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# Effect of TNBS-induced morphological changes on pharmacological contractility of the rat ileum

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**Abstract**

Intraluminal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS) in ethanol is a classical model of colitis in the rat. Little is known about the time-related effect of TNBS on the contractility and morphology of the rat ileum. After 36 h, TNBS induced acute ileitis. Spontaneous activity of longitudinal muscle strips was decreased, as were receptor- and nonreceptor-mediated contractions and contractions induced by electrical stimulation. After 1 week, mucosal integrity was restored, although the thickness of both mucosal and muscle layers was increased. Spontaneous activity, receptor- and nonreceptor-mediated contractions and electrically induced contractions of longitudinal muscle strips were increased due to hypertrophy and hyperplasia of smooth muscle cells. This was confirmed in the contractility study of individual muscle cells. Functional alterations after 1 week were restricted to a decreased response to substance P. TNBS-ileitis in the rat lacks a chronic phase and is accompanied by functional hypocontractility of longitudinal smooth muscle cells during the acute inflammation, whereas the contractility of the longitudinal muscle layer is increased in the postinflammation phase due to structural alterations. There is a selective inhibition of the response to substance P in the postinflammation phase. 

*Keywords:* Contractility; Ileum; Inflammation; Morphology; (Rat); Smooth muscle

## 1. Introduction

Gastrointestinal inflammation is accompanied by structural and functional changes of the gut, leading to gastrointestinal motility disturbances (Collins, 1996). These motility disturbances contribute to the generation of symptoms, such as nausea, dyspepsia, abdominal cramps and diarrhea.

Intraluminal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS) in ethanol is a classical model of intestinal inflammation, mimicking some aspects of Crohn's disease (Elson et al., 1995). Initially, TNBS was administered in an enema to develop a rat model of colitis (Morris et al., 1989). Nowadays, the model of TNBS-induced inflammation has been further adapted to the mouse colon (Neurath et al., 1995), the guinea pig small intestine (Miller et al., 1993), the rabbit small intestine (Sjogren et al., 1994) and also the rat small intestine (Goldhill et al., 1999).

The model of TNBS-induced colitis is used in several studies to investigate motility disturbances occurring in the inflamed rat colon. Dysmotility was shown in vivo as an initial increase in colonic transit and a decrease in colonic myoelectrical activity within the first few hours after TNBS instillation, followed by a progressive decrease of the transit and an increase in the colonic myoelectrical activity (Pons et al., 1992; Morteau et al., 1993). In vitro studies evidenced functional changes at the colonic neuromuscular level. The contractility of the circular smooth muscle layer is increased 4 h after TNBS instillation, whereas after 1 week, the contractility of both the circular and the longitudinal muscle layer is decreased, irrespective of the type of the contractile stimulus (Grossi et al., 1993; Hosseini et al., 1999). Also, the expression and the release of specific neurotransmitters from the myenteric plexus are disturbed

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(Jacobson et al., 1997; Miampamba and Sharkey, 1998). Finally, TNBS-induced inflammation of the rat colon leads to morphological changes of the neuromuscular apparatus. Important structural changes of the rat colonic wall have been shown to appear from 1 week onward after TNBS administration. The thickness of the muscularis externa is significantly increased, which is partially due to smooth muscle cell hypertrophy (Morris et al., 1989; Miampamba and Sharkey, 1998; Hosseini et al., 1999). This increase in muscle mass may contribute to the inflammation-induced dysmotility.

Although little information is as yet available on the effect of TNBS on the rat small intestine, it appears that the inflammatory response to TNBS in the small intestine is different from the well-characterized response in the rat colon. The cellular components of the inflammatory infiltrate are comparable in the colon and the small intestine (Morris et al., 1989; Grossi et al., 1993; Morteau et al., 1993; Coméra et al., 1999; Hosseini et al., 1999). However, the time course of the inflammatory response is different. Whereas TNBS-induced colitis persists for several weeks (Morris et al., 1989; Pons et al., 1992), preliminary reports of different groups show that the transmural inflammation of the rat small intestine recovers 1 week after TNBS (Demedts et al., 1999a,b; Moreels et al., 1999). Furthermore, it was shown that the myoelectrical activity of the small intestine was disrupted with a loss of phase I quiescence and decreased phase III activity 1 week after TNBS (Demedts et al., 1998). Contractility studies of the acutely inflamed rat ileum show that the response of circular muscle strips to substance P was selectively increased (Goldhill et al., 1999). However, in the acutely inflamed colon, circular muscle contractility is increased irrespective of the contractile stimulus used, with the exception of the selectively decreased response to neurokinin A (Hosseini et al., 1999). At this moment, no data are available on the TNBS-induced modulation of ileal longitudinal muscle contractility in the rat.

Therefore, the present study was undertaken to investigate the effect of TNBS-induced ileitis on the structural and contractile properties of the longitudinal muscle layer of the rat ileum during the course of inflammation. To study receptor-specific alterations, different contractile receptor agonists were used. To determine the portion of the contractile response that was accounted for by mere structural changes of the muscle layer, longitudinal muscle strip contractility was always presented in g contraction and in g contraction normalized to the cross-sectional area of the longitudinal muscle layer. Using the two methods to express the contractile data enables to differentiate between structural and functional alterations of the neuromuscular apparatus during TNBS-induced ileitis. In addition, we compared the contractility of longitudinal muscle strips with the contractility of individual longitudinal smooth muscle cells. The inflammatory response was assessed both histologically and biochemically.

## 2. Material and methods

### 2.1. Animals

Before the ileal treatment with TNBS or saline, male Wistar rats (Iffa Credo, St.-Germain sur l'Abresle, France) weighing 300–400 g were fasted for 48 h with free access to drinking water. This prolonged fasting period was necessary for the ileum to empty its content completely into the caecum. Preliminary experiments showed that TNBS treatment of chyme-containing ileum failed to induce a pronounced inflammatory response. Before tissues were harvested for histology, myeloperoxidase activity assay and pharmacology, rats were fasted for 12 h. This fasting period enabled us to study ileal smooth muscle contractility in fasting conditions.

Rats were randomly divided into four groups. The first two groups were studied 36 h after the treatment with TNBS ($n = 7$) or saline ($n = 4$). The third and fourth groups were studied 1 week after the treatment with TNBS ($n = 6$) or saline ($n = 3$). Rats treated with saline served as time controls for the rats treated with TNBS. When the contractility of individual longitudinal smooth muscle cells was studied, two groups were used: 1 week after TNBS treatment ($n = 5$) and 1 week after saline treatment ($n = 5$). The local Ethics Committee of the University of Antwerp approved all described experiments.

