Nuclear gastroenterology: novel techniques in clinical and experimental gastrointestinal mobility, IBD and hepatology
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Chapter 6

Application of gastric-emptying scintigraphy in mice:

Postoperative ileus is maintained by intestinal immune infiltrates that activate inhibitory neural pathways in mice

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Abstract

Postoperative ileus following abdominal surgery largely contributes to patient morbidity and prolongs hospitalization. We aimed to study its pathophysiology in a murine model by determining gastric emptying after manipulation of the small intestine.

Methods. Gastric emptying was determined at 6, 12, 24 and 48 h after abdominal surgery using scintigraphic imaging. Intestinal or gastric inflammation was assessed by immune-histochemical staining and measurement of tissue myeloperoxidase activity. Neuromuscular function of gastric and intestinal muscle strips was determined in organ baths.

Results. Intestinal manipulation resulted in delayed gastric emptying up to 48 h after surgery; gastric half-emptying time 24 h after surgery increased from 16.0 ± 4.4 min after control laparotomy to 35.6 ± 5.4 min after intestinal manipulation. The sustained delay in gastric emptying was associated with the appearance of leukocyte infiltrates in the muscularis of the manipulated intestine, but not in untouched stomach or colon. The delay in postoperative gastric emptying was prevented by inhibition of intestinal leukocyte recruitment. In addition, postoperative neural blockade using hexamethonium (1 mg/kg, i.p.), or guanethidine (50 mg/kg, i.p.) normalized gastric emptying, without affecting small-intestinal transit. The appearance of intestinal infiltrates after intestinal manipulation was associated with increased c-fos protein expression in sensory neurons in the lumbar spinal cord.

Conclusion. Sustained postoperative gastroparesis following intestinal manipulation is mediated by an inhibitory enterogastric neural pathway that is triggered by inflammatory infiltrates recruited to the intestinal muscularis. These findings reveal new targets to shorten the duration of postoperative ileus pharmacologically.
Application: Gastric emptying scintigraphy in mice

Introduction

Postoperative ileus is characterized by a transient hypomotility of the gastrointestinal tract, which occurs after essentially every abdominal operation.\(^1\) It is a major contributor of postoperative discomfort as it results in prolonged hospitalization and increased patient morbidity.\(^2\) The pathophysiology of postoperative ileus is unclear, and as a result current treatment is limited to supportive procedures, such as nasogastric suction, early postoperative feeding,\(^3,4\) and minimal use of opioid analgesics, that are known to intensify ileus.\(^5,6\) Earlier pharmacological means of accelerating postoperative intestinal motility, for instance by antiadrenergic,\(^7\) or cholinergic\(^8\) agents or by inhibiting peripheral opioid effects on gastrointestinal transit\(^4\) have had limited success.\(^4,6,9\) Therefore, more insight into the mechanism mediating postoperative ileus is required for the development of new pharmacological strategies to treat postoperative ileus.

Most previous experimental animal studies have focused on the pathophysiology of instant hypomotility during or directly after abdominal surgery.\(^10-13\) This early component of postoperative ileus results from the activation of mechanoreceptors, nociceptors, or both by bowel manipulation during surgery. The subsequent stimulation of afferent fibers triggers both spinal and supraspinal reflexes, inhibiting gastrointestinal motility and causing an acute generalized postoperative ileus.\(^10\) However, because mechanical activation of mechanoreceptors and nociceptors will cease shortly after closure of the wound, this mechanism cannot explain the prolonged nature of postoperative ileus. In previous reports, it has been demonstrated that the sustained phase of postoperative intestinal hypomotility due to bowel handling results from inflammatory, rather than neuronal, mechanisms.\(^14\) Previously, it has been shown that intestinal handling during abdominal surgery led to an impaired in vitro contractility and a delayed transit of the manipulated small intestine. The latter resulted from activation of resident macrophages and the subsequent establishment of a neutrophilic infiltrate in the muscularis of the small intestine after bowel handling.\(^14\) Although this phenomenon can account for the impaired propulsive motility of the small intestine, it does not explain the hypomotility of the entire gastrointestinal tract, as observed in postoperative ileus.\(^15\) It should also be emphasized that in human postoperative ileus, small-intestinal motility recovers within
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12 h after surgery, whereas gastric and colonic motility remains disturbed for 3-5 days.\textsuperscript{1,6,15} Therefore, mechanisms other than local intestinal inflammation determine the long-term hypomotility of untouched parts of the gastrointestinal tract.

