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The ICAM-1 antisense oligonucleotide ISIS-3082 prevents the development of postoperative ileus in mice

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1 Intestinal manipulation (IM) during abdominal surgery triggers the influx of inflammatory cells, leading to postoperative ileus. Prevention of this local muscle inflammation, using intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-1-specific antibodies, has been shown to shorten postoperative ileus. However, the therapeutic use of antibodies has considerable disadvantages.

2 The aim of the current study was to evaluate the effect of ISIS-3082, a mouse-specific ICAM-1 antisense oligonucleotide, on postoperative ileus in mice.

3 Mice underwent a laparotomy or a laparotomy combined with IM after treatment with ICAM-1 antibodies, 0.1–10 mg kg^{-1} ISIS-3082, saline or ISIS-8997 (scrambled control antisense oligonucleotides, 1 and 3 mg kg^{-1}). At 24 h after surgery, gastric emptying of a ^{99m}TC labelled semi-liquid meal was determined using scintigraphy. Intestinal inflammation was assessed by myeloperoxidase (MPO) activity in ileal muscle whole mounts.

4 IM significantly reduced gastric emptying compared to laparotomy. Pretreatment with ISIS-3082 (0.1–1 mg kg^{-1}) as well as ICAM-1 antibodies (10 mg kg^{-1}), but not ISIS-8997 or saline, improved gastric emptying in a dose-dependent manner. This effect diminished with higher doses of ISIS-3082 (3–10 mg kg^{-1}).

5 Similarly, ISIS-3082 (0.1–1 mg kg^{-1}) and ICAM-1 antibodies, but not ISIS-8997 or higher doses of ISIS-3082 (3–10 mg kg^{-1}), reduced manipulation-induced inflammation. Immunohistochemistry showed reduction of ICAM-1 expression with ISIS-3082 only.

6 ISIS-3082 pretreatment prevents postoperative ileus in mice by reduction of manipulation-induced local intestinal muscle inflammation. Our data suggest that targeting ICAM-1 using antisense oligonucleotides may represent a new therapeutic approach to the prevention of postoperative ileus.
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Abbreviations: ICAM-1, intercellular adhesion molecule-1; IM, intestinal manipulation; LFA-1, leukocyte function-associated antigen-1

Introduction

Postoperative ileus is characterised by a generalised hypomotility of the gastrointestinal tract, and is observed after almost every abdominal surgical procedure (Prasad & Matthews, 1999). Although self-limiting, postoperative ileus is responsible for increased morbidity and prolonged hospitalisation, leading to extra costs of between 750 million and 1 billion US dollars (Livingston & Passaro, 1990; Prasad & Matthews, 1999). Mainly due to a lack of pathophysiological insight, treatment is limited to supportive and conservative measures such as no oral feeding and intravenous (i.v.) fluids (Kehlet & Holte, 2001).

Acute studies have convincingly shown that a laparotomy, but especially handling of the intestine, inhibits gastrointestinal motility by activation of spinal and supraspinal inhibitory pathways (Plourde *et al*., 1993; Barquist *et al*., 1996; De Winter *et al*., 1997a, b; Boeckxstaens *et al*., 1999; 2000). Recently, it became clear that manipulation of the intestine also triggers the influx of inflammatory cells. This process becomes prominent several hours after abdominal surgery and is now accepted to play a crucial role in the prolonged inhibition of gastrointestinal motility (Kalff *et al*., 1999a, b; de Jonge *et al*., 2003). This local inflammation not only leads to impaired contractility of the diseased intestinal segment but also triggers an adrenergic inhibitory neural pathway, explaining the more generalised aspect of postoperative ileus (Kalff *et al*., 1999b; de Jonge *et al*., 2003; Kreiss *et al*., 2003). Leukocyte function-associated antigen-1 (LFA-1) and its ligand intercellular adhesion molecule-1 (ICAM-1) are two adhesion molecules that are crucial in the process of transmigration and recruitment of leukocytes (Smith *et al*., 1989; Issekutz *et al*., 1999). ICAM-1, normally only moderately expressed on vascular endothelium, is strongly upregulated in response to inflammatory stimuli, including intestinal manipulation (IM) (Dustin *et al*., 1986; Rothlein *et al*., 1986; Kalff *et al*., 1999b).

