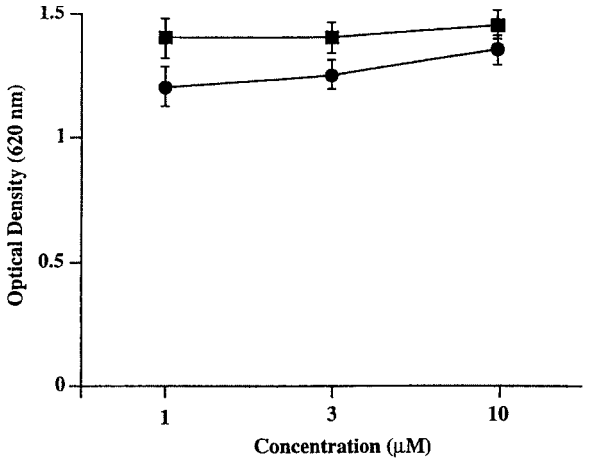


Figure 3. DMP 777 does not directly inhibit H^+,K^+-ATPase. H^+,K^+-ATPase activity in isolated rabbit tubulovesicles was measured as a release of phosphate as detected by malachite green in the absence (●) or presence (■) of 10 μmol/L DMP 777. Each measurement is represented as a mean (±SEM) of quadruplicate determinations. The results are representative of 3 separate experiments. DMP 777 had no effect on H^+,K^+-ATPase activity.

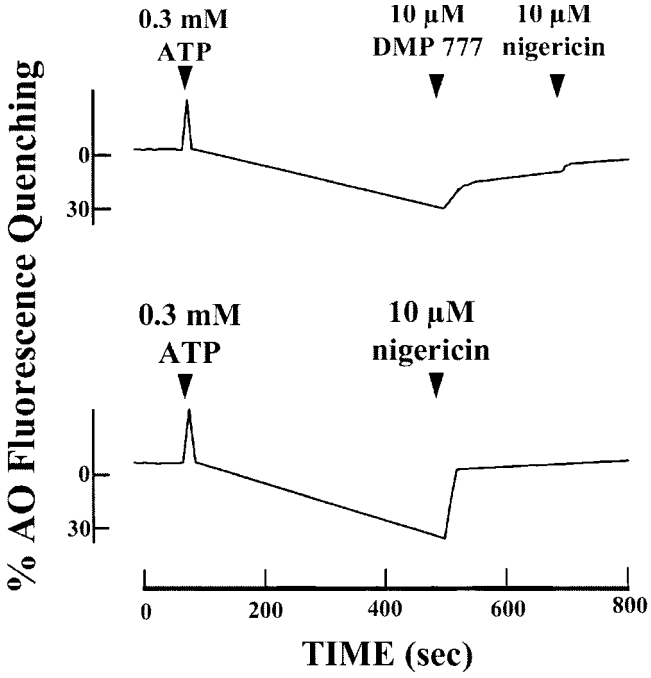


Figure 4. DMP 777 can abolish an established proton gradient in isolated tubulovesicles. Tubulovesicles were equilibrated with acridine orange (AO), and then a proton gradient was initiated with the addition of 0.3 mmol/L ATP. After 400 seconds, 10 μmol/L DMP 777 (*top*) or 10 μmol/L nigericin (*bottom*) was added. At 180 seconds after DMP 777, 10 μmol/L nigericin was added (*top*). The results are representative of 6 identical experiments. Both DMP 777 and nigericin reversed the proton gradient in tubulovesicles.

daily by gavage with 15, 50, or 200 mg · kg^{-1} · day^{-1} of DMP 777 over 3 months. Gastric mucosa was then examined using both histological and immunohistochemical stains. Figure 5 shows that DMP 777 at 200 mg · kg^{-1} · day^{-1} elicited a marked change in the fundic mucosa. Gastric changes were minimal in rats dosed with 50 mg · kg^{-1} · day^{-1} and absent in rats dosed with 15 mg · kg^{-1} · day^{-1} (data not shown). H&E staining