### 2.2. Induction of inflammation

After a fasting period of 48 h with free access to drinking water, rats underwent a laparotomy under ether anesthesia. The terminal ileal loop was gently exteriorized on a sterile gauze. Subsequently, 30 mg of TNBS dissolved in 0.25 ml of 40% (v/v) ethyl alcohol was injected transmurally into the lumen, 10 cm proximal to the ileocolonic junction. Controls received 0.25 ml of 0.9% saline. The site of injection was stained with a permanent marker. The laparotomy was closed in two layers. Sixty minutes after the operation, the rats were allowed to eat and drink ad libitum and were weighed daily.

Thirty-six hours or 1 week later, the rats were euthanized by decapitation and bleeding after an overnight fast. Tissue samples were harvested for histology, myeloperoxidase activity assay and in vitro smooth muscle-contractility studies. Preliminary experiments showed that TNBS-induced inflammation reached out over an ileal segment of about 4 cm proximally to the site of injection.

### 2.3. Tissue preparation

After the rats were sacrificed, the 4-cm-long treated ileal segment, located 10 cm proximal to the ileocolonic junction, was rapidly removed and placed in ice-cold Krebs-Ringer solution (118.3 mM NaCl, 4.7 mM KCl, 1.2

mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 2.5 mM $CaCl_2$, 25 mM $NaCHO_3$, 0.026 mM CaEDTA and 11.1 mM glucose). This 4-cm segment was used in all experiments in the following way. The most distal ~1.3 cm was used for the myeloperoxidase activity assay. The middle ~1.3 cm was prepared as a 1-cm-long longitudinal muscle strip for pharmacological experiments. The most proximal ~1.3 cm was used for histological examination. In a separate set of experiments, in which the contractility of the individual smooth muscle cells was studied, the entire 4-cm segment was used.

### 2.4. Morphometric analysis

#### 2.4.1. Staining

A 1.3-cm-long segment of the terminal ileum immediately proximal to that used for the contractility studies was fixed in 4% formaldehyde and embedded in paraffin. Routine 4 μm transverse sections were examined after Sirius red and haematoxilin-eosin staining. Morphometric analysis was performed in a blinded fashion using a computer-assisted color image analysis system (PC-image Colour, Foster Findlay Associates, Newcastle-upon-Tyne, United Kingdom). The thickness of the different layers of the gut wall, the cross-sectional area of the longitudinal muscle layer and the cell number and size of longitudinal smooth muscle cells were determined. All morphometric measurements were performed at three points around the circumference separated by ~120°. The average of the three measurements was used for statistical analysis. The cross-sectional area of a section was determined only once.

#### 2.4.2. Cell number

To determine the role of hyperplasia (increased cell number), the total number of smooth muscle nuclei in each cross-section of the ileum was determined in three regions of a known area (Blennerhassett et al., 1992). The average of these numbers of nuclei per area was then used to extrapolate to the total number of nuclei in the longitudinal muscle layer using the following equation: nuclei/area × cross-sectional area = total number of nuclei. The contractility experiments of single smooth muscle cells showed no multinucleated cells. Hence, the nuclei number represents the number of smooth muscle cells.

#### 2.4.3. Cell size

To determine the role of hypertrophy (increased cell size), the number of longitudinal smooth muscle cells was determined per standard area (1000 $\mu m^2$). A decreased number in cells per standard area reflects an increased cell size and, thus, hypertrophy (Blennerhassett et al., 1992).

Due to the transmural inflammatory infiltrate 36 h after TNBS treatment, counting smooth muscle nuclei was only performed in ileal sections of control rats and of rats 1 week after TNBS treatment, when the inflammatory infiltrate was no longer present.

### 2.5. Myeloperoxidase activity assay

Tissue myeloperoxidase activity, which is directly related to the number and activity of myeloid cell infiltrate in the inflamed tissue, was assayed to monitor the degree of inflammation (Bradley et al., 1982). Ileal segments of ~1.3 cm long were taken at the site of injection. Full thickness tissue samples were blotted dry, weighed and placed in a potassium-phosphate buffer pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide in a proportion of 5 g tissue per 100 ml buffer. The samples were placed on ice, minced and homogenized for 30 s (PRO 200, PRO Scientific, Monroe, CT, USA). The homogenate was subjected to two sonication and freeze–thawing cycles. The suspension was centrifuged at 15,000 × *g* for 15 min at 4 °C. Aliquots (0.1 ml) of the supernatant were added to 2.9 ml of an *o*-dianisidine solution (16.7 mg of *o*-dianisidine in 1 ml methyl alcohol, 98 ml 50 mM potassium-phosphate buffer pH 6.0 and 1 ml of a 0.05% $H_2O_2$ solution as a substrate for the myeloperoxidase enzyme). The change in absorbance was read at 460 nm over 60 s using a Spectronic Genesys 5 Spectrophotometer (Milton Roy, Rochester, NY, USA). One unit of myeloperoxidase activity was defined as the quantity able to convert 1 μmol $H_2O_2$ to $H_2O$ per min at 25 °C and was expressed in units per gram tissue.

### 2.6. Pharmacology of ileal longitudinal smooth muscle strips

#### 2.6.1. Tissue preparation

The middle ~1.3 cm ileal segment was used for pharmacological experiments. After removal of the mucosa by sharp dissection under a stereomicroscope, longitudinal muscle strips of exactly 1.0 cm in length and 0.3 cm wide were mounted in organ baths (5 ml) filled with Krebs-Ringer solution maintained at 37 °C and aerated with a mixture of 5% $CO_2$ and 95% $O_2$.

#### 2.6.2. Isometric tension recording

One end of the muscle strip was anchored to a glass rod and pulled through two platinum ring electrodes. The other end was connected to a strain gauge transducer (Statham UC2) for continuous recording of isometric tension. The muscle strips were brought at their optimal point of length–tension relationship in a stepwise fashion using 0.1 μM carbachol and then allowed to equilibrate for at least 60 min before experimentation. During the equilibration period, the muscle strips were washed every 15 min with fresh Krebs-Ringer solution. Next, passive tension and active tension to 50 mM KCl was determined at the optimal length ($L_0$) of the muscle strip.