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An important role for ICAM-1 in the development of the inflammatory infiltrate mediating postoperative ileus is suggested by the observation that administration of a combination of blocking antibodies to LFA-1 and ICAM-1 prior to abdominal surgery prevented the recruitment of inflammatory cells in manipulated tissue and postoperative ileus (Kalff *et al*., 1999b; de Jonge *et al*., 2003). Although it has not been studied whether blockade of only one of these adhesion molecules has a similar effect, these data indicate that ICAM-1 may be an important target to prevent postoperative ileus. However, the use of antibodies as therapeutic strategy in humans still has considerable downsides, such as the formation of neutralising antibodies or the development of hypersensitivity reactions (Shawler *et al*., 1985; LoBuglio *et al*., 1989).

Antisense oligonucleotides are 15–25-base long oligomers designed to hybridise to the specific mRNA encoding for the target protein. As such, it prevents the translation of mRNA, thereby downregulating the expression of the respective protein (Crooke, 1992; Stein & Cheng, 1993). ISIS-3082 is a murine ICAM-1-specific antisense oligonucleotide with anti-inflammatory properties in experimental models of colitis, and a human-specific form, ISIS-2302 (alicaforsen), is currently being tested in a clinical trial to evaluate this drug as potential new treatment in patients with inflammatory bowel disease (Miner *et al*., 2004; van Deventer *et al*., 2004). In the present study, we investigated the efficacy of the antisense oligonucleotide ISIS-3082 to shorten postoperative ileus in our experimental mouse model.

Methods

Animals

Female Balb/C mice (Harlan Nederland, Horst, The Netherlands), 12–15 weeks old, were kept under environmentally controlled conditions (light on from 08:00 till 20:00 h; water and rodent nonpurified diet *ad libitum*; temperature 20–22°C; 55% humidity).

All experiments were performed after approval of the Ethical Animal Research Committee of the University of Amsterdam and according to their guidelines.

Surgical procedure

Mice were anaesthetised by intraperitoneal (i.p.) injection of $10\,ml\,kg^{-1}$ of an anaesthetic solution containing $0.078\,mg\,ml^{-1}$ fentanyl citrate, $2.5\,mg\,ml^{-1}$ fluanisone (Hypnorm; Janssen, Beerse, Belgium) and $1.25\,mg\,ml^{-1}$ midazolam (Dormicum; Roche, Mijdrecht, The Netherlands). Surgery was performed under sterile conditions. Mice underwent a laparotomy, or a laparotomy followed by small IM, as described previously (de Jonge *et al*., 2003).

In short, a midline incision was made and the peritoneal cavity was opened along the linea alba. The small intestine was carefully exteriorised from the distal duodenum until the cecum and gently manipulated for 5 min using sterile moist cotton applicators. Contact or stretch of stomach or colon was strictly avoided. After repositioning of the intestinal loops, the abdomen was closed using a two-layer continuous suture (Mercilene Softsilk 6-0). Mice recovered from surgery in a temperature-controlled cage at 32°C with free access to water, but not to food. At 24 h after surgery, gastric emptying was measured. Thereafter, mice were anaesthetised and killed by cervical dislocation. The small intestine was removed, flushed in ice-cold phosphate-buffered saline (PBS), and snap frozen in liquid nitrogen or fixed in ethanol for further analysis.

Drug preparation and treatment

ICAM-1 antibody (anti-CD54; IgG2b; clone YN1/1.7) (Lub *et al*., 1996) was kindly provided by Professor Y. van Kooyk (Department of Molecular Cell Biology & Immunology, VU University Medical Center, Amsterdam, The Netherlands). Antibodies were dissolved in sterile 0.9% NaCl and injected i.p. 1 h prior to the surgical intervention in a dose of $10\,mg\,kg^{-1}$ (de Jonge *et al*., 2003).

ICAM-1 antisense oligonucleotide (ISIS-3082) and its scrambled control oligonucleotide (ISIS-8997) were kindly provided by Dr Frank Bennett (ISIS-Pharmaceuticals, Carlsbad, CA, U.S.A.). The specific sequences of the oligonucleotides used in this study were: ISIS-3082, 5′-TGCATCCCCCAGGCCAC CAT-3′ and ISIS-8997, 5′-CAGCCATGGTTCCCCCCAA C-3′. The final concentration of the oligonucleotide was determined using spectrometry (Nanodrop ND-1000, Nanodrop Technologies Inc., Wilmington, DE, U.S.A.). ISIS-3082, ISIS-8997 or their vehicle (sterile 0.9% NaCl) was injected subcutaneously (s.c.) once daily starting 6 days prior to the surgical procedure. As intracellular localisation of the drug is only achieved after 24 h, the onset of action of antisense oligonucleotide is not instant (Butler *et al*., 1997). Therefore, ISIS-3082 or ISIS-8997 was administered by s.c. injection once a day for 6 days to achieve a steady-state concentration (approximately five half-lives) prior to surgery (Crooke *et al*., 1996). ISIS-3082 was administered in a pharmacological range of 0.1, 0.3, 1.0, 3.0 or $10\,mg\,kg^{-1}$, which has been shown to be effective in DSS-colitis (Bennett *et al*., 1997). As the most effective dose of ISIS-3082 was $1\,mg\,kg^{-1}$, the control oligonucleotide, ISIS-8997, was tested in the same dose, as well as a higher dose of $3\,mg\,kg^{-1}$.

