Original Paper

Intestinal Wall Damage in Simple Ileus in Rabbits: Immune-modulator Role of Somatostatin

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ABBREVIATIONS:
Somatostatin (SS); Tumor Necrosis Factor α (TNF); Interleukin 2 (IL-2); Interleukin 6 (IL-6); Simple Mechanical Intestinal Obstruction (SMIO); Hematoxylin-Eosin (H&E); Shifts Periodic Acid-Alcian Blue (SPAB); Analysis Of Variance (ANOVA); Neuman-Keuls Test (NK Test); Lymphokine Activated Killer (LAK); A Group (AG); B Group (BG); Control Group (CG)

ABSTRACT

Background/Aims: We aim to determine in which way the local immune system would be responsible for the structural changes in intestinal obstruction, and how these are influenced by Somatostatin, an intestinal peptide with immunomodulatory properties.

Simple ileus causes a series of functional and anatomical changes, which have been related to the peptidergic neural system, and inflammatory mediators. These changes are reversible with the use of Somatostatin.

Methodology: 27 rabbits divided into three groups, were subjected to the same procedure, in which a simple closed loop obstruction is caused by means of jejunum ligatures. The three groups are perfused with physiologic saline during 24 hours post-obstruction; one of them is perfused with Somatostatin from the time of intervention, and other after 8 hours. Samples of the intestinal wall are taken for histological analysis, and of the intraluminal liquid to determine the tumor necrosis factor α, interleukin 2, interleukin 6, and serotonin.

Results: Both group treated with Somatostatin show a wall which is in good condition, while the untreated group showed lesions. These lesions are related to higher levels of tumor necrosis factor α, and interleukin 2, while there were no changes in the levels of interleukin 6.

Conclusions: The Somatostatin in perfusion shows a cytoprotective activity in the intestinal wall, and a blockage of the production of mediators of cellular immunity, while humoral immunity does not appear to be involved in these phenomena.

INTRODUCTION

Intestinal obstruction is an old problem which the surgeon must often resolve, as it makes up 5% of the ordinary admissions, and 20% of the emergency admissions, it generates an important morbidity and a 5% mortality, which is increased when the viability of the wall is jeopardized (1), and which has barely changed in recent years (2,3).

Simple mechanical intestinal obstruction (SMIO), initiated by the mechanical problem which leads to the detention of intestinal transit, is able to generate a succession of events, first local and later systemic, which are only reversible initially. In the pathophysiology of SMIO in animal models, there is a relation between the accumulation of the intestinal contents proximal to the obstruction, the distension which at first is moderate and then stimulates the secretion of fluids leading to more distension, and the evolution towards ischemia and gangrene when the increase of the pressure prevents parietal blood flow (4).

The explosion of knowledge in the last decades, about the complex interactions between the nutritional and immune functions and the neuropeptidergic system and the intestinal hormones, have provided new pathophysiological evidence of the involvement of local changes in SMIO, such as: the action of intestinal peptides (5), the prostaglandins (6), and the mediators of inflammation (7).

Somatostatin (SS) is a peptide, produced, among other places, at the intestinal level, it shows both endocrine and paracrine activity, with a broad spectrum of intestinal actions, such as the inhibition of other intestinal hormones, secretion and intestinal motility (8), which justify its use experimentally in the first phases of the local changes generated by the SMIO (9). Surprisingly, its use leads to a better conservation of the intestinal wall which appears to be independent of the volume, and therefore the intraluminal pressure, and this could be due to its immunomodulatory properties (10).

To verify this hypothesis, we measured the intraluminal levels of tumor necrosis factor alpha (TNF), Interleukin 2 (IL-2), and Interleukin 6 (IL-6), as mediators of the immune response in an experimental model of closed loop acute jejunum obstruction, with preserved blood flow, in rabbits submitted to a periph-

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eral perfusion of Somatostatin 14 (SS). We also studied the intraluminal levels of Serotonin produced by the intestinal enterochromaffin cells, as a control for the functional lesion. The control of the tissue damage is done by means of a microscopic study of the wall, with different techniques.

METHODOLOGY

Experimental Animals

27 New Zealand rabbits (Granjas Jordy B&K, UL, Barcelona), female, weight between 3 and 3.5kg, without evidence of disease in 20 days of acclimatization in the animal research house, and fed with water and rabbit food on demand during the same period, were admitted to the experiment, with the international norms on maintenance and protection of experimental animals being observed at all times.