In this study, our aim was to show in a murine model for postoperative ileus that leukocyte infiltrates recruited in the intestinal muscularis by selective small-intestinal manipulation affect the motility of parts of the gastrointestinal tract, distant from the site of manipulation, by triggering an inhibitory neural pathway.
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Materials and Methods

Animals

Mice (female BALB/c; Harlan) were kept under environmentally controlled conditions (light on from 8:00 AM to 8:00 PM; water and rodent nonpurified diet ad libitum, 20-22 °C, 55% humidity). Mice were used at 8-12 wk of age. All animal experiments were performed with approval of the Animal Research Ethics Committee of the University of Amsterdam and following its guidelines.

Surgical procedures

Mice were used at 6-10 wk of age. After an overnight fast, mice were anesthetized by an intraperitoneal (i.p.) injection of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). Surgery was performed under sterile conditions. Mice (10-12 per treatment group) underwent control surgery of only laparotomy, or laparotomy followed by intestinal manipulation. The surgery was performed as follows. A midline abdominal incision was made, and the peritoneum was opened over the linea alba. The small bowel was carefully exteriorized, layered on a sterile moist gauze pad, and manipulated from the distal duodenum to the cecum during 5 min using sterile moist cotton applicators. Contact or stretch on stomach or colon was strictly avoided. After the surgical procedure, the abdomen was closed by a continuous 2-layer suture (Mersilene, 6-0 silk). After closure, mice were allowed to recover for 4 h in a heated (32 °C) recovery cage. After 4 h mice were completely recovered from anesthesia. At 6, 12, 24, and 48 h after surgery, gastric emptying rate was measured using gastric scintigraphy (see below). Thereafter, mice were quickly anesthetized and killed by cervical dislocation, and stomach and small intestine were removed for histological analysis.

Treatments

Monoclonal antibodies against intracellular cell adhesion molecule-1 (anti-CD54 [ICAM-1]; immunoglobulin IgG2b; clone YN1/1.7; 4.5 mg/kg) and lymphocyte function associated antigen-1 (CD11a [LFA-1]; IgG2a; H154.163; 2.3 mg/kg) were dissolved in dialyzed saline (0.9% sodium chloride) and given by i.p. injection 1 h before
surgery. Identical quantities of nonspecific isotype-matched IgGs were administered as controls.

Hexamethonium (1 mg/kg) or guanethidine (50 mg/kg) were dissolved in sterile 0.9% sodium chloride and administered by single i.p. injection. Hexamethonium was administered 10 min, and guanethidine 1 h before the onset of the gastric emptying tests.

**Gastric emptying and transit**

To determine the gastric emptying rate of a noncaloric semiliquid test meal, mice were orally administered 0.1 mL of a 30 mg/mL methylcellulose solution containing 10 MBq of $^{99m}$Tc-Albures (albumin micro colloid; Nycomed-Amersham) in water. Caloric solid test meals were prepared by baking 4 mL of egg yolk mixed with 1 mL of water containing 400 MBq of $^{99m}$Tc-Albures. Mice were offered 100 mg of the baked egg yolk, which was consumed within 1 min. Immediately after administration (semiliquid) or consumption (solid) of the test meal, mice were scanned using a gamma camera (Philips ARC3000) set at 140 keV with 20% energy windows, fitted with a pinhole collimator equipped with a 3-mm tungsten insert. A series of static images of the entire abdominal region was obtained by scanning for 30 sec at 16 min intervals. Static images were obtained at 1, 16, 32, 48, 64, 80, 96 (semiliquid) and 112 min (solid) after administration of the test meal. The scanning frequency applied (once every 16 min) elicited no delay in gastric emptying due to handling stress. Static images were analyzed using Hermes computer software (Nuclear Diagnostics). To determine the gastric-emptying rate, a region of interest (ROI) was drawn around the gastric and total abdominal region in each image obtained. Gastric emptying was measured by determining the percentage of activity present in the gastric ROI, compared with the total abdominal ROI, for each image. Subsequently, the gastric half-emptying time (T½) and gastric retention at 64 min (Ret$_{64}$) were determined for each individual mouse using DataFit software (Oakdale Engineering). To this end, a modified power exponential function $y(t) = 1-(1-e^{-kt})^B$ was used, where $y(t)$ is the fractional meal retention at time $t$, $k$ is the gastric emptying rate in min$^{-1}$, and $B$ is the extrapolated $y$-intercept from the terminal portion of the curve.

For determination of gastro-intestinal transit at 24 h after surgery, animals were killed at 80 min after consumption of the solid test meal. The abdomen was opened and the
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stomach clamped. Stomach, small intestine, cecum and colon were carefully exteriorized, and small intestine was divided in 6 fragments of equal length. The amount of $^{99m}$Tc present in the stomach, small-intestinal fragments, cecum, and colon was subsequently counted in a gamma counter. The geometric center was calculated from each experimental group according to the following formula: $\Sigma(\%\text{ radioactivity per segment} \times \text{segment number})/100$.