Measurement of gastric emptying

As previously described, gastric emptying rate was determined after gavage of a semi-liquid, noncaloric test meal (0.1 ml of 3% methylcellulose solution containing 10 MegaBecquerel (MBq) of ^{99m}Tc-Albures. Mice were scanned using a gamma camera set at 140 keV (Bennink *et al*., 2003). The entire abdominal region was scanned for 30 s, immediately and 80 min after gavage. During the scanning period, mice were conscious and manually restrained. The static images obtained were analysed using Hermes computer software (Hermes, Stockholm, Sweden). Gastric retention was calculated by determining the percentage of activity present in the gastric region of interest compared to the total abdominal region of interest.

Whole-mount preparation

Ileal segments (4–6 cm proximal of cecum) were quickly excised. The mesentery was removed from the intestine, which was cut open along its border. Faecal content was washed out in ice-cold PBS, after which tissue segments were fixed in

100% ethanol for 10 min. Fixed preparations were kept in 70% ethanol at 4°C until further analysis.

Before final analysis, segments were stretched 1.5 times to their original size and pinned down on a glass dish filled with 70% ethanol, after which the mucosa was carefully removed.

Assessment of leukocyte infiltration of the intestinal muscle

Fixed preparations were rehydrated by incubation in 50% ETOH and PBS, pH 7.4, for 5 min. To visualise MPO-positive cells, preparations were incubated for 10 min with 3-amino-9-ethyl carbazole (Sigma, St Louis, MO, U.S.A.) as substrate and dissolved in sodium acetate buffer (pH 5.0), to which 0.01% H_2O_2 was added (de Jonge *et al*., 2003).

Immunohistochemistry

Immunohistochemical staining for ICAM-1 was performed on acetone fixed transverse ileal segments. Endogenous peroxidase activity was eliminated by incubation of segments in methanol containing 0.3% H_2O_2. Nonspecific protein-binding sites were blocked by incubation in PBS, pH 7.4, containing 10% of normal goat serum for 10 min. Sections were incubated overnight with biotinylated hamster anti-mouse ICAM-1 antibodies (Pharmingen, San Diego, CA, U.S.A.) (dilution 1 : 1000). Next, sections were incubated with ABComplex/HRP (DAKOCytomation, Glostrup, Denmark) for 30 min. HRP was visualised using SigmaFast DAB (Sigma-Aldrich, St Louis, MO, U.S.A.), incubating 5 min, and contra-stained with 2% methyl green for 2 min.

Statistical analysis

A sample size of eight animals was used for each treatment group. Statistical analysis was performed using SPSS 12.02 software for Windows. The data were expressed as mean ± s.e.m. Owing to the sample size, data were considered nonparametrically distributed. The nonparametric Kruskal–Wallis test was used to analyse the cohort of independent variables. If the difference between the multiple variables was statistically significant, the Mann–Whitney test was performed to compare the individual treatment groups, identifying the specific statistical differences. $P<0.05$ was considered statistically significant.

Results

Effect of IM on gastric emptying and local intestinal muscle inflammation 24 h after abdominal surgery

At 24 h after abdominal surgery, IM resulted in a significant increase of gastric retention 80 min after gavage of a noncaloric test meal, compared to a laparotomy (Figure 1). The observed delay in gastric emptying after IM coincided with a profound local intestinal muscle inflammatory cell influx compared to laparotomy (Figure 2).