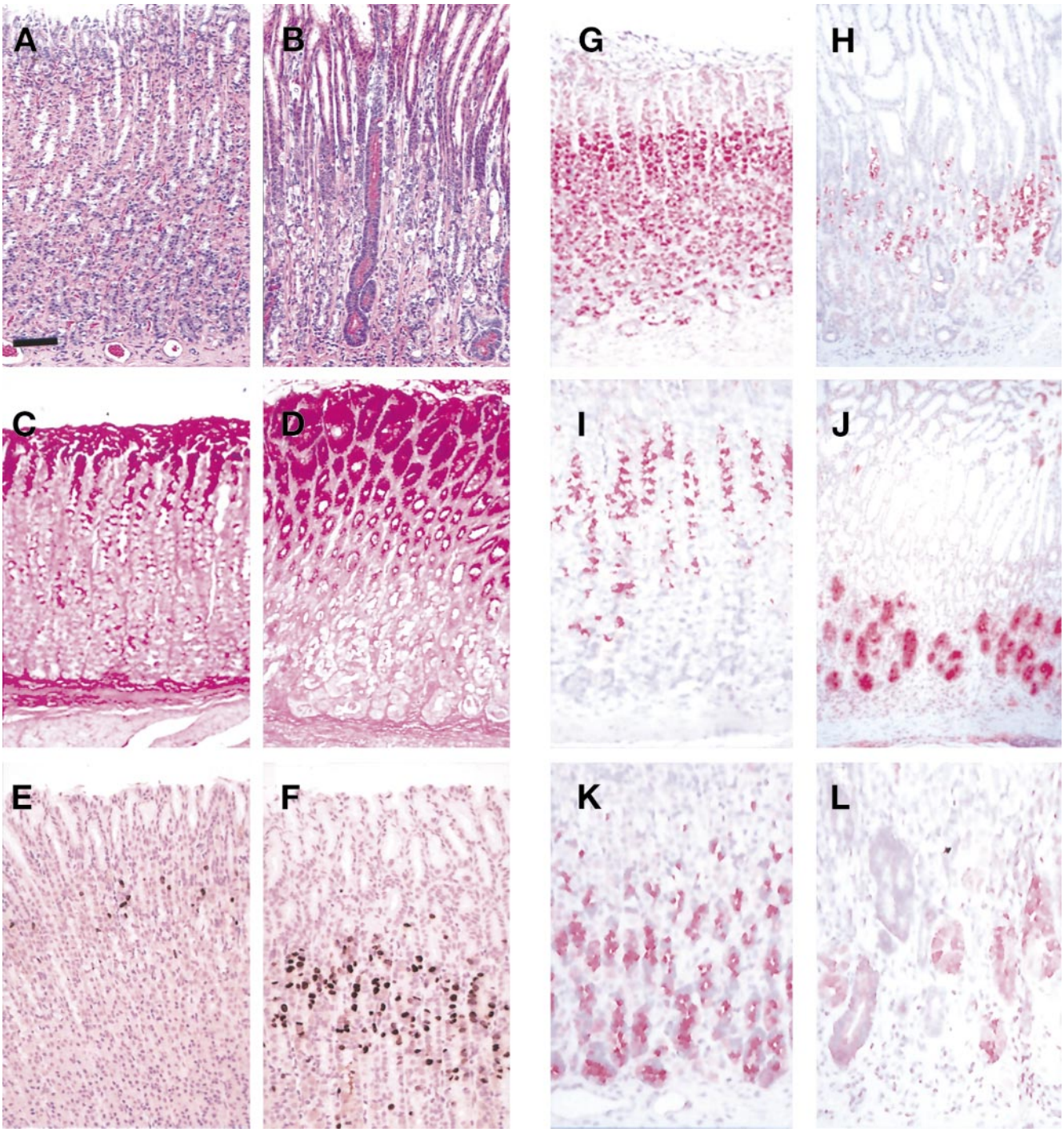


Figure 5. Effects of 3 months of DMP 777 treatment on gastric cell lineages. Paraffin sections of gastric fundic mucosa from rats treated with (*A*, *C*, *E*, *G*, *I*, and *K*) vehicle solution or (*B*, *D*, *F*, *H*, *J*, and *L*) DMP 777 (200 mg · kg^{-1} · day^{-1}) for 3 months were stained with H&E (*A* and *B*), diastase-resistant PAS (*C* and *D*), BrdU (*E* and *F*), H^+,K^+-ATPase (*G* and *H*), spasmolytic polypeptide (*I* and *J*), and intrinsic factor (*K* and *L*). Note the prominent foveolar hyperplasia (*B* and *D*), presence of a novel eosinophilic glandular lineage (*B*), increase in BrdU-labeled nuclei in DMP 777–treated animals (*F*), and reductions in parietal cell (*H*) and chief cell (*L*) numbers in DMP 777–treated animals. Spasmolytic polypeptide–staining cells in DMP 777–treated animals were displaced toward the bottom of glands and showed a nodular staining pattern. *Bar* = 60 μm (*A–D*, *G–J*) and 30 μm (*E*, *F*, *K*, *L*).

showed a loss of parietal cells and a marked increase in foveolar surface mucous cells (Figure 5*B*). Fundic mucosa from DMP 777–dosed rats also showed the presence of an abnormal eosinophilic glandular cell lineage located at the bases of gastric glands. This lineage sometimes dominated most of the length of a gland unit (Figure 5*B*). The fundic mucosa from DMP 777–treated animals showed a marked expansion of diastase-resistant periodic acid–Schiff (PAS)-staining surface mucous cells characteristic of foveolar hyperplasia (Figure 5*D*). The increase in foveolar hyperplasia was accompanied by an increase in BrdU labeling of S-phase nuclei (Figure 5*F*). In addition, the position of the BrdU-labeled progenitor zone cells was displaced downward in the gland and resided beneath the region of foveolar hyperplasia (Figure 5*F*).

Immunohistochemical staining was used to identify more precisely alterations in specific gastric fundic cellular lineages in DMP 777–treated animals. Figure 5*H* shows that DMP 777–treated rats showed a marked decrease in H^+,K^+-ATPase–staining parietal cells. The parietal cells that were present in the gastric mucosa were generally present at the bases of glands and were abnormally vacuolated, suggestive of an expanded secretory canaliculus. Spasmolytic peptide–staining cells were present as glandular nodules in the middle and basal regions of the glands (Figure 5*J*). The spasmolytic peptide–staining cells showed a more open apical morphology than normal mucous neck cells. We also observed a decrease in the number of normal-appearing intrinsic factor–staining chief cells in the mucosa (Figure 5*L*). Chromogranin A–staining ECL cells were markedly decreased in numbers throughout the fundic mucosa compared with sham controls (Table 4). DMP 777–treated fundic mucosa also showed a significant decrease in somatostatin-staining endocrine cells (Table 4).