Immunohistochemistry and in vitro measurements

Immunohistochemistry. Immunohistochemistry was performed as follows: after rehydration, endogenous peroxidase activity was eliminated by incubating section in 150 mmol/L sodium chloride, pH 7.4 and 50% methanol, containing 3% (wt/vol) H$_2$O$_2$. Nonspecific protein binding sites were blocked by incubation for 30 min in TENG-T buffer (10 mmol/L Tris, 5 mmol/L EDTA, 150 mmol/L sodium chloride, 0.25% gelatin, 0.05% Tween-20, pH 8.0). Serial sections were incubated overnight with an appropriate dilution of rat monoclonal antibodies raised against LFA-1, CD3, and CD4. Binding of the primary antibodies was visualized using 3-amino-9-ethyl carbazole (AEC; Sigma) as a substrate, dissolved in Sodium Acetate buffer (pH 5.0) to which 0.01% H$_2$O$_2$ was added.

C-fos immunohistochemistry. C-fos immunohistochemistry was performed according to Bonaz et al., with modifications. Mice were anaesthetized using a mixture of hypnorm and midazolam at either 90 min or 24 h after surgery. Mice were then transcardially perfused (1.6 mL/min) with 8 mL of a 0.9% NaCl solution, followed by 50 mL of 4% paraformaldehyde in phosphate buffer (0.1 mol/L, pH 7.4). After perfusion, the spinal cord was rapidly removed, post fixed overnight in the same fixative at 4 °C, and cryoprotected for 24 h in 30% sucrose solution containing 0.05% sodium azide. After fixation, part of the lumbar spinal cord (L1-L6) was embedded in OCT (Tissue-Tek; Sakura Finetek Inc.). Forty micron transversal sections were cryostat cut and free floating sections were incubated overnight at 4 °C with the primary polyclonal sheep antibody (0.3 μg/mL; Sigma Genosys) in 0.25% gelatine and 0.5% Triton X-100 in Tris-buffered saline (TBS, pH 7.4). Sections were washed in TBS and incubated with biotinylated anti-sheep antiserum (Vector Laboratories) for 1.5 h at room temperature. After washing in TBS, sections were processed for avidin-biotin-peroxidase (Vectorstain; Vector Laboratories), and peroxidase was visualized using diaminobenzidine in 0.02% nickel sulphate in TBS as the chromogen. For quantification of the number of c-fos expressing nuclei, 30 sections were counted per lumbar spinal cord analyzed.

Whole Mount preparation and immunohistochemistry. Whole mounts of ileal segments were prepared as previously described, with slight modifications. In short, ileal segments (1-6 cm distal from the cecum) were quickly excised and mesentery was removed. Intestinal segments were cut open along the mesentery border, fecal content was washed out in ice-cold phosphate-buffered saline, and segments were pinned flat in a glass-dish filled with pre-oxygenated Krebs-Ringer solution (pH 7.4). Mucosa was removed and the
remaining full-thickness sheet of muscularis externa was fixed for 10 min in 100% ethanol. Muscularis preparations were stored on 70% ethanol at 4 °C until analysis.

**Myeloperoxidase activity assay.** Tissue myeloperoxidase (MPO) activity was determined as follows: either full thickness ileal segments, or isolated ileal muscularis, was blotted dry, weighed, and homogenized in a 20 X volume of a 20 mmol/L potassium phosphate buffer (pH 7.4). The suspension was centrifuged (8000g for 20 min at 4 °C) and the pellet was taken up in 1 mL of a 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.5% of hexadecyltrimethylammoniumbromide (HETAB) and 10 mmol/L ethylenediaminetetraacetic acid (EDTA) and stored in 0.1 mL aliquots at -70 °C until analysis. Fifty μL of the appropriate dilutions of the tissue homogenate was added to 445 μL of assay mixture, containing 0.2 mg/ml tetramethylbenzidine in 50 mg potassium phosphate buffer (pH 6.0), 0.5% HETAB, and 10 mmol/L EDTA. The reaction was started by adding 5 μL of a 30 mmol/L H2O2 to the assay mixture, and the mixture was incubated for 3 min at 37 °C. After 5 min., 30 μL of a 300 μg/mL catalase solution was added to each tube, and tubes were placed on ice for 3 min. The reaction was ended by adding 2 mL of 0.2 mol/L glacial acetic acid and incubating at 37 °C for 3 min. Absorbance was read at 655 nm. One unit of MPO activity was defined as the quantity of MPO activity required to convert 1 μmol of H2O2 to H2O per min at 25 °C using purified MPO activity as a standard (Sigma) and activity was given in Units per gram tissue.