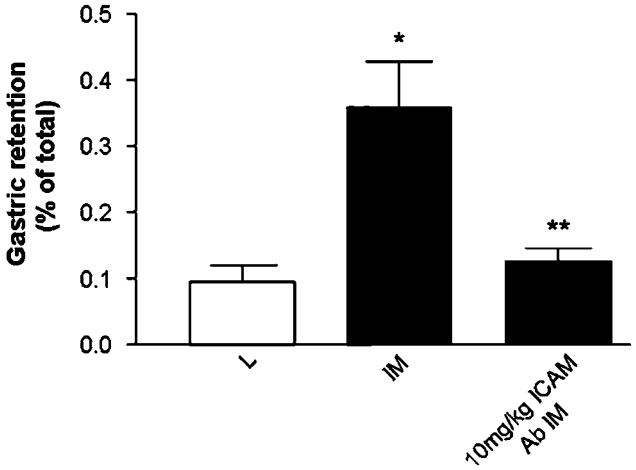

Figure 1 Effect of IM on gastric retention 24 h after abdominal surgery compared to laparotomy only (L), or IM after treatment with ICAM-1 antibodies (anti-CD54 IgG2b clone 1/1.7). Each individual group consisted of eight animals. Data are mean ± s.e.m. gastric retention 80 min after gavage of semi-liquid test meal; $*P<0.05$ compared to L control; $**P<0.05$ compared to IM control.

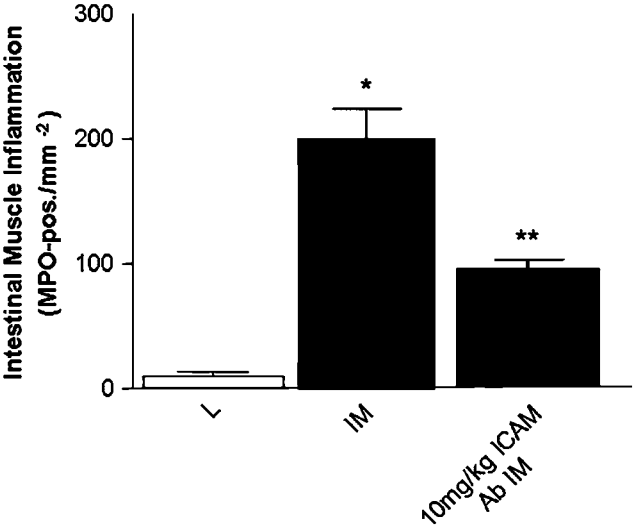

Figure 2 Effect of IM, laparotomy only (L) or IM pretreated with ICAM-1 antibodies (anti-CD54 IgG2b clone 1/1.7) on local inflammatory cell influx 24 h after abdominal surgery. Each individual group consisted of eight animals. Data are mean ± s.e.m. number of MPO-positive cells mm^{-2}; $*P<0.05$ compared to L control; $**P<0.05$ compared to IM control.

Effect of ICAM-1 antisense oligonucleotide (ISIS-3082) pretreatment on gastric emptying and intestinal muscle inflammation 24 h after abdominal surgery

Pretreatment with ISIS-3082 (0.1–1 mg kg^{-1}) reduced gastric retention in a dose-dependent manner, restoring gastric emptying 24 h after IM at a dosage of 1 mg kg^{-1} (Figure 3). This effect was not observed with higher dosages (3–10 mg kg^{-1}) (Figure 3). Moreover, ISIS-3082 did not affect gastric emptying 24 h after a laparotomy in the absence of IM in mice treated with 1 mg kg^{-1}, compared to their vehicle control. In contrast, 1 and 3 mg kg^{-1} ISIS-8997, the scrambled

control antisense oligonucleotides, did not improve gastric retention 24 h after IM (Figure 3).

The number of MPO-positive cells in muscle whole mounts diminished dose-dependently (0.1–1 mg kg^{-1}) in mice treated with ISIS-3082 (Figure 4). Higher doses (3–10 mg kg^{-1}), however, did not elicit reduction of the cellular infiltrate, nor did the scrambled control antisense oligonucleotide (1 and 3 mg kg^{-1}). To evaluate whether administration of high doses of ISIS-3082 had a local proinflammatory effect (Pisetsky & Reich, 1994; Bennett *et al*., 1997), we also studied the effect of 10 mg kg^{-1} on animals who only underwent laparotomy. 10 mg kg^{-1} of ISIS-3082 did not show an increase in MPO-positive cells after laparotomy (Figure 4).

Similar to 1 mg kg^{-1} ISIS-3082, administration of ICAM-1-specific antibodies (10 mg kg^{-1} i.p.) 1 h before IM resolved the impaired gastric emptying observed 24 h after surgery, and significantly reduced the manipulation-induced leukocyte influx (Figures 1, 2, 5a–f).