Foveolar hyperplasia has been noted in the presence of increased TGF-α expression in mucosal cells.[5,7–9] We did observe intense TGF-α staining in the few vacuolated parietal cells that remained after DMP 777 administration (data not shown), but there was no gross increase in TGF-α staining as is seen in patients with Ménétrier's disease or in MT–TGF-α mice.[5,7–9]

Profound changes were observed in the fundic mucosa, but no gross changes in the antral mucosa stained with H&E. However, staining of the antral sections from rats treated with 200 mg · kg^{-1} · day^{-1} DMP 777 for gastrin showed an increase in gastrin cell numbers (Table 4).

DMP 777 Causes Acute Parietal Cell Necrosis

Long-term DMP 777 dosing appeared to lead to oxyntic atrophy in a number of animals, but the actual cause of the loss of parietal cells was not clear. We therefore sought to assess the acute effects of DMP 777 on gastric cells. Rats were dosed with 200 mg kg^{-1} · day^{-1} orally for 1–10 days, and gastric mucosae were examined for acute changes. One day after dosing with 200 mg/kg DMP 777, severe parietal cell necrosis was observed in all rats (Figure 6*B*). Electron micrographs of fundic mucosa taken 24 hours after a single dose of 200 mg/kg DMP 777 showed dilatation of secretory canaliculi, with loss of microvilli and accumulation of membrane aggregates of microvillar material in canalicular lumina (Figure 6*C*). Evidence of parietal cell necrosis was observed with cytoplasmic condensation, complete loss of microvilli, cell shrinkage, and marginalization of nuclear chromatin (Figure 6*D*). In contrast, chief cells (Figure 6*B*) and ECL cells (data not shown) were unaffected. In addition, during this acute period, there was little inflammatory infiltrate except an increase in the number of mucosal eosinophils.

During the 10 days of acute treatment, loss of parietal cells was accompanied by a rapid expansion of diastase-resistant PAS–positive surface mucous cells (Figure 7*A–C*) and a marked increase in BrdU-staining S-phase cells (Figure 7*D–F*). After 2 days of treatment, we observed increased BrdU staining in the region of the progenitor zone, but also the presence of BrdU-staining mucosal cells deep in the gastric glands. The BrdU-staining cells were associated with the emergence of a weakly PAS-positive mucous cell lineage at the base of the fundic glands. By 10 days of treatment, BrdU-staining cells were observed throughout the lower two thirds of the gastric glands and the weakly PAS-positive lineage was

Table 4. Endocrine Cell Numbers in DMP 777–treated Rats

	Chromogranin A positive		Somatostatin positive		G cells	
	Control	DMP 777	Control	DMP 777	Control	DMP 777
3 mo treatment	74.1 ± 28.1	17.3 ± 11.9[a]	8.2 ± 5.5	1.5 ± 1.6[a]	1.2 ± 0.6	5.2 ± 3.9
3 mo treatment + 3 mo recovery	100.7 ± 46.0	71.6 ± 31.3	10.8 ± 3.2	9.7 ± 3.7	1.5 ± 0.8	1.6 ± 0.9

NOTE. Male rats were treated with DMP 777 (200 mg · kg^{-1} · day^{-1} per gavage) for 3 months followed by a 3-month drug-free recovery period in half of the animals. The mean numbers of chromogranin-immunoreactive and somatostatin-immunoreactive cells in the oxyntic mucosa and gastrin immunoreactive G cells in the antrum were assessed per 20× microscopic field (±SD).

[a]$P < 0.01$ compared with controls.