#### 2.6.3. Experimental protocol

Contractions of the ileal muscle strips were induced in three different ways. First, nonreceptor-mediated contrac-

tions were induced by 50 mM KCl in the presence of the neurotoxin tetrodotoxin (1 μM). Secondly, receptor-mediated contractions were studied by means of different contractile receptor agonists. They were randomly induced by cumulative concentrations of acetylcholine (0.1 nM–1 mM), serotonin (0.1 nM–0.1 mM), prostaglandin $F_{2\alpha}$ (0.1 nM–1 μM) and substance P (0.1 nM–0.1 μM). Between the responses to the different contractile receptor agonists, tissues were washed four times with an interval of 15 min. Receptor-mediated contractions were expressed by their respective $pD_2$ (negative logarithm of the molar concentration inducing 50% of the maximal contractile response) and $E_{max}$ values (maximal contraction). Thirdly, contractility was studied by means of electrical field stimulation (0.25–16 Hz, 1-ms pulse duration, 10-s pulse trains) of the myenteric neurons. Preliminary experiments showed that these electrical field stimulation parameters selectively depolarized enteric neurons, since the elicited contractions were completely blocked by pretreatment with 1 μM tetrodotoxin. The responses to stimulation at higher frequencies were not completely blocked. Electrical field stimulation-induced contractions were expressed by their respective $EF_{50}$ (frequency inducing 50% of the contractile response to 16 Hz) and $E_{max}$ values. Responses were always measured at the top of the contractile peak. All contractions are expressed in g contraction and in g contraction normalized to the cross-sectional area ($mm^2$), which was measured on a section of the adjacent 1.3 cm ileal segment. The contractility of longitudinal muscle strips 36 h and 1 week after TNBS treatment was compared with the muscle strip contractility of age-matched control rats.

### 2.7. Pharmacology of isolated longitudinal smooth muscle cells

In a separate group of animals, longitudinal smooth muscle cells were isolated by collagenase digestion from the 4 cm ileal segment proximal to the injection site of saline or TNBS, as previously described (Botella et al., 1992). Briefly, after decapitation, this 4 cm ileal segment was rapidly removed and the longitudinal muscle layer was carefully dissected from the rest of the ileal wall. Small sheets from the longitudinal muscle layer were incubated for 30 min at 31 °C, in 15 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered medium (25 mM), containing 150 U/ml collagenase (Type II) and 0.01% soybean trypsin inhibitor and gassed with a mixture of 95% $O_2$ and 5% $CO_2$. The medium consisted of NaCl, 132.0 mM; KCl, 5.4 mM; $NaH_2PO_4$, 1.0 mM; $Na_2HPO_4$, 5.0 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 1 mM; glucose, 11.5 mM; bovine serum albumin, 0.2% (w/v) and was supplemented with sodium pyruvate, 5 mM; sodium fumarate, 5 mM; sodium glutamate, 5 mM; glutamin, 2 mM; amino acid mixture, 1% (v/v); vitamin mixture, 1% (v/v); penicillin G, 50 μg/ml; and streptomycin, 50 μg/ml. The pH of the buffered medium was adjusted to 7.4. At the end of the incubation, the medium was filtered through a 500-μm Nitex filter and the partly digested tissues were washed with 30 ml enzyme-free medium, after which they were allowed to disperse spontaneously in enzyme-free medium for 30 min. Finally, the spontaneously dissociated muscle cells were harvested by filtration and used for functional measurements.

Cell suspensions were studied usually within 30 min at 31 °C. Concentration–response curves were constructed for acetylcholine (0.001 nM–0.1 μM) by adding an aliquot (250 μl) of cell suspension to collagenase-free HEPES medium (250 μl) containing the concentration of acetylcholine to be tested. The reaction was terminated after 30 s by adding glutaraldehyde (pH 7.4) to a final concentration of 2.5%. Cells receiving no acetylcholine served as controls.

The length of the isolated smooth muscle cells was determined by Image Splitting. An aliquot of 50 μl treated cell suspension was placed on a Malassez slide. The first 50 randomly encountered and morphologically intact cells were measured using a Carl Zeiss eyepiece at a magnification of at least 200 times. For the vials with control cells, two different aliquots were taken and two times 50 cells were measured. The absolute cell-length measurement was performed with a scale mask placed on a video screen, connected to a video camera. Magnification due to the video camera had been first calculated by use of a micrometer. The contractile response to acetylcholine of the isolated smooth muscle cells was expressed as the percentage decrease in cell length from controls receiving no acetylcholine. The following formula was used: $[(L_0 - L_x)/L_0] \times 100$, where $L_0$ is the mean length of cells in control state and $L_x$ the mean length of acetylcholine-treated cells. Individual smooth muscle cell contractility 1 week after TNBS treatment was compared with the smooth muscle cell contractility of age-matched controls receiving saline.

### 2.8. Drugs used

Acetylcholine chloride, carbachol, hexadecyltrimethylammonium bromide, *o*-dianisidine dihydrochloride, penicillin G, sodium fumarate, sodium glutamate, sodium pyruvate, streptomycin, substance P, vitamin mixture (Sigma, St. Louis, MO, USA); collagenase (Worthington Biochemical, Freehold, NJ, USA); essential amino acid mixture (ICN Pharmaceuticals, Costa Mesa, CA, USA); *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), soybean trypsin inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA); hydrogen peroxide 30% (Merck, Darmstadt, Germany); serotonin creatinine sulfate monohydrate (Janssen Chimica, Geel, Belgium); tetrodotoxin (Alomone Labs, Jerusalem, Israel); 2,4,6-trinitrobenzene sulfonic acid solution (Fluka, Neu-Ulm, Germany); prostaglandin $F_{2\alpha}$ (Dinolytic® purchased from Upjohn (Puurs, Belgium), as a sterile aqueous solution containing 5 mg/ml prostaglandin $F_{2\alpha}$ and 9 mg/ml benzyl alcohol).

All drugs were dissolved in distilled water and further dilutions were made in physiological salt solution on the day of the experiment.

### 2.9. Presentation of results and statistical analysis

The body weight of the rats is expressed in grams. The morphometric analysis of the ileal wall thickness is expressed in micrometers. Cross-sectional area is expressed in $mm^2$. Cell number is expressed as number of cells per cross-sectional area. Cell size is expressed as the ratio cells/1000 $\mu m^2$. Myeloperoxidase activity is expressed in units per g tissue. Spontaneous activity and contractions of muscle strips are expressed both in g contraction and in g contraction/$mm^2$ cross-sectional area. $EF_{50}$, $pD_2$ and $E_{max}$ values are calculated by Graphpad Prism software (GraphPad Software, San Diego, CA, USA). The contractile response of the isolated smooth muscle cells was expressed as the percentage decrease in cell length from untreated controls.