Small-intestinal ICAM-1 expression

Figure 6 shows the immunohistochemical staining for ICAM-1 on transverse ileal tissue segments to assess the *in situ* effect

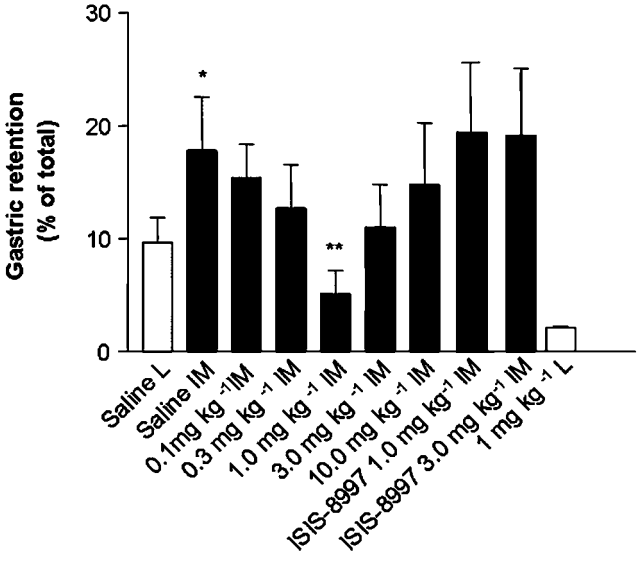

Figure 3 Effect of ISIS-3082, ISIS-8997 or vehicle on gastric retention 24 h after laparotomy (L) or laparotomy with IM. Each individual group consisted of eight animals. Data are mean ± s.e.m. gastric retention 80 min after gavage of semi-liquid test meal; $*P<0.05$ compared to L control; $**P<0.05$ compared to vehicle IM control.

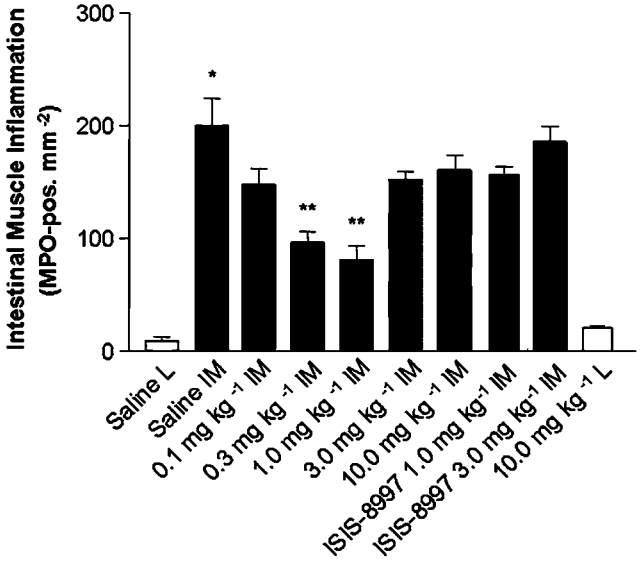

Figure 4 Effect of ISIS-3082, ISIS-8997 or vehicle on local inflammatory cell influx 24 h after laparotomy (L) or laparotomy with IM. Each individual group consisted of eight animals. Data are mean ± s.e.m. number of MPO-positive cells mm^{-2}; $*P<0.05$ compared to L control; $**P<0.05$ compared to vehicle IM control.