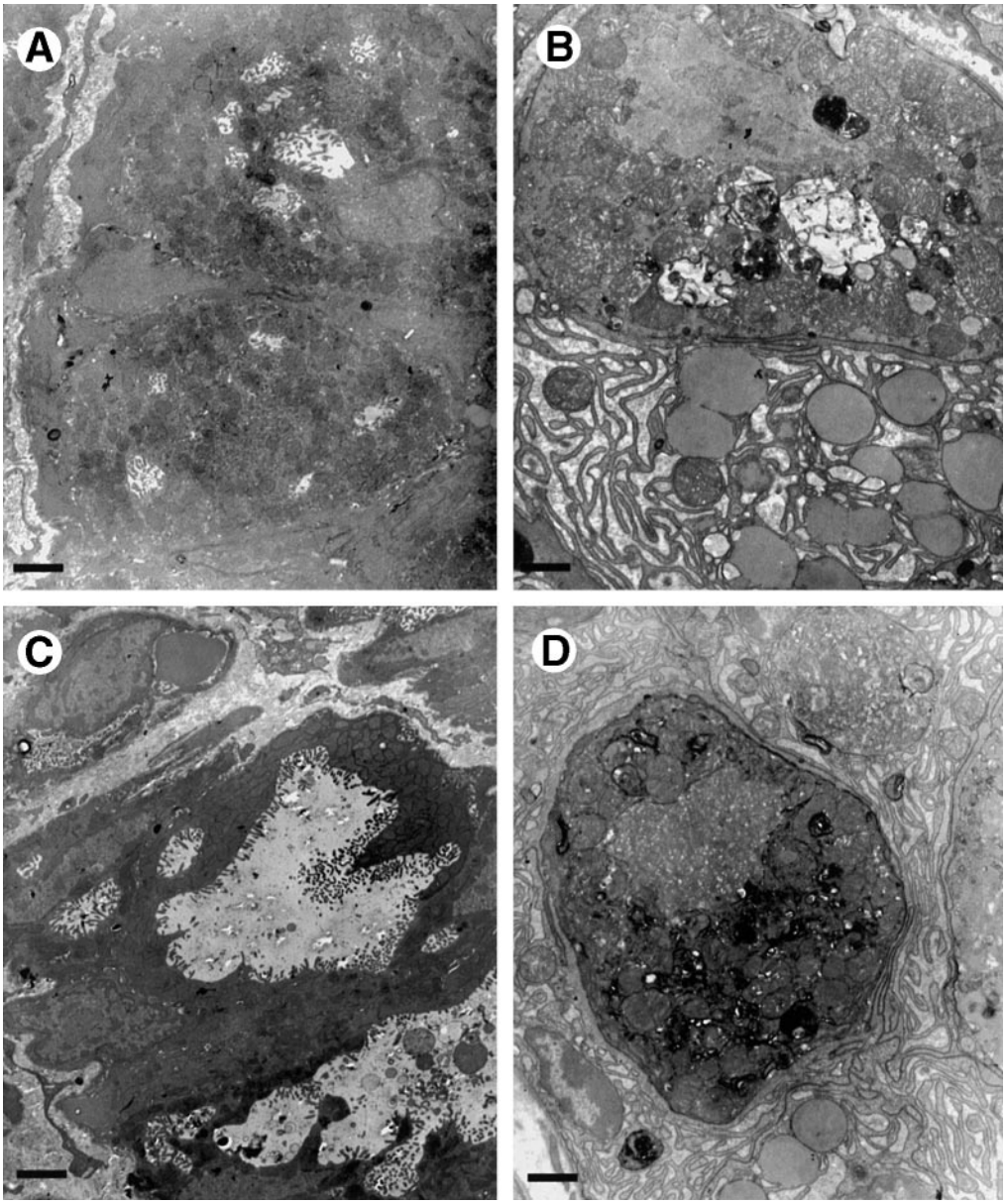
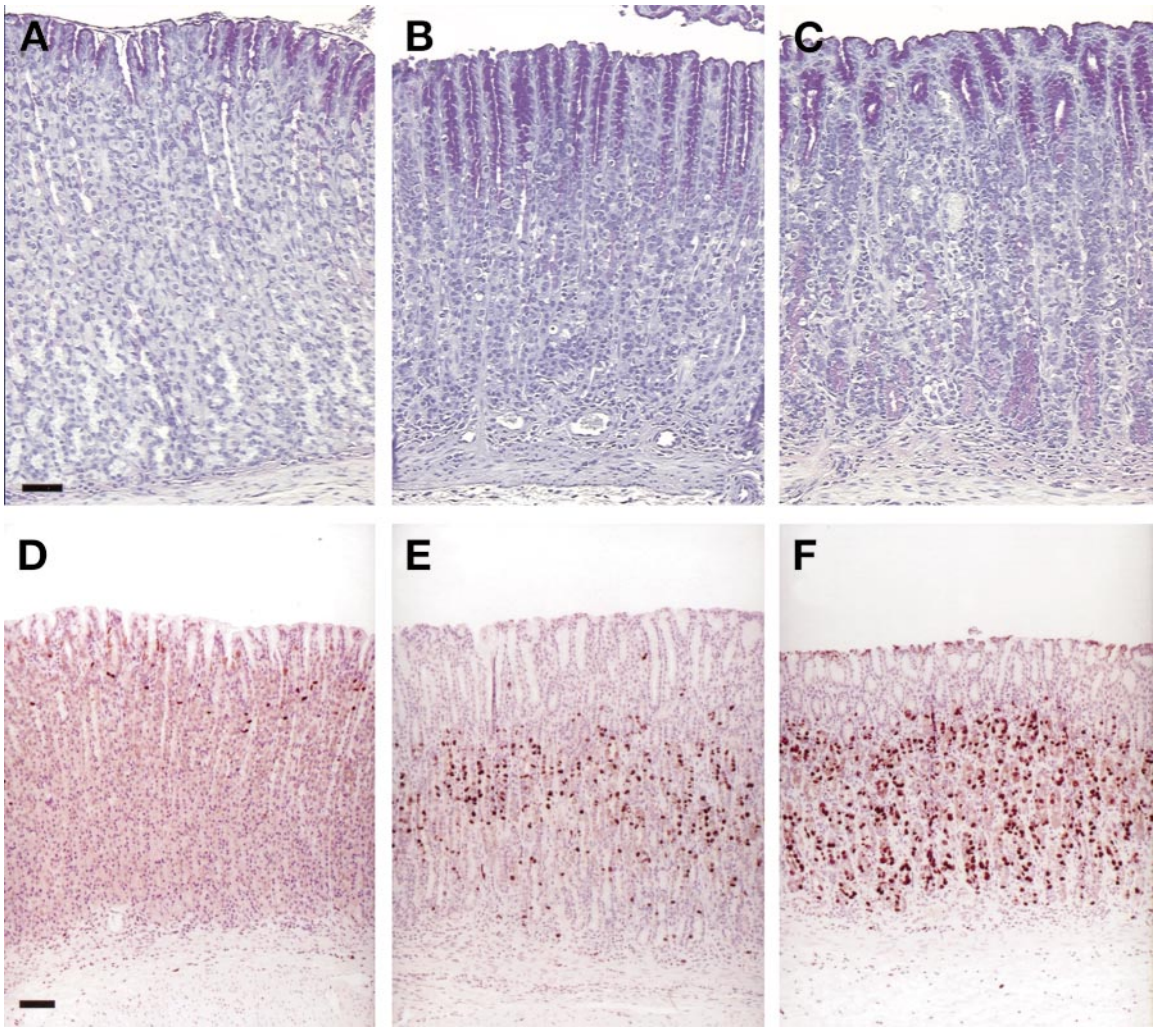