**In vitro contractility measurements.** Stomach and ileum was quickly excised, cut open, and fecal content was flushed with ice-cold Krebs-Ringer solution (pH 7.4). Tissues were pinned down flat on a dissecting dish. After removal of the mucosa, longitudinal muscle strips (approximately 10 x 5 mm) of the gastric fundus and antrum, circular muscle strips (approximately 0.7 x 5 mm) from the antrum, and circular muscle strips of the ileum (approximately 1.0 x 5.0 mm) were mounted in organ baths (25 mL) filled with Krebs-Ringer solution (pH 7.4) maintained at 37 °C and continuously aerated with a mixture of 5% CO2 and 95% O2. One end of each muscle strip was anchored to a glass rod and placed between two platinum electrodes. The other end was connected to a strain gauge transducer (type GM2/GM3; Scaimge) for continuous recording of isometric tension. Recording and analysis of muscle contractions was performed using Acknowledge software (Biopac systems Inc.). The gastric and ileal muscle strips were brought to their optimal point of length-tension relationship using 3 μmol/L acetylcholine and then allowed to equilibrate for at least 60 min before experimentation. Neuromediating contractions of the muscle strips of both the gastric fundus and antrum were induced by means of electrical field stimulation (EFS; 0.5 - 16 Hz, 1 and 2-msec pulse duration, 10-sec pulse trains). Responses were always measured at the top of the contractile peak. In a second series of experiments, contractions were evoked by the muscarinic receptor agonist carbachol (0.1 nmol/L. to 3 μmol/L) and prostaglandin F2a (0.1 nmol/L. to 3 μmol/L). Between the responses to the different contractile receptor agonists, tissues were washed 4 times with an interval of 15 min. At the end of each experiment, muscle strips were blotted dry and weighed. Contractions were calculated in grams contraction per g of tissue dry weight.
Application: Gastric emptying scintigraphy in mice

Results

Intestinal manipulation generates a sustained gastroparesis

At 6, 12, 24, and 48 h after laparotomy or laparotomy combined with intestinal manipulation, gastric emptying of a noncaloric semiliquid test meal was measured by scintigraphic imaging. Examples of such an abdominal scan series of mice that underwent laparotomy (L) or intestinal manipulation (IM) are presented in Figure 1. The anesthetics used during abdominal surgery (ketamine 100 mg/kg and xylazine 20mg/kg) did not alter postoperative (> 6 h) gastric emptying. Also, as shown in Figure 2A and B, laparotomy alone had no effect on the rate of gastric emptying at any time after surgery. After intestinal manipulation, however, gastric emptying was significantly delayed (Fig. 1 and 2). The delay was especially pronounced 6 h after surgery; intestinal manipulation increased Ret<sub>64</sub> by 2.5-fold compared with laparotomy only (Fig. 2A). The T½ was increased even 3-fold (Fig. 2A). Gastric emptying after intestinal manipulation remained significantly delayed at 12 and 24 h after surgery (Fig. 2A), although the animals were fully recovered from surgery at these time points. At 48 h after surgery, Ret<sub>64</sub> and T½ in intestinal manipulation-treated mice had recovered to normal (Fig 2A). Similar results were obtained using caloric solid test meal (Fig 2B). At 24 h after surgery, gastric emptying of a caloric solid test meal was delayed to an extent similar to that of the semiliquid test meal: intestinal manipulation increased the T½ 2.5 fold, compared with laparotomy (Fig 2B).

Intestinal manipulation recruits leukocytes into intestinal muscularis

The delayed gastric emptying at 12, 24 and 48 h after intestinal manipulation coincided with an enhanced activity of the neutrophil indicator MPO in transmural ileal homogenates. At 24 and 48 h after surgery, intestinal manipulation, but not laparotomy alone, resulted in a significant ($P < 0.05$) increase in MPO activity measured in homogenates of ileal tissue, or in ileal homogenates from which the mucosa was stripped off. No increase in MPO activity was observed at earlier time points after surgery.
Figure 1. Gastric-emptying scintigraphy in mice

A representative series of planar pinhole scintigraphic scans of mice that underwent laparotomy (L) or intestinal manipulation (IM) is shown. The position of the stomach is indicated (st) with a dotted circle. From these scans, gastric emptying could be repetitively assessed for each mouse individually by determining the amount of radioactivity present in the gastric region compared to the total abdominal region. Note the difference in radioactivity in the abdominal region between L and IM mice (arrows) at t = 80 min.