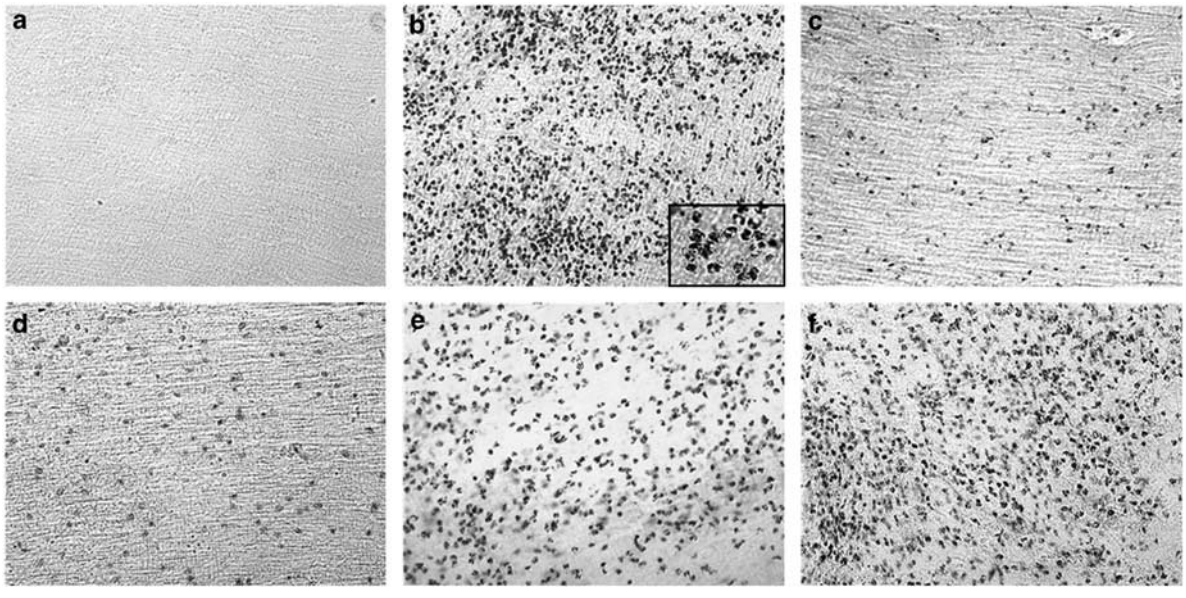

Figure 5 MPO staining of muscle whole mounts from mice that underwent a laparotomy after pretreatment with saline (a), or a laparotomy with intestinal manipulation after pretreatment with saline (b), ICAM-1 antibodies (10 mg kg^{-1}) (c), 1 mg kg^{-1} ISIS-3082 (d), 10 mg kg^{-1} ISIS-3082 (e) or 1 mg kg^{-1} ISIS-8997 (f). Magnification × 20; insertion × 65.

of ISIS-3082. Overall, ICAM-1 was predominantly expressed on the endothelium of vascular structures. IM resulted in a marked increase of ICAM-1 expression in the submucosal and muscle layers, which was not observed after laparotomy. This increase of ICAM-1 expression after IM was reduced in mice pretreated with 1 mg kg^{-1} ISIS-3082, but not 1 mg kg^{-1} of the control antisense oligonucleotide ISIS-8997.

Discussion

In the present study, we show that both ICAM-1 antibodies and the antisense oligonucleotide ISIS-3082, targeted against ICAM-1, attenuate postoperative ileus by reducing manipulation-induced inflammation. These findings illustrate the importance of ICAM-1 in the pathogenesis of postoperative ileus, and suggest that ISIS-3082 may represent a potential new pharmacological approach to prevent postoperative ileus.

Postoperative ileus complicates abdominal surgical intervention and causes prolonged hospitalisation (Prasad & Matthews, 1999). With regard to its pathophysiology, it has been shown that intestinal handling during abdominal surgery activates mast cells and resident macrophages, initiating the recruitment of neutrophils into the intestinal muscle layer (Kalff *et al*., 1999a, b; de Jonge *et al*., 2003; 2004). This local infiltrate of leukocytes is now recognised as a crucial player in postoperative ileus, as it has been shown to activate inhibitory neural pathways that lead to a generalised hypomotility of the