Figure 6. DMP 777 treatment leads to acute parietal cell necrosis. Fundic mucosal samples from (*A*) vehicle-treated and (*B–D*) DMP 777–treated (200 mg/kg, single dose) animals were fixed in glutaraldehyde, embedded in plastic resin, and sectioned thin for transmission electron microscopy. (*A*) normal parietal cell morphology is observed in vehicle-treated animals. Treatment with DMP 777 leads to necrotic parietal cell death with (*B*) vacuolation and cytoplasmic condensation, (*C*) sloughing of membranous material into expanded canaliculi, and (*D*) and frank evidence of necrosis with nuclear and cytoplasmic degeneration. The morphology of neighboring chief cells is normal (*B*). *Bar* = 1 μm.

present prominently in the mucosa. These results suggest that, although the direct effects of DMP 777 are exerted specifically on the parietal cell, the loss of parietal cells elicits an immediate change in differentiation and/or turnover of other mucosal cell lineages.

The Effects of DMP 777 Are Reversible

To assess the reversibility of the effects of DMP 777, animals were treated with the drug (200 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}) for 3 or 6 months and then the drug was withdrawn for a further 3 months. Figure 8 shows that removal of drug therapy led to a complete reversal of foveolar hyperplasia and oxyntic atrophy. After the drug-free period, there was complete restitution of both the normal fundic architecture and the normal repertoire of fundic mucosal lineages. Histology and immunostaining for H^+,K^+-ATPase, spasmolytic polypeptide, and intrinsic factor demonstrated normal cell numbers and cellular distribution within gland units. Endocrine cell numbers in both the fundus and the antrum also returned to normal levels (Table 4). In addition, there was a complete absence of the mucous cell metaplasia evident


A
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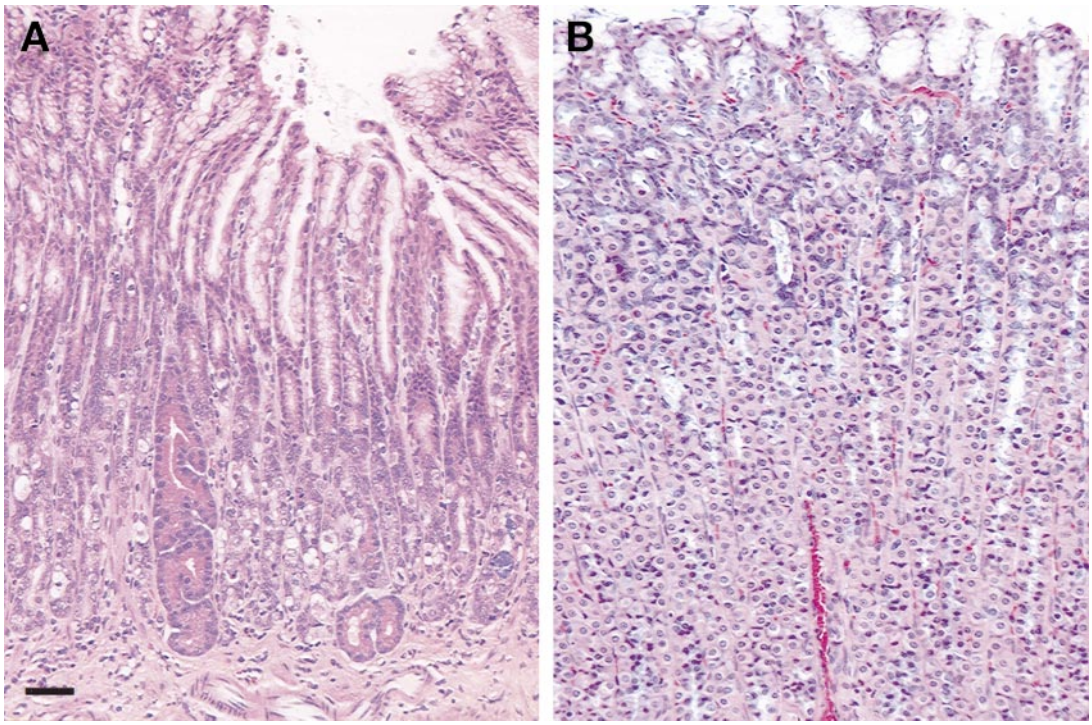
A
B

in treated rats. These results indicate that the effects of DMP 777 are completely reversible.