All values are shown as mean ± S.E.M. for the number of rats indicated. For statistical analysis, one-way analysis of variance followed by Student–Newman–Keuls test was used to compare the results of the two TNBS-treated groups of rats and the control rats. $P$ values of less than 0.05 were considered to be significant. Since there were no statistically significant differences between the two age-matched control groups, all control rats were pooled into one group. Smooth muscle cell count and the contractility experiments of isolated smooth muscle cells were only performed in control rats and in rats 1 week after TNBS treatment. For comparison between these two groups, Student's $t$-test for unpaired values was used. $P$ values of less than 0.05 were considered to be significant.

## 3. Results

### 3.1. Induction of ileitis

After TNBS treatment, rats developed black loose stools for 2 to 3 days. Thereafter, the fecal pellets regained normal colour and consistency. This short period of diarrhea was followed by a significantly slower increase in body weight as compared to rats treated with saline (Fig. 1). Although by the time of 1 week and after a fasting period of 12 h before experimentation, this difference in body weight between controls and TNBS-treated rats was no longer significant.

### 3.2. Histology

No inflammatory infiltrates were seen in the ileum of control rats (Fig. 2A). Thirty-six hours after TNBS treatment, diffuse ulcerative inflammation and epithelial exfoliation of the ileal mucosa in the treated segment were seen (Fig. 2B). There was no macroscopic evidence of inflammation in any part of the ileum proximally or distally to the 4 cm harvested segment. The inflammatory infiltrate consisted of polymorphonuclear and mononuclear leukocytes. Morphometric analysis showed that the total ileal wall thickness of TNBS-treated rats was significantly decreased, due to a decrease in villus height, resulting in a decreased villus crypt ratio. Cross-sectional area of the longitudinal smooth muscle layer was not different from control values. (Table 1). One week after TNBS treatment, a proliferative regeneration of the mucosal integrity was seen. Infiltrated inflammatory cells were only scarcely present (Fig. 2C). At that time, the total ileal wall thickness of TNBS-treated rats was increased due to an increase of thickness of both mucosal and muscular layers. Also, the cross-sectional area of the longitudinal smooth muscle layer was significantly increased. Counting the nuclei of smooth muscle cells showed both hyperplasia and hypertrophy of the longitudinal smooth muscle layer, since the amount of muscle cells per cross-sectional area was significantly increased and the amount of muscle cells per 1000 $\mu m^2$ was significantly decreased as compared to control values. The villus crypt ratio was not different from control values (Table 1).

### 3.3. Myeloperoxidase activity

In control rats, the mean ileal myeloperoxidase activity was 2.67 ± 1.16 U/g tissue ($n = 6$). Thirty-six hours after TNBS treatment, myeloperoxidase activity was more than 20-fold increased to 62.40 ± 12.14 U/g tissue ($P < 0.01$,

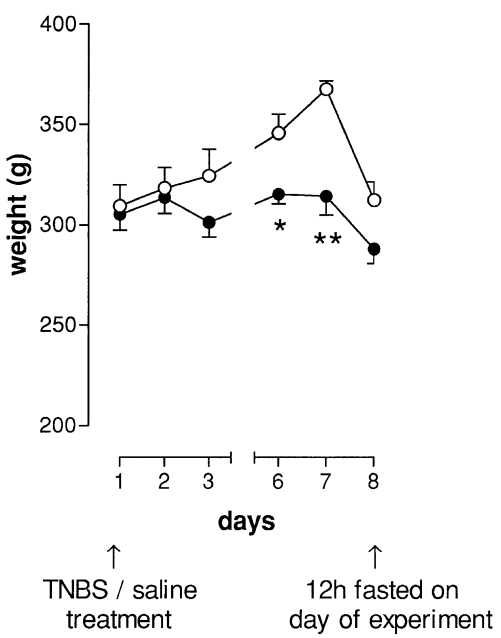


Fig. 1. Evolution of body weight of control rats (○) and TNBS-treated rats (●). After 1 week, body weights did not significantly differ after an overnight fast. *, $P < 0.05$, **, $P < 0.01$ significantly different from controls, Student's $t$-test for unpaired values ($n = 6$).

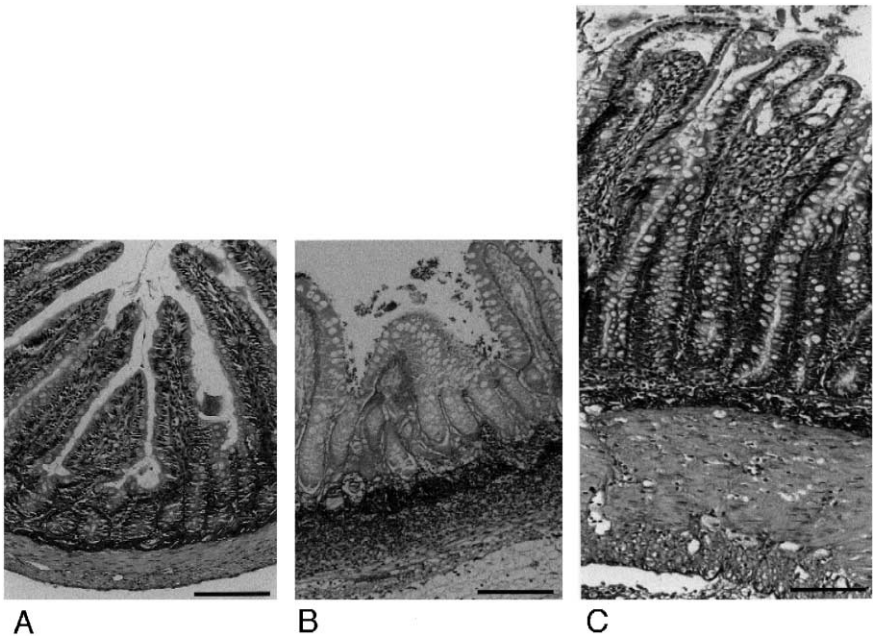


Fig. 2. Cross-sections from the ileum of control rats (A) and of rats 36 h (B) and 1 week (C) after TNBS treatment. The images are printed at the same final magnification. The villus crypt ratio of the ulcerative mucosa is decreased 36 h after TNBS treatment, whereas the mucosal and smooth muscle layer thickness is increased 1 week after TNBS. Note the increased cellular infiltrate 36 h after TNBS treatment. Bar represents 100 μm. (Sirius red staining, ×92).

$n = 6$). However, 1 week after TNBS treatment, myeloperoxidase activity ($2.87 \pm 0.71$ U/g tissue, $P < 0.01$, $n = 6$) returned to control levels.