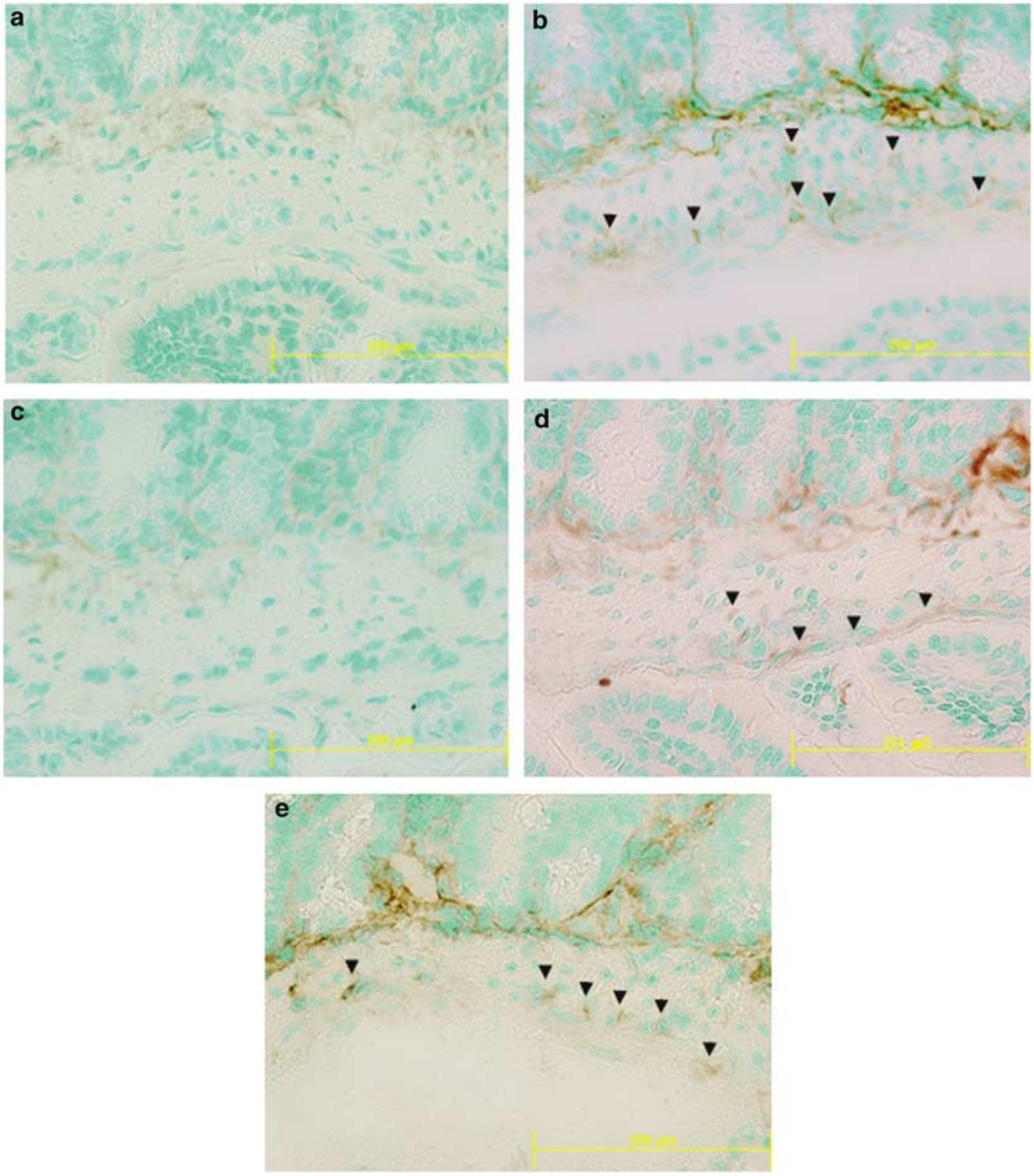

Figure 6 ICAM-1 staining of ileal transverse segments from mice pretreated with saline that underwent a laparotomy (a), and from mice that underwent a laparotomy with IM after pretreatment with saline (b), 1 mg kg^{-1} ISIS-3082 (c), 10 mg kg^{-1} ISIS-ISIS-3082 (d) or 1 mg kg^{-1} ISIS-8997 (e). Note the increased ICAM-1 expression in the densely vascularised submucosa, but also in the blood vessels, visible in the muscularis propria after IM (arrow heads). Only pretreatment with 1 mg kg^{-1} ISIS-3082 reduces the ICAM-1 expression (c).

gastrointestinal tract (de Jonge *et al*., 2003; Kreiss *et al*., 2003). Although it is not known to what extent the same mechanism is responsible for the development of postoperative ileus in patients, Kalff *et al*. (2003) observed an increase in mRNA expression in the human intestine for several proinflammatory proteins like LFA-1, iNOS, IL-6 and TNF-α after abdominal surgery.

Upregulation of adhesion molecules such as LFA-1 and ICAM-1 are necessary for the extravasation of leukocytes. Here, we show that ICAM-1 expression is clearly increased after IM, being most profound in the vasculature between the submucosal and the muscle layers, but also in the muscularis propria. This observation confirms that manipulation of the small intestine indeed increases the expression of ICAM-1, facilitating local infiltration of inflammatory cells (Kalff *et al*., 1998; 1999b). Previous studies demonstrated that pretreatment with a combination of antibodies against LFA-1 and ICAM-1 prevented postoperative ileus by blocking of this manipulation-induced infiltrate (de Jonge *et al*., 2003). In the present study, we show that pretreatment with antibodies targeted to ICAM-1 alone also results in a reduction of inflammatory cell influx and the prevention of delayed gastric emptying. These results illustrate that ICAM-1 is an important target to prevent postoperative ileus.