Discussion

The results of this study represent the first demonstration of reversible oxyntic atrophy. Rats treated with DMP 777 demonstrate a rapid loss of parietal cells through the ability of the drug to act directly as a protonophore with selectivity for parietal cell secretory membranes. Loss of parietal cells is accompanied by rapid expansion of foveolar surface mucous cells. With continued dosing, further changes in fundic lineages occur with decreases in ECL, somatostatin, and chief cells. All of these changes were reversed after cessation of drug administration.

Unlike compounds such as nigericin, DMP 777 is not a general protonophore. This would explain its general lack of other systemic side effects. Although DMP 777 is a potent cell-permeant inhibitor of neutrophil elastase, its effects on parietal cells and the resulting sequelae in the gastric fundus do not appear to be related to its elastase inhibitor activity. Indeed, a comparison of a number of DMP 777 analogues showed no correlation of elastase inhibition with protonophore activity. Thus, the parietal cell protonophore activity seems to be due to the backbone structure of this series of compounds. We have previously described a similar mode of action for another compound, the calmodulin-dependent protein kinase inhibitor, KN-93.[25] KN-93 also displayed a specific protonophore activity for parietal cell membranes. The exact mechanism by which DMP 777 leads to parietal cell death is not clear, but the association of the drug with parietal cell secretory membranes suggests that, during the process of acid secretion, the drug is delivered through the fusion of tubulovesicles to the apical membrane, where it may cause backwash of acid into the cell. A similar mode of toxicity may account for the loss of parietal cells in mice deficient in Na^+/H^+ exchanger isoform 2 (NHE-2).[26] Interestingly, in preliminary studies, we found that treatment of animals with omeprazole mitigated DMP 777 effects on the gastric mucosa (B. D. Car, unpublished results). The precise intracellular mechanisms responsible for pronophore-induced parietal cell death remain to be determined.

The lineages of the normal gastric fundic mucosa arise from the progenitor zone, which is located approximately one third of the gland length down from the lumen. This position of the progenitor zone near the lumen suggests that an orderly program of differentiation must be followed to maintain the progenitor region at this point. The cells of the fundus arise from second-order stem cells.[1] Parietal cells are the only cell type present both above and below the progenitor region. Surface cells migrate toward the lumen and have a short lifetime.[2] Parietal cells, mucous neck cells, enteroendocrine cells, and chief cells all are found below the progenitor zone and have longer lifetimes of 60 days or more.[3,4,27] Mucous neck cells redifferentiate into chief cells as they migrate toward the base of the glands.[3] Many studies over the past decade have provided evidence that parietal cells are critical regulators of cell lineage maturation from the gastric mucosal progenitor zone.[7–9,17,18] Parietal cells, in addition to secreting acid, also are responsible for the secretion of many growth factors including TGF-α and HB-EGF.[5,28,29] Loss of parietal cells leads to other mucosal cell changes observed in the setting of chronic gastritis including foveolar hyperplasia.

A critical feature of DMP 777–induced oxyntic atrophy is its complete reversibility after a 3-month drug-free interval. We have observed complete recovery of normal mucosal histological phenotype in as little as 1 month (B. D. Car, unpublished observations, 1999). The loss of parietal cells after DMP 777 dosage at 200 $mg \cdot kg^{-1} \cdot day^{-1}$ is rapid and sustained as long as the drug dosing is maintained. Several previous animal models have produced absence or loss of parietal cells. MT–TGF-α mice,which overexpress TGF-α, demonstrate a near-complete loss of parietal cells, as well as foveolar hyperplasia and loss of both chief cells and ECL cells.[5,7–9,30] A similar pattern of TGF-α overexpression in surface

Figure 7. DMP 777 treatment causes acute expansion of surface cell compartment and emergence of a basal metaplastic mucous cell lineage. Rats were treated with DMP 777 (200 $mg \cdot kg^{-1} \cdot day^{-1}$) for (*A* and *D*) 0 days, (*B* and *E*) 2 days, and (*C* and *F*) 10 days. Before death, all animals received an injection of BrdU to label S-phase cells. Paraffin-embedded sections of gastric mucosa were stained with PAS (*A–C*) or with antibodies against BrdU (*D–F*). Loss of parietal cells and expansion of the foveolar region are readily apparent after 2 days of treatment (*B*). This is accompanied by an expansion of the S-phase cells staining the normal progenitor region as well as the appearance of BrdU-staining cells deep in the gland (*E*). By 10 days of treatment, there is prominent appearance of a weakly PAS-positive mucous lineage in the deep gland region (*C*), and BrdU-staining nuclei are observed throughout the gland region deep to the foveolar cells. *Bar* = 60 μm.