(data not shown). Histological analysis of transverse sections of ileal tissue indeed showed the presence of LFA-1⁺ leukocytes in the ileal muscularis 24 h after intestinal manipulation, but not after laparotomy alone. Further immunohistochemical staining showed that these leukocytes were MPO⁺, but CD3⁻ and CD4⁻ (data not shown). Examination of the presence of inflammatory cells containing MPO activity in whole-mount preparations and in isolated ileal muscularis tissue confirmed the presence of leukocyte infiltrates in muscularis of manipulated ileum only. It is important to note that no increased presence of LFA-1⁺ leukocytes was found in the muscularis of gastric antrum or in colonic tissue at any time point after surgery (data not shown).
Figure 2. Gastric-emptying results

Panel A shows the half emptying time (T½, open symbols) and gastric retention after 64 min (Ret64, closed symbols) as a function of time after laparotomy (L, squares) or intestinal manipulation (IM, circles). Intestinal manipulation, performed at t = 0 h, resulted in a significant (P < 0.05) increase in T½, as well as Ret64, compared to laparotomy at t = 6, 12, and 24 h after surgery. Similar results were obtained using a caloric, solid test meal; T½ was significantly increased after intestinal manipulation, compared to mice that underwent laparotomy only (Panel B). Asterisks indicate significant difference from laparotomy using a one-way ANOVA, followed by Dunnett's multiple comparison test.
Figure 3. Gastroparesis after intestinal manipulation is prevented by blocking leukocyte infiltration or neural blockade by hexamethonium or guanethidine treatment (part I)

Gastric emptying, determined by scintigraphic imaging of the abdomen after oral administration of a semiliquid noncaloric meal at 6 h (left) and 24 h (right) after IM, compared to laparotomy alone. Values are given as relative gastric content compared to the total abdominal region. Preoperative treatment with anti-ICAM-1 and anti-LFA-1 antibodies (IM+MAb) normalized gastric emptying of the semiliquid test meal at 24 h postoperatively. Postoperative injections of hexamethonium (IM+hex) or guanethidine (IM+gua) normalized gastric emptying at 6 h, as well as 24 h. Significant differences (P < 0.05), determined by one-way ANOVA with treatment group as variants, are indicated.
**Figure 4.** Gastroparesis after intestinal manipulation is prevented by blocking leukocyte infiltration or neural blockade by hexamethonium or guanethidine treatment (part 2)

Gastric emptying, determined by scintigraphic imaging of the abdomen after oral administration of a semiliquid noncaloric (gray bars), as well as a caloric solid (white bars) test meal at 6 h and 24 h after IM, compared to laparotomy alone. Gastric T½ values using a semiliquid noncaloric test meal are significantly (P < 0.05) increased at 6 h and 24 h after IM, compared to L. Preoperative treatment with anti-ICAM-1 and anti-LFA-1 antibodies (IM+MAb) normalized T½ of semiliquid, as well as solid test meals at 24 h postoperatively. Postoperative injections of hexamethonium (IM+hex) or guanethidine (IM+gua), normalized T½ at 6 h, as well as 24 h. Values are averages ± SEM. Significant differences (P < 0.05), determined by one-way ANOVA with treatment group as variants, are indicated.
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**Occurrence of delayed gastroparesis depends on intestinal leukocyte influx**

To evaluate the role of the small-intestinal infiltrate in the development of gastroparesis, intestinal manipulation mice received a preoperative bolus with monoclonal blocking antibodies against ICAM-1 and LFA-1 to prevent leukocyte recruitment during the postoperative period. Analysis of MPO-containing leukocytes in ileal muscularis or MPO activity in ileal muscularis homogenates at 24 h after intestinal manipulation showed that antibody treatment inhibited the leukocyte recruitment down to 30% \((P < 0.05)\) of untreated ileal segments. Prevention of the postoperative inflammatory infiltrate did not affect the delay in gastric emptying 6 h after surgery but normalized gastric emptying 24 h after intestinal manipulation (data not shown). This effect was seen using a noncaloric liquid, as well as a caloric solid test meal (data not shown). Treatment with identical quantities of isotype-matched control IgG did not affect leukocyte recruitment or the observed postoperative delay in gastric emptying. These observations show that the later phase of postoperative gastric ileus is mediated by an intestinal inflammatory infiltrate. The antibody regimen could not prevent gastroparesis 6 h after surgery, which is in line with the observation that the intestinal MPO activity was not increased at this time point.