The use of antisense oligonucleotides is a novel approach to block the synthesis of regulatory peptides. These 15–25-base-long oligomers hybridise to the specific mRNA, preventing its translation, thereby downregulating the expression of the respective protein (Crooke, 1992; Stein & Cheng, 1993). ISIS-3082 is a mouse-specific ICAM-1 antisense oligonucleotide, which has been shown to be effective in experimental murine models for heart allograft rejection and inflammatory bowel disease (Stepkowski *et al*., 1994; Bennett *et al*., 1997). We used ISIS-3082 to study its anti-inflammatory effects in our experimental model for postoperative ileus. Similar to the ICAM-1 antibody (anti-CD54 IgG2b clone YN1/1.7) (Lub *et al*., 1996; Pruijt *et al*., 1998), ISIS-3082 reduces the IM-induced inflammatory cell influx and improves gastric emptying in a dose-dependent manner, with a maximum effect at $1\,mg\,kg^{-1}$, restoring delayed gastric emptying. As the non-sense control oligonucleotide (ISIS 8997) in a dose of 1 as well $3\,mg\,kg^{-1}$ did not have these effects, a sequence unspecific effect of the phosphorothioate backbone can be excluded. Therefore, we conclude that the anti-inflammatory effect of ISIS-3082 observed results from a sequence-specific reduction in ICAM-1 mRNA translation and protein expression. The latter is supported by the immunohistochemical staining showing a reduction of ICAM-1 expression by ISIS-3082, but not by ISIS-8997 or saline.

In the pharmacological range tested, the anti-inflammatory effect of ISIS-3082 diminished in higher doses (3 and $10\,mg\,kg^{-1}$). Bennett *et al*. (1997) observed a similar dose-dependent effect in a study evaluating ISIS-3082 in a DSS-colitis model. The lack of effect of higher dosages may be explained by the pro-inflammatory properties of the phosphorothioate backbones in antisense oligonucleotides like ISIS-3082 (Pisetsky & Reich, 1994; Zhao *et al*., 1996; Bennett *et al*., 1997). However, ICAM-1 expression was not reduced in the presence of the local muscle inflammation, making this possibility less likely. A more plausible explanation might be the biphasic response of ribonuclease H activity on phosphorothioate antisense oligonucleotide concentration. Low concentrations of phosphorothioate oligonucleotides increase ribonuclease H activity, whereas high concentrations have the opposite effect, leading to increased stability of the antisense-bound mRNA (Gao *et al*., 1992). The latter leads to decreased breakdown of ISIS-3082-bound (ICAM-1-specific) mRNA by ribonuclease H, and a diminished effect on ICAM-1 protein synthesis.

At present, treatment of postoperative ileus consists of supportive measures such as nothing by mouth, nasogastric suction, i.v. fluids, and the use of prokinetic and antiemetic drugs. Unfortunately, this approach has been rather disappointing (Kehlet & Holte, 2001; Luckey *et al*., 2003). Based on the current data, pretreatment of patients with antibodies or antisense oligonucleotides targeted against ICAM-1 are possible new preventive strategies to shorten postoperative ileus. One of the risks of using antibody treatment is the potential formation of neutralising antibodies (Shawler *et al*., 1985; LoBuglio *et al*., 1989). Antisense oligonucleotides could represent an alternative to antibody treatment. The human equivalent of ISIS-3082 (ISIS-2302) is currently being tested in a clinical trial as a putative new treatment for inflammatory bowel disease. Based on the bell-shaped dose–response curve, it should be emphasised that the therapeutic range is narrow, compromising its clinical use. In addition, one should also consider that leukocyte recruitment to traumatised tissues is needed for healing of the surgical wound. Both ISIS-3082 and ISIS-2302 have been extensively tested in several, also surgery-involving, models, disorders and clinical trials. None of theses studies reported impairment of wound healing or other postsurgical complications (Stepkowski *et al*., 1994; Kahan *et al*., 2004; Chen *et al*., 2005).

In conclusion, ICAM-1 antisense pretreatment prevented postoperative ileus in mice by reduction of manipulation-induced intestinal muscle inflammation. Our data encourage further clinical evaluation of ICAM-1 antisense oligonucleotides as tool to prevent postoperative ileus.

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