Figure 8. Cessation of DMP 777 treatment leads to complete restoration of the normal mucosal phenotype. Rats were treated for 3 months with DMP 777 (200 $mg \cdot kg^{-1} \cdot day^{-1}$); then (*A*) one group was killed, whereas (*B*) a second group was allowed a 3-month drug-free recovery period. Paraffin-embedded sections of gastric mucosa were examined by H&E staining. The sections show that cessation of drug treatment allowed complete restoration of the normal repertoire of fundic mucosal lineages. *Bar* = 30 μm.

mucous cells is observed in patients with Ménétrier's disease.[5] Although the foveolar hyperplasia and oxyntic atrophy in MT–TGF-α mice were exacerbated by administration of zinc, the mucosal changes were not reversible. In that model, foveolar hyperplasia seemed to occur as a result of the TGF-α–induced stimulation of surface cell production from the progenitor zone at the expense of other lineage differentiation.[7] We did observe some intense staining for TGF-α in the few remaining parietal cells, but DMP 777–treated rats did not have an increase in TGF-α expression sufficient to account for the foveolar hyperplasia and oxyntic atrophy. This picture of foveolar hyperplasia and oxyntic atrophy in the absence of TGF-α overexpression is similar to the mucosal pattern in the fundic mucosa of patients after antrectomy and gastrojejunostomy.[31] These patients show fundic foveolar hyperplasia and oxyntic atrophy. Importantly, although postantrectomy patients and MT–TGF-α mice have low or normal gastrin levels, the DMP-treated rats are hypergastrinemic. Thus, foveolar hyperplasia seems to occur in response to a number of different inciting events including loss of parietal cells or overexpression of TGF-α.

Canfield et al.[17] targeted expression of herpes simplex thymidine kinase to parietal cells in transgenic mice using the promoter for the β-subunit of H^+,K^+-ATPase. While thymidine kinase was specifically expressed in parietal cells, ganciclovir treatment elicited a rapid death of parietal cells as well as the other lineages in the mucosa. Although this ablation of gastric lineages was reversible 3 months after withdrawal of ganciclovir, the dramatic rapid loss of all mucosal cells questioned the specificity of the model. Recent studies have shown that adducts of ganciclovir can pass through gap junctions to neighboring cells, eliciting a field kill effect.[32] These results likely explain the phenotype of ganciclovir treatment in H^+,K^+–thymidine kinase mice. Thus, while the mucosal cell loss is reversible, its phenotype is difficult to control.

Gordon et al.[18,33,34] recently developed several transgenic mouse models to examine the process of lineage development in the stomach. In particular, they have targeted the expression of tetanus toxin to parietal cells, again using the H^+,K^+-ATPase promoter.[18] These mice demonstrate oxyntic atrophy with a mild foveolar hyperplasia and a decrease in chief cell numbers. The hallmark of this model is a marked expansion in the number of preparietal cells in the gastric glands. In contrast, in the DMP 777 model, we did not observe a significant increase in the preparietal cell populations. The differences may reflect species differences in response to the loss of parietal cells; however, mice treated with DMP 777 also demonstrate a similar phenotype of oxyntic atrophy and foveolar hyperplasia (data not shown). It is more likely that divergent patterns of lineage expression stem from differences in the timing of parietal cell loss during lineage ontogeny. In the case of the H^+,K^+–tetanus toxin mice, toxin-induced death probably occurs almost immediately after the onset of H^+,K^+-ATPase expression, at the earliest point of parietal cell maturation. In the case of DMP 777 treatment, our results suggest that a mature acid-secreting parietal cell is required for the action of the drug. Indeed, we found that pretreatment of animals with omeprazole to block acid secretion significantly ameliorates the DMP 777–induced phenotype (B. D. Car, unpublished results). It is therefore possible that the H^+,K^+–tetanus toxin mouse has eliminated some local feedback from maturing parietal cells to the progenitor zone that regulates the production of preparietal cells. In any case, as in other transgenic models, the phenotype in H^+,K^+–tetanus toxin mice is not reversible.

One final model of oxyntic atrophy bears significantly on the complexity of oxyntic atrophy. C57BL/6 mice infected with *H. felis* develop a prominent chronic gastritis with oxyntic atrophy and foveolar hyperplasia.[35,36] Importantly, in these mice, the expansion of the foveolar region is not accounted for by surface cells, but rather by the expansion of a novel metaplastic mucous cell lineage that expresses spasmolytic polypeptide (SPEM). However, in contrast with the small normal mucous neck cells, SPEM has a morphology similar to that of a Brunner's gland cell with a wide apical surface and abundant mucous granules.[36] This spasmolytic polypeptide–expressing metaplasia (SPEM) is also observed in humans with chronic *H. pylori* gastritis and with gastric adenocarcinoma.[37] DMP 777 treatment did induce mucous cell metaplasia with characteristics similar to SPEM, but the metaplasia was less extensive than that in *Helicobacter felis*–infected mice. Thus, although *H. felis* infection leads to oxyntic atrophy, the manifestations of lineage alterations in the presence of the *Helicobacter* species infection appear to be different in magnitude compared with primary parietal cell loss as in the DMP 777 model. It is not clear whether eradication of *H. felis* in infected mice can reverse the changes in mucosal lineage development. In the present model, we did not observe a prominent mononuclear infiltrate in the face of acute parietal cell loss. The lack of infiltrate may be caused in part by the neutrophil elastase inhibition by DMP 777. In that case, it is possible that the influence of inflammatory cytokines is required for the expansion of SPEM lineages as seen in the *H. felis*–infected mice.