### 3.4. Effect of TNBS treatment on the contractility of ileal longitudinal smooth muscle strips

The effect of intraluminal instillation of TNBS on the spontaneous activity and the mechanical properties of the longitudinal muscle strips of the rat ileum after 36 h and 1 week is shown in Table 2. To determine the portion of the contractile response that was accounted for by mere structural changes of the muscle layer, contractions of longitudinal muscle strips were always presented in g contraction and in g contraction normalized to the cross-sectional area of the longitudinal muscle layer. Using the two methods to express the contractile data enables to differentiate between structural and functional alterations of the neuromuscular apparatus during TNBS-induced ileitis. The basal tension at $L_0$ of the longitudinal muscle strips was significantly increased 1 week after TNBS (Table 2). However,

Table 1
Effect of TNBS treatment on ileal morphology
When muscle cells were counted, Student's *t*-test for unpaired values was used to compare means of controls and TNBS 1W.

| | Control ($n = 7$) | TNBS 36H ($n = 7$) | TNBS 1W ($n = 6$) |
|---|---|---|---|
| Wall thickness (μm) | $674 \pm 31$ | $504 \pm 39^{a}$ | $1071 \pm 82^{a,b}$ |
| Villus height (μm) | $325 \pm 28$ | $184 \pm 26^{a}$ | $529 \pm 58^{a,b}$ |
| Crypt depth (μm) | $176 \pm 14$ | $165 \pm 12$ | $283 \pm 19^{a,b}$ |
| Villus crypt ratio | $1.92 \pm 0.24$ | $1.13 \pm 0.15^{a}$ | $1.85 \pm 0.10^{b}$ |
| Longitudinal muscle layer (μm) | $43 \pm 4$ | $42 \pm 3$ | $79 \pm 10^{a,b}$ |
| Cross-sectional area ($mm^2$) | $0.51 \pm 0.06$ | $0.68 \pm 0.09$ | $1.27 \pm 0.17^{a,b}$ |
| Muscle cells/cross-sectional area | $4936 \pm 421$ | ND | $7264 \pm 998^{a}$ |
| Muscle cells/1000 $\mu m^2$ | $9.91 \pm 0.40$ | ND | $6.28 \pm 1.19^{a}$ |

ND: not determined due to interference of infiltrated inflammatory cells.
TNBS 36H: 36 h after TNBS treatment.
TNBS 1W: 1 week after TNBS treatment.
Results are shown as mean ± S.E.M. for the number of rats indicated.
[a] $P < 0.05$ significantly different from the control group.
[b] $P < 0.05$ significantly different from TNBS 36 H, one-way analysis of variance followed by Student–Newman–Keuls test.

Table 2
Effect of TNBS treatment on spontaneous activity and mechanical properties of longitudinal muscle strips of the rat ileum
To determine the portion of the contractile response that was accounted for by mere structural changes of the muscle layer, contractions of longitudinal muscle strips were presented in g contraction and in g contraction normalized to the cross-sectional area of the longitudinal muscle layer. Contractions to 50 mM KCl were always studied in the presence of 1 μM tetrodotoxin.

| | Control ($n = 7$) | TNBS 36H ($n = 7$) | TNBS 1W ($n = 6$) |
|---|---|---|---|
| *g Contraction* | | | |
| Amplitude of spontaneous activity | $0.28 \pm 0.04$ | $0.12 \pm 0.02$[a] | $0.67 \pm 0.09$[a,b] |
| Passive tension at $L_0$ | $0.68 \pm 0.09$ | $0.55 \pm 0.07$ | $1.29 \pm 0.24$[a,b] |
| Active tension to 50 mM KCl at $L_0$ | $2.89 \pm 0.20$ | $1.12 \pm 0.24$[a] | $6.70 \pm 0.65$[a,b] |
| *g Contraction / mm² cross-sectional area* | | | |
| Amplitude of spontaneous activity | $0.53 \pm 0.12$ | $0.17 \pm 0.03$[a] | $0.70 \pm 0.12$ |
| Passive tension at $L_0$ | $1.65 \pm 0.52$ | $0.77 \pm 0.09$ | $1.47 \pm 0.33$ |
| Active tension to 50 mM KCl at $L_0$ | $5.62 \pm 0.52$ | $1.52 \pm 0.27$[a] | $5.76 \pm 0.40$ |

Results are shown as mean ± S.E.M.
TNBS 36H, 36 h after TNBS treatment; TNBS 1W, 1 week after TNBS treatment.
[a] $P < 0.05$ significantly different from controls.
[b] $P < 0.05$ significantly different from TNBS 36H, one-way analysis of variance followed by Student–Newman–Keuls test ($n = 6$–7).

when data were normalized to the cross-sectional area of the longitudinal muscle layer, no differences in basal tension were found between the three groups, indicating that the increase in basal tension 1 week after TNBS was completely accounted for by the increase in muscle mass without changes in the tissue intercellular medium. On the other hand, the active tension to 50 mM KCl of the same preparations was significantly decreased 36 h after TNBS compared to controls (Table 2). One week later, the active tension was significantly increased compared to controls. Normalization of the data to the cross-sectional area of the longitudinal muscle layer resulted in a significant reduction of the active tension 36 h after TNBS and normal active tension 1 week after TNBS. Also, spontaneous basal activity of ileal longitudinal muscle strips was significantly decreased 36 h after TNBS treatment, even after normalization to the cross-sectional area of the longitudinal muscle layer (Table 2). One week after TNBS, spontaneous basal activity was significantly increased. This was due to an increase in muscle mass, as shown by the normalization of the amplitude to the cross-sectional area of the longitudinal muscle layer.

Receptor-mediated contractions of the longitudinal muscle strips were studied by means of different contractile receptor agonists. In control rats, all contractile agonists induced concentration-dependent contractions. Contractile responses to acetylcholine were the most potent (Table 3). The maximal response to acetylcholine was significantly decreased 36 h after TNBS, whereas it was increased after 1 week as compared to controls (Figs. 3 and 4A). No changes in $pD_2$ values were found. However, the $E_{max}$ normalized to the cross-sectional area 1 week after TNBS was no longer different from control values, whereas the decreased response to acetylcholine 36 h after TNBS was more pronounced (Fig. 4B and Table 3). Similar results were obtained when contractions were induced by the contractile receptor agonists serotonin and prostaglandin $F_{2\alpha}$, although the respective maximal contractile responses were smaller compared to the maximal response to acetylcholine (Table 3). In contrast to all other used contractile receptor agonists, the maximal contractile response to substance P 1 week after TNBS was significantly decreased after normalization to the cross-sectional area (Table 3).