**Postoperative inflammatory infiltrates in the intestinal muscularis activate spinal afferents resulting in gastric ileus**

Next, we investigated whether the small-intestinal infiltrate induced gastroparesis by activation of an inhibitory neural pathway. To evaluate afferent neurotransmission in this context, we measured the induction of the immediate-early gene c-fos within the spinal cord 24 h after laparotomy or laparotomy with intestinal manipulation. Intestinal manipulation significantly \((P < 0.05)\) increased the number of nuclei expressing c-fos protein in the lumbar dorsal horn of the spinal cord compared with laparotomy alone (data not shown). Most positively labeled nuclei were found in laminae I of the lumbar dorsal horn. Treatment with neutralizing antibodies against ICAM-1 and LFA-1 before intestinal manipulation prevented the increase in spinal c-fos expression, showing that
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Figure 5. Postoperative hexamethonium treatment accelerates gastric emptying but not intestinal transit after intestinal manipulation

Transit measured as a percentage distribution of the nonabsorbable $^{99m}$Tc-Albures over the gastrointestinal tract after oral intake of a caloric solid test meal. Stomach and 6 equal segments of small bowel, cecum and colon were isolated 80 min after oral ingestion of the caloric test meal (baked egg yolk), and radioactivity present was counted in each segment. In mice that underwent intestinal manipulation and received vehicle (saline) (IM + saline; dark gray bars) the distribution of radioactivity indicates a delayed gastric emptying and an impaired small-intestinal transit time, compared with control mice that underwent only laparotomy (L + saline; black bars). The geometric center is significantly lower ($P < 0.05$; one-way ANOVA) in mice that received IM + saline. Postoperative treatment with hexamethonium prevented the surgery-induced delay in gastric emptying (IM + hexamethonium; light gray bars), but not intestinal transit. Consequently, the geometric center was not different from mice that underwent IM + saline. The impaired intestinal transit after manipulation is highlighted by higher percentage of radioactivity found in the intestinal fragments 1 and 2 in manipulated intestine compared to laparotomy, and the lower percentage of radioactivity in fragments 5 and 6 (indicated by the dotted boxes). Numbers shown are the averages ($\pm$ SEM).

Intestinal leukocyte infiltrates mediate spinal afferent activation (data not shown). Treatment with control IgG antibodies did not prevent the increased c-fos expression after intestinal manipulation.
To further examine whether the sustained phase of delayed gastric emptying after intestinal manipulation was neurally mediated, mice were treated either with hexamethonium, an antagonist of nicotinic receptors (1 mg/kg, 10 min before gastric scintigraphy), or with guanethidine, an adrenergic blocker (50 mg/kg, 1 h before gastric scintigraphy) at 24 h after abdominal surgery. These treatments did not affect gastric emptying (T½ or Ret₆₄) in control mice that underwent control laparotomy. Furthermore, the treatment with hexamethonium or guanethidine did not affect the leukocyte recruitment in the ileal muscularis after intestinal manipulation at 24 h (data not shown). After intestinal manipulation however, treatment with these neural blockers either partially (6 h after surgery) or completely (24 h after surgery) prevented the delay in gastric emptying, compared to treatment with vehicle control (Fig. 3 and 4).

**Hexamethonium ameliorates postoperative gastric emptying, but not intestinal transit**

Because normalization of gastric emptying could also be secondary to improvement or acceleration of intestinal transit, we evaluated the effects of hexamethonium on intestinal transit. Figure 5 shows that, in mice that underwent intestinal manipulation, the radiolabeled test meal accumulates in the stomach, and that the small-intestinal transit is delayed compared with control mice that underwent laparotomy only. As indicated in Figure 5, intestinal manipulation and vehicle (saline) treatment led to a significant decrease of the geometric center ($P < 0.05$). Postoperative treatment with hexamethonium prevented this surgery-induced delay in gastric emptying but did not prevent the delay in small-intestinal transit. Consequently, the geometric center was not different from mice that underwent intestinal manipulation and received saline (Fig. 5). The finding that hexamethonium treatment normalizes gastric emptying even though intestinal transit is still delayed implies that the delayed gastric emptying is not secondary to a functional obstruction of the small intestine. To further evaluate the effect of hexamethonium on the delay in intestinal transit induced by manipulation, we tested the in vitro contractility of intestinal circular muscle strips. Intestinal manipulation led to an impaired contractile activity of circular smooth muscle in response to carbachol.
The addition of hexamethonium ($3 \times 10^{-5}$ mol/L) did not reverse the impaired contraction response (data not shown).