During short-term treatment with DMP 777, in addition to foveolar hyperplasia, we also observed the

emergence of BrdU-labeled cells deep in the fundic glands coincident with the development of mucous cell metaplasia. These results indicate that cryptic progenitor cells, distinct from the cells of the more luminally positioned normal progenitor zone, are present in the basal regions of fundic gastric glands. Loss of parietal cells leads to the expansion of the cells and development of mucous cell metaplasia. Thus, mucous cell metaplasia in humans[37] and rodents[36] may result from activation of such cryptic progenitor cells. According to this hypothesis, metaplastic lineages such as SPEM would be the product of the cryptic progenitor zone, rather than the normal luminally positioned gastric progenitor zone. In the normal stomach, parietal cells may provide a negative influence to inhibit the proliferation of these cryptic progenitor cells. Alternatively, paracrine factors may be released in response to parietal cell loss that elicit the expansion of these cells. Nomura et al.[38] recently provided evidence for the polyclonality of a significant population of gastric fundic glands even in adult mice. All these data provide strong evidence that a second progenitor cell class exists in the fundic mucosa separate from the previously identified progenitor zone.[1]

A major feature of the acute response to the loss of parietal cells is a rapid increase in serum gastrin level. Previously, serum gastrin increases have been associated with both increases in parietal cell mass (Zollinger–Ellison syndrome in humans)[10] and ECL cell hyperplasia.[39] We did not observe any evidence of ECL hyperplasia in DMP 777–treated animals; indeed, we observed a significant loss of chromogranin A–immunoreactive cells. At the same time, we observed a rapid onset of foveolar hyperplasia. Previous studies have suggested that gastrin may stimulate gastric mucosal proliferation.[40,41] The rapid onset of foveolar cell differentiation may be stimulated by the rapid and sustained hypergastrinemia in these rats. Nevertheless, gastrin cannot be an absolute requirement for foveolar hyperplasia, because MT–TGF-α mice have massive foveolar hyperplasia without hypergastrinemia.[5] Interestingly, MT–TGF-α mice also have decreased numbers of chromogranin A–labeled cells.[7,42] Similarly, mice with targeted disruption of the gastrin gene exhibit an expansion of their surface cell compartment.[43] The foveolar hyperplasia may be a general response to significant injury. Thus, acute injury from acetic acid denudation of the gastric mucosa elicits a rapid up-regulation of the pathways leading to production of surface mucous cells.[44] This response to injury seems logical because, unlike the rest of the gastric cell lineages, surface mucous cells have a lifespan of only approximately 4 days.[2]

Oxyntic atrophy, as one of the hallmarks of chronic gastritis, has been associated with the development of gastric adenocarcinoma. Patients with pernicious anemia have a significantly increased risk of gastric adenocarcinoma.[45,46] However, although several rodent models have elicited phenotypes with glandular dysplasia and submucosal invasion, adenocarcinoma in the setting of oxyntic atrophy has not been observed. Similarly, although DMP 777 produces profound oxyntic atrophy and an increased proliferative index in the gastric mucosa, no evidence of adenocarcinoma was observed, even in rats dosed with 200 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} for 6 months. No evidence for adenocarcinoma has been observed in dogs and monkeys administered the drug for more than 1 year. Two-year carcinogenicity studies with DMP 777 in rats and mice are currently ongoing. In animals dosed for up to 1 year, the loss of parietal cells alone does not appear to lead to gastric adenocarcinoma. One important difference between the gastric lesion induced by DMP 777 in rats and oxyntic atrophy in humans is the presence of *H. pylori*–induced inflammatory infiltrates. No significant mononuclear infiltrates were observed in DMP 777–treated rats. Thus, cytokine mediators or other by-products of *H. pylori* infection, as well as dietary or environmental factors, are probably required in addition to oxyntic atrophy for development of gastric adenocarcinoma.

In summary, DMP 777 acts as a parietal cell–targeted protonophore, and oral administration of high doses leads to a rapid cytotoxic death of parietal cells. Acute loss of parietal cells elicits a rapid increase in mucosal proliferative rate and foveolar hyperplasia. With extended dosing, there is a loss of endocrine cells and chief cells and the development of mucous cell metaplasia. All of the effects of DMP 777 on the gastric lineages are completely reversed after withdrawal of treatment. The rapid action of this compound and the reversibility of its effects should allow further study of the mechanisms responsible for the regulation of gastric mucosal lineage ontogeny.

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Received October 27, 1999. Accepted February 3, 2000.

Address requests for reprints to: James R. Goldenring, M.D., Ph.D., Institute for Molecular Medicine and Genetics, CB-2803, Medical College of Georgia, 1120 Fifteenth Street, Augusta, Georgia 30912-3175. e-mail: jgolden@mail.mcg.edu; fax: (706) 721-7915.

Supported by the DuPont Pharmaceutical Corp.; by Department of Veterans Affairs Merit Award and National Institutes of Health (NIH) grants NIDDK DK48370 and DK43405 (to J.R.G.); and by NIH grant CA46413 (to R.J.C.).

The authors thank Ms. Jenetta Smith for technical assistance and Raymond Meade for electron microscopy.