To study the effect of TNBS treatment on the neuronal function of the ileum, contractions were induced by electrical field stimulation of the myenteric neurons. In control rats, electrical field stimulation of the myenteric neurons induced frequency-dependent contractions with an $EF_{50}$ value of $4.62 \pm 0.86$ Hz and an $E_{max}$ value of $2.31 \pm 0.31$ g contraction ($n = 4$). They were completely blocked by 1 μM tetrodotoxin. They were mainly of cholinergic origin since they were almost completely blocked by the muscarinic receptor antagonist atropine (1 μM) (data not shown). The maximal contractile response to electrical field stimulation was significantly decreased 36 h after TNBS treatment to $0.64 \pm 0.24$ g contraction ($P < 0.01$, $n = 4$), whereas it was significantly increased 1 week after TNBS treatment to $4.46 \pm 0.33$ g contraction ($P < 0.001$, $n = 4$). Normalizing these data to the cross-sectional area of the longitudinal smooth muscle layer showed a decreased contractile response 36 h after TNBS, whereas the response after 1 week was not different compared to controls (Table 3).

### 3.5. Effect of TNBS treatment on the contractility of individual longitudinal smooth muscle cells

More smooth muscle tissue could be collected for dissociation from the 4 cm ileal segment 1 week after TNBS treatment than from the ileum of control rats. On microscopy, no multinucleated longitudinal smooth muscle cells were found in either group. The mean length of

Table 3
$E_{max}$ and $pD_2$ values of different contractile receptor agonists and $E_{max}$ and $EF_{50}$ values of electrically induced contractions in ileal longitudinal smooth muscle strips of control rats and of rats 36 h and 1 week after TNBS treatment
Contractile responses are expressed in g contraction and in g contraction/mm$^2$ cross-sectional area. The receptor-mediated contractions are characterized by their respective $pD_2$ (negative logarithm of mean effective molar concentration) and $E_{max}$ (maximal contraction) values. Electrically induced contractions are characterized by the $EF_{50}$ (mean effective frequency in Hertz) and $E_{max}$ value.

| | | Control | g contraction | | g contraction/mm$^2$ cross-sectional area | | |
|---|---|---|---|---|---|---|---|
| | | | TNBS 36H | TNBS 1W | Control | TNBS 36H | TNBS 1W |
| ACh | $pD_2$ | 5.83 ± 0.14 | 6.28 ± 0.50 | 6.35 ± 0.15 | 5.94 ± 0.19 | 6.20 ± 0.51 | 5.69 ± 0.18 |
| | $E_{max}$ | 5.15 ± 0.32 | 1.12 ± 0.23[a] | 6.62 ± 0.40[a,b] | 8.80 ± 0.73 | 1.63 ± 0.34[a] | 8.84 ± 0.65 |
| 5-HT | $pD_2$ | 6.70 ± 0.17 | 7.18 ± 0.42 | 7.11 ± 0.25 | 6.76 ± 0.21 | 7.21 ± 0.42 | 6.18 ± 0.86 |
| | $E_{max}$ | 3.92 ± 0.29 | 0.74 ± 0.11[a] | 6.57 ± 0.64[a,b] | 7.31 ± 0.66 | 1.03 ± 0.16[a] | 9.55 ± 3.23 |
| SP | $pD_2$ | 7.94 ± 0.21 | 7.89 ± 0.42 | 8.01 ± 0.24 | 7.94 ± 0.25 | 7.76 ± 0.37 | 8.01 ± 0.27 |
| | $E_{max}$ | 2.95 ± 0.34 | 0.67 ± 0.15[a] | 4.04 ± 0.48[a,b] | 6.18 ± 0.85 | 0.97 ± 0.20[a] | 3.58 ± 0.48[a,b] |
| $PGF_{2\alpha}$ | $pD_2$ | 7.66 ± 0.19 | 7.96 ± 0.41 | 7.65 ± 0.19 | 7.66 ± 0.24 | 7.82 ± 0.44 | 7.67 ± 0.32 |
| | $E_{max}$ | 2.44 ± 0.19 | 0.72 ± 0.12[a] | 5.82 ± 0.44[a,b] | 5.06 ± 0.49 | 1.03 ± 0.18[a] | 4.69 ± 0.59 |
| EFS | $EF_{50}$ | 4.63 ± 0.86 | 2.60 ± 0.65 | 3.21 ± 0.96 | 4.62 ± 0.86 | 2.61 ± 0.53 | 3.20 ± 0.98 |
| | $E_{max}$ | 2.31 ± 0.31 | 0.64 ± 0.24[a] | 4.46 ± 0.33[a,b] | 4.12 ± 0.80 | 0.92 ± 0.34[a] | 5.54 ± 0.42 |

Results are shown as mean ± S.E.M.
TNBS 36H, 36 h after TNBS treatment; TNBS 1W, 1 week after TNBS treatment; ACh, acetylcholine; 5-HT, serotonin; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; SP, substance P; EFS, electrical field stimulation.
[a] $P < 0.05$ significantly different from controls.
[b] $P < 0.05$ significantly different from TNBS 36H, one-way analysis of variance followed by Student–Newman–Keuls test ($n = 4–7$).

longitudinal smooth muscle cells in control rats was 128.10 ± 1.99 μm ($n = 5$). One week after TNBS treatment, the length was significantly increased to 139.40 ± 4.17 μm ($P < 0.05$, $n = 5$). These mean cell lengths were taken as reference values. Incubation with increasing concentrations of acetylcholine (0.001 nM–0.1 μM) for 30 s, contracted the cells in a concentration-dependent manner, with an $E_{max}$ value of 20.23 ± 2.30% shortening in con-