**Neuromuscular properties of gastric fundus and antrum are not affected by intestinal manipulation**

To exclude the possibility that the delayed gastric emptying resulted from impaired local neuromuscular function, in vitro contractility of isolated muscle strips from gastric fundus and antrum was investigated in organ baths. The isometric contractile responses were determined from longitudinal or circular muscle strips isolated from gastric fundus and antrum to increasing concentrations of the muscarinic receptor agonist carbachol (0.1 nmol/L to 3 µmol/L), and of prostaglandin F2α (0.1 nmol/L to 3 µmol/L). Intestinal manipulation did not affect the dose-dependent contractile response to stimulation of gastric muscle strips with prostaglandin F2α, or carbachol, compared to mice that underwent laparotomy alone (data not shown). In addition, contractions evoked by nerve stimulation (0.5 – 16 Hz, 1-msec pulse duration, 10-sec pulse trains) in gastric fundus and antrum from mice that underwent intestinal manipulation were not significantly different from contractions in those that underwent control laparotomy.

**Discussion**

Postoperative ileus is associated with vomiting, bloating, nausea, and abdominal pain and contributes considerably to postoperative patient morbidity. In addition, it has a major economical effect due to prolonged hospitalization and increased costs of health care. The annual economic cost resulting from the occurrence of postoperative ileus in the U.S. population has been estimated to be $750,000,000,² and this may even be a gross underestimation, because drug costs and indirect costs were not measured. Until now, treatment of postoperative ileus has been rather disappointing, mainly because of a lack of pathophysiological insight. Here we provide data clarifying the underlying mechanisms of the sustained phase of postoperative ileus. First, we confirmed¹⁴ that bowel manipulation induces the local influx of inflammatory cells. Subsequently, we
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showed that the recruitment of this muscular infiltrate is associated with the activation of an inhibitory adrenergic neural pathway leading to prolonged postoperative gastroparesis. Our data suggest that this mechanism is responsible for the generalized hypomotility observed in postoperative ileus.

Most previous studies have evaluated only the acute effects of abdominal surgery on gastrointestinal motility. However, we show here that, in mice, intestinal manipulation, but not laparotomy alone, delays gastric emptying up to 48 h after surgery. Two phases can be distinguished in the period of postoperative gastric hypomotility: a first acute phase that is not related to any inflammatory event and a second, later-onset, and more sustained phase that is temporally associated with a leukocyte influx into the intestinal muscularis. Abundant evidence has been reported indicating that the mechanism underlying the first, acute phase is a neurally mediated phenomenon: chemical neural blockade with capsaicin, hexamethonium or adrenergic antagonists reduced the rate of postoperative ileus in animal models. In addition, surgical procedures interrupting neural input to the investigated gastrointestinal region, such as vagotomy or splanchnectomy, prevented or reduced the postoperative hypomotility. Furthermore, studies evaluating neuronal c-fos expression showed that both spinal and supraspinal pathways synapsing in the brainstem are activated during abdominal surgery. The inhibitory efferent pathways involved have been shown to be adrenergic and nonadrenergic noncholinergic in nature.

In this study, we confirmed that the acute phase of postoperative ileus is mediated by a neural inhibitory mechanism: the nicotinic antagonist hexamethonium as well as the adrenergic blocker guanethidine improved the manipulation-induced delayed gastric emptying. The observation that guanethidine only partially normalized the gastric emptying after intestinal manipulation is in concert with the involvement of a nonadrenergic mechanism in the efferent pathway mediating this phenomenon. These findings clearly indicate that bowel manipulation activates neural pathways, most likely via activation of mechanoreceptors or nociceptors. However, mechanisms other than mechanical activation of these receptors must be involved after closure of the abdomen to explain for the prolonged phase of postoperative ileus lasting up to 24 h, as observed in this study.
In this respect, Kalf et al. previously described that intestinal manipulation initiated the up-regulation of ICAM-1 and LFA-1, and subsequent recruitment of leukocytes into the intestinal muscularis, leading to impaired contractility of circular muscle strips of jejunum. It was suggested that these functional changes in the intestinal muscularis resulting from a local inflammatory response were directly responsible for the sustained paralysis of the gastrointestinal tract. In this study, we showed that the occurrence of an inflammatory infiltrate was confined to the manipulated small intestine and was absent in the nonmanipulated stomach or colon. In addition, although the in vitro contractility of ileal circular muscle strips was impaired after intestinal manipulation (compare with Kalf et al.), that of gastric muscle strips was unaffected by intestinal manipulation. The latter finding shows that the delayed gastric emptying 24 h after intestinal manipulation is not due to impaired gastric neuromuscular function related to inflammation.