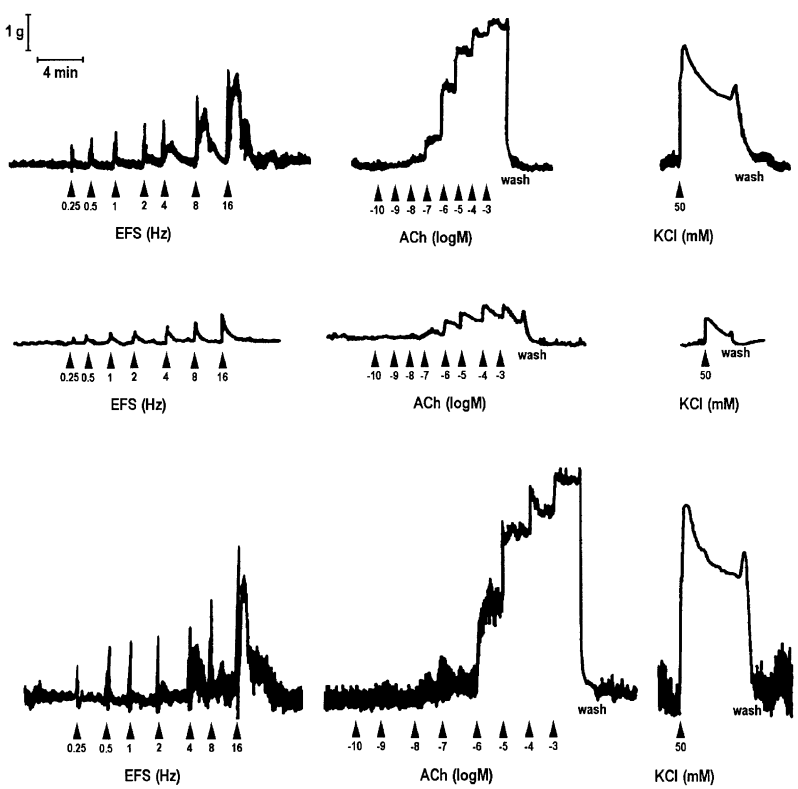


Fig. 3. Typical tracing of ileal longitudinal smooth muscle strips of control rats (upper) and of rats 36 h (middle) and 1 week (lower) after TNBS treatment. Spontaneous, electrically (EFS), KCl- and acetylcholine (ACh)-induced contractility of muscle strips is decreased 36 h after TNBS treatment, whereas they are increased 1 week after TNBS treatment.

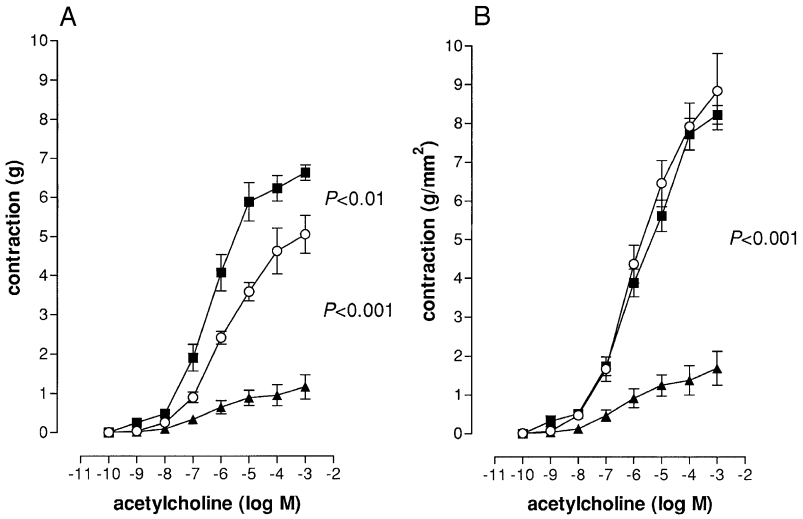


Fig. 4. Effect of TNBS treatment on acetylcholine-induced contractions of ileal longitudinal smooth muscle strips. Contractions are expressed in g contraction (A) and in g contraction/mm$^2$ cross-sectional area (B). $^{**}$ $P < 0.01$, $^{***}$ $P < 0.001$, $E_{max}$ significantly different from other groups, one-way analysis of variance followed by Student–Newman–Keuls test ($n = 6–7$). ○ controls; ▲ 36 h after TNBS; ■ 1 week after TNBS.

trols and $18.05 \pm 2.46\%$ shortening after TNBS treatment ($P > 0.05$, $n = 5$). Similarly, no differences were found in the $pD_2$ values for acetylcholine in both groups ($9.20 \pm 0.54$ in controls and $9.07 \pm 0.44$ after TNBS treatment, $P > 0.05$, $n = 5$).

## 4. Discussion

An enema of TNBS in ethanol is a well-characterized model of acute and chronic colitis in the rat (Morris et al., 1989). Recently, this model was adjusted to the rat small intestine (Goldhill et al., 1999). However, it appears that the inflammatory response of the rat ileum to TNBS differs from the response in the rat colon. In the present study, we elaborated the knowledge on TNBS-induced ileitis in the rat by studying the structural and functional alterations of the longitudinal muscle layer.

Intraluminal instillation of TNBS into the ileum resulted in loose stools and a retarded body weight gain. However, the stool pattern and body weight were restored within 1 week. These clinical observations were paralleled by the histological and biochemical characteristics of the inflamed ileum. Thirty-six hours after TNBS, the ileum was acutely inflamed and myeloperoxidase activity was more than 20-fold increased. However, 1 week later, only histological signs of recovery were found and myeloperoxidase activity was normal, indicating that there was no spontaneous evolution to chronic inflammation. This quick restitution of the mucosal integrity was previously reported and is in sharp contrast to the development of a chronic inflammatory phase of TNBS-induced colitis in the rat (Demedts et al., 1999a,b; Moreels et al., 1999). Several hypotheses may be put forward to explain this discrepancy, such as differences in the dose of TNBS, a different rat strain or more likely a different gut segment with its specific characteristics. The dose of TNBS and the ethanol concentration in the present study are equal to the ones that lead to chronic colitis. Differences in rat strain have been suggested to explain the differences in the inflammatory response (Coméra et al., 1999). TNBS-induced colitis lasts up to 8 weeks in Sprague–Dawley rats (Morris et al., 1989), whereas in Wistar rats, the time course of the colitis tends to be reduced (Coméra et al., 1999; Kankuri et al., 1999). However, the quicker recovery of TNBS-induced colitis in Wistar rats is not a general finding (Wallace and Keenan, 1990). The most plausible explanation of the recovery of TNBS-induced ileitis in the rat after 1 week is the difference in gastrointestinal segment. The rat ileum may be less sensitive to TNBS-induced inflammation. However, we showed that 36 h after TNBS installation, acute transmural inflammation is present with a 20-fold increase of myeloperoxidase activity, indicating that the ileal mucosal layer is not less susceptible to TNBS-induced injury than the colon. On the other hand, a vast amount of data emphasizes the important role of the intraluminal microbiological flora in the induction and maintenance of gastrointestinal inflammation (Sartor et al., 1996). The bacterial load of the rat gastrointestinal tract is highest in the colon and lowest in the small intestine (Raibaud et al., 1966; Rath et al., 1999). The microbial composition of gastrointestinal bacteria is different in the small intestine and the colon (Raibaud et al., 1966). Therefore, the resident gut flora may be necessary to maintain the inflammatory response to TNBS in the rat. Indeed, Videla et al. (1994) showed that the chronic phase of TNBS-induced colitis in rats is absent after treatment with broad spectrum antibiotics and Garcia-Lafuente et al. (1997) showed that colonic anaerobes play a key role in transmural TNBS-induced colitis in the rat. The underlying

mechanism is unclear. However, it is possible that changes in intraluminal pH by specific microflora will explain the difference in inflammatory response (Yamada et al., 1992). Based on these results, the quick recovery of TNBS-induced ileitis and the absence of chronic inflammation is likely due to the relative absence in the ileum of specific resident bacteria which are found throughout the colon.

Histology and myeloperoxidase activity assay demonstrated that 36 h after TNBS instillation, there was acute transmural inflammation of the ileum. Morphometric analysis showed a decreased villus crypt ratio, but no significant alterations of the muscle mass. When normalized to the cross-sectional area of the longitudinal muscle layer, the contractility study showed an inhibition of the spontaneous activity without alterations in passive tension of the muscle strips. The active tension to KCl and different contractile receptor agonists was significantly decreased, indicating that the hypocontractility of the longitudinal muscle strips was clearly due to functional alterations of the muscle cells at the postreceptor level, since both receptor- and nonreceptor-mediated contractions were equally inhibited. These findings in longitudinal muscle strips are in contrast to the selectively increased response to substance P and the unaltered nonreceptor-mediated contractility of circular muscle strips of the rat during acute TNBS-induced ileitis (Goldhill et al., 1999). Studies in other models of acute gut inflammation in the guinea pig and the dog also show differential receptor-mediated alterations of the contractility of the circular and the longitudinal muscle layer (Martinolle et al., 1997; Shi and Sarna, 1999). The present study indicates that during acute TNBS-induced ileitis in the rat, the functional integrity of the contractile apparatus of the longitudinal muscle layer is lost, whereas it is conserved in the circular muscle layer (Goldhill et al., 1999).

Histology and myeloperoxidase activity assay demonstrated that TNBS-induced ileitis was recovered after 1 week. On the other hand, the mass of the longitudinal muscle layer was significantly increased after 1 week, as shown by the morphometric analysis of the cross-sectional area. The increase in muscle mass was due to both hypertrophy and hyperplasia of longitudinal smooth muscle cells, as shown by the morphometric analysis of histological sections and by the determination of the individual smooth muscle cell length. Increased muscle mass of the intestinal wall may contribute to altered gut motility, since increased muscle mass will exaggerate the consequences of muscle contraction and amplify the effect of excitatory stimuli (Blennerhassett et al., 1992). To study the role of the increased muscle mass in the contractility of longitudinal muscle strips, we first compared the raw strip chart contractility with the contractility normalized to the muscle mass. Secondly, we studied the contractility of individual longitudinal muscle cells. When preparing the muscle strips for the contractility studies, care was taken to cut strips of equal size. Raw strip chart recordings showed an increased passive tension and increased responses to all contractile stimuli 1 week after TNBS. To investigate whether these alterations in contractility resulted from structural and/or functional changes of the neuromuscular apparatus, we normalized passive tension and receptor- and nonreceptor-mediated contractions to the cross-sectional area of the longitudinal muscle layer. After normalization, both passive tension and contractile responses were comparable to controls, indicating that the hypercontractility, observed 1 week after TNBS, was completely due to structural changes of the muscle layer without modifications of the functional properties. These findings on longitudinal smooth muscle strips are in accordance with our findings on the contractility of individual smooth muscle cells. Although the mean length of longitudinal muscle cells was significantly increased in previously inflamed ileum, the $pD_2$ and $E_{max}$ of acetylcholine were similar in muscle cells from control and previously inflamed ileum. Therefore, our findings indicate that in the rat model of TNBS-induced ileitis the postinflammation hypercontractility of longitudinal muscle strips was attributable to an increase in smooth muscle mass, without changes in the contractile capacity of individual smooth muscle cells. Also, in the vascular system, it has been demonstrated that changes in smooth muscle mass may directly act upon the contractility of the smooth muscle layer (Owens et al., 1988). Furthermore, the increased $E_{max}$ values of all receptor agonists, without alterations of the respective $pD_2$ values, suggest that there is no alteration of agonist receptor number or sensitivity. Thus, our results indicate that in the postinflammation period of the rat model of TNBS-induced ileitis, the maximal force-developing capability of the longitudinal smooth muscle layer is increased, although the force development per unit contractile mass appears to be unaltered.

Tachykinins are important regulators of gastrointestinal motility and contributory factors in inflammatory disturbances of the gut (Holzer and Holzer-Petsche, 1997a,b). However, inflammatory changes in the expression and the distribution of tachykinins largely depend on the species and the region of the gastrointestinal tract under study (Holzer, 1998). In the rat model of TNBS-induced ileitis, the contractile response of the longitudinal muscle layer to the tachykinin substance P, normalized to the cross-sectional area, was significantly decreased 1 week after TNBS, whereas this was not the case for the other contractile agents. Using a rat model of TNBS-induced colitis, Evangelista et al. (1996) have shown a downregulation of substance P receptors lasting up to 1 week, whereas Miampamba and Sharkey (1998) have shown an acute, but transient, decrease in neuronal substance P immunoreactivity. Our results seem to reflect the functional consequence of such a receptor downregulation, and indicate that selective functional changes persist, even after histological and biochemical recovery of TNBS-induced inflammation. The existence of postinflammation dysmotility is illustrated by the fact that the relative risk to develop irritable bo-

wel syndrome is more than 10 times increased after an episode of bacterial gastroenteritis (Garcia-Rodriguez and Ruigomez, 1999). Similarly, gastrointestinal motility is disturbed in the vast majority of patients with inactive inflammatory bowel diseases (Isgar et al., 1983; Annese et al., 1997). We provide evidence that a persistent disturbance of substance P-mediated contractility may participate in postinflammation dysmotility.

Since trophic changes of intestinal smooth muscle cells can be the result of myenteric plexus destruction (Hadzijahic et al., 1993), and because altered morphology of the myenteric plexus is also a characteristic finding in patients with Crohn's disease (Geboes and Collins, 1998), we investigated whether TNBS-induced ileitis modulated enteric neuronal function. Although, in our experimental setup, limited alterations in the release of contractile neurotransmitters may be concealed by the extensive alterations in smooth muscle contractility, no clear evidence was found for neuronal dysfunction after TNBS treatment.

In conclusion, our results demonstrate that the rat model of TNBS-induced ileitis is characterized by an acute transmural inflammation, which is self-limiting after 1 week. This model does not develop a chronic inflammatory phase. During acute ileitis, the contractile integrity of the longitudinal muscle cells is lost. In the postinflammation phase of the rat model of TNBS-induced ileitis, the maximal force-developing capability of the longitudinal smooth muscle layer is increased, although the force development per unit contractile mass is unaltered. Functional alterations were shown as a selective disturbance of the response to substance P. No direct evidence for myenteric neuron dysfunction was found.

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