Instead, our results provide evidence that gastric ileus is the result of activation of an inhibitory adrenergic neural pathway triggered by manipulation-induced leukocyte infiltrates in the intestinal muscularis. This evidence is based on 2 main findings. First, the neuronal blockers guanethidine and hexamethonium normalized postoperative gastric emptying. Second, we confirmed that the occurrence of muscular infiltrates was associated with the activation of c-fos expression in spinal sensory neurons. Furthermore, blockade of manipulation-induced intestinal leukocyte recruitment by treatment with neutralizing antibodies against LFA-1 and its main cellular ligand, ICAM-1, prevented postoperative activation of spinal neurons and normalized gastric emptying. These findings indicate that the activation of the adrenergic inhibitory pathway is most probably maintained by the leukocyte infiltrate in the small-intestinal muscularis. The finding that ICAM-1 treatment did not normalize the delay in gastric emptying 6 h after surgery further corroborates this notion, because no infiltrate was yet present at that time. What specific cell population, leukocyte derived mediator, or afferent nerve receptor is responsible for the neuroimmune interaction leading to the activation of the adrenergic pathway remains to be established.

Alternatively, impaired gastric emptying may simply be secondary to stasis of chyme in the intestine. The intestinal malfunction resulting from the manipulation-induced muscular inflammation could theoretically back up the emptying of the stomach.
However, we showed that hexamethonium did normalize gastric emptying even though intestinal transit remained delayed, making this possibility less likely. The independent modulation of gastric emptying and intestinal transit is in agreement with previous reports. The finding that hexamethonium normalized only gastric emptying and not intestinal transit does not imply that the inhibitory neural input is confined to the stomach. Rather, the delay in intestinal transit being resistant to hexamethonium can be explained by the local effect of manipulation-induced muscular inflammation on intestinal motility. Indeed, we found that hexamethonium did not prevent the occurrence of the infiltrate and had no effect on the impaired in vitro contractility of the manipulated small intestine. To what extent the inhibitory neural input contributes to the impaired intestinal transit cannot be determined from our experiments.

Finally, intestinal inflammation could affect gastric motility via enhanced release of circulating inflammatory mediators from the site of inflammation, such as the cytokines IL-1β, TNFα, or IL-6, prostaglandines, bradykinin, or mediators released by activated mast cells that potentially may affect gastric motility. However, in our current study, hexamethonium or guanethidine administered 24 h after surgery could prevent gastroparesis, which implies that neuronal activity, rather than circulating mediators, determines the delay in gastric emptying.

Several pathophysiological mechanisms may explain the inflammatory events observed in surgically manipulated bowel tissue. Mechanical manipulation of the bowel during surgery leads to intense activation of nerve fibers in the gut wall. This may result in local release of substances with potent proinflammatory properties, such as substance P or calcitonin gene-related peptide, which can potentially induce neurogenic inflammation. In addition, recruitment of leukocytes may also be initiated via the release of proinflammatory mediators by activated resident intestinal muscularis macrophages or mast cells. The latter are known to be activated by neurally released substance P, and massive mast cell activation has been described in response to manipulation of the gut. These leads, together with our current data, suggest that the antiinflammatory effects of mast cell stabilization may be instrumental in shortening the duration of postoperative ileus.
Conclusion

We conclude that postoperative ileus is a neurally mediated disorder consisting of an early phase, which results from the triggering of afferents by activation of mechanoreceptors, nociceptors, or both after bowel manipulation or trauma, and a second, prolonged phase, in which an adrenergic inhibitory pathway is triggered by a local infiltrate. In the rat, incremental degrees of surgical intestinal manipulation and trauma have been shown to be proportional to the increase in recruitment of leukocyte infiltrates and the severity of intestinal paralysis. This positive correlation may also explain the relation between the extent, site, and length of intra-abdominal manipulation duration and the severity of postoperative ileus found in human studies. These findings indicate that in order to accelerate resumption of postoperative gastrointestinal motility and patient recovery, bowel manipulation and the consequent recruitment of leukocytes should be kept minimal during abdominal surgery, i.e., during laparoscopy. However, our study also shows important new targets in reducing the duration and severity of postoperative ileus pharmacologically by inhibiting postoperative recruitment of leukocytes to the intestinal wall, for instance, by using blocking antibodies or antisense nucleotides against ICAM-1. Shortening postoperative ileus is clinically, and socio-economically highly desired, and we anticipate that temporal, perioperative prevention of the influx of inflammatory cells may evolve as a new approach to reduce postoperative patient morbidity.
References


Application: Gastric emptying scintigraphy in mice