Randomly divided into three equal groups (n=9), the control group (CG) was only given saline solution; the second group was given a Somatostatin perfusion (Somiaton, Serono SA, Madrid) immediately following the surgical intervention [A group (AG)], and the third group was given a Somatostatin perfusion 8 hours after the end of the intervention [B group (BG)].

Intervention

The animals were anaesthetized with 100mg/kg of body weight of Ketamine IM. (Ketolar, Parke-Davis SA, Barcelona), and 0.5mL/kg of Droperidol with Fentanyl (Thalamonal, Syntex Latino SA, Barcelona), also IM. The marginal vein of the rabbits ear was canalized once the animal was anaesthetized, for the intravenous administration of saline solution at a rate of 6mL/kg/h, during the entire intervention.

The three groups were subjected to a surgical intervention, through an abdominal access, in which the distal jejunum was identified. Four cm from the jejunum-ileum junction, in the proximal direction, an intestinal obstruction is made by means of a ligature through an avascular mesenteric window with (number 2) silk, until the lumen is completely occluded. Taking care not to damage any mesenteric vessels during the manipulation, 15cm of jejunum cephalic from the first ligature are identified, and by means of soft pressure of the wall, the content of the lumen is moved proximally, and another ligature with the same characteristics of the first is applied. This makes up a closed and empty loop 15cm long, in which the perfusion through mesenteric vessels is guaranteed, and a simple obstruction proximally to the last ligature with the same conditions.

Up to the next intervention the animals are kept on an absolute diet while they are perfused with saline solution at 4mL/kg/h. Somatostatin was added at a dose of 3.5μg/kg/h immediately following the intervention in AG; and 8 hours after the end of the intervention in BG, at the same dose.

After 24 hours, the animals are again anaesthetized and re-laparotomized, and the obstructed loop is identified. Samples of the intestinal fluid are taken by means of an intravenous needle through the wall, and afterwards the intestinal loop along with its attached mesenterium is removed. Immediately after this the animals are sacrificed while they remain anaesthetized, by means of an intravenous administration of 100mg of Ketamine.

Processing of the Samples

The samples of intestinal fluid are centrifuged at 3500 rpm, to discard the solid particles in suspension, and immediately divided into 250-mL aliquots and stored at -80°C. The intestinal wall is completely submerged in a 10% formaldehyde solution until its processing, which shall never be longer than three days.

Determination of IL2, TNF, IL6 and Serotonin

The aliquots of centrifuged intestinal liquid are thawed out to room temperature and are subsequently subjected to Enzyme immune sandwich type analysis (ELISA), using kits with anti-IL-2 antibody, anti-TNF antibody, anti-IL-6 antibody and anti-Serotonin antibody, supplied by Immunotech International (130 Avenue de Lattre de Tassigny, France), in which in the case of IL-2 it was necessary to remake the standard curves with greater dilutions than those suggested by the manufacturer, given the low levels in the intestinal liquid. As a control measure, the results are randomly repeated up to three times.

Processing of the Intestinal Wall

Several paraffin cuts of the intestinal wall are made and later dyed with hematoxylin-eosin (H&E) and Shifts periodic acid-alcian blue (SPAB), the latter to obtain an adequate coloring of the mucus of the mucous cells of the intestinal epithelium.

The lesions observed with light microscopy are quantified by means of a semiquantitative method in 4 degrees, in which the degree 0 indicates an absence of lesion, while degrees 1 to 3 indicate an increase in the damage of the intestinal wall for 6 categories previously established: the integrity of the mucous layer, the number of mucous cells dyed in the epithelium, the edema of the submucous layer, the infiltration by inflammatory cells, the indemnity of the muscular layer, and the involvement of the peritoneum. All the samples were seen by two pathologists, who scored them without knowledge in each case of which group it belonged to.

Statistical Procedures

All data are expressed as mean values (standard deviation), the statistical significance was obtained by means of analysis of variance for three samples (ANOVA), and the Neuman-Keuls test (NK Test) for the 2 by 2 comparison of the samples with sufficient statistical significance in the previous test. The results of the pathological analysis are expressed in numerical values which are the result of multiplying by the degree which was assigned to each of the categories, and the sum of the scores of all the categories for each individual separately. The correlation analysis was done between the values of IL-2 and TNF, and the histopathological lesion. As a minimum level of statistical significance we accepted $p<0.01$.

TABLE 1 Initial Weights, Total Volumes of Saline Administered, and Intervention Times

Group	Weight (mg)	Volume of saline (mL)	Intervention time (minutes)
CG	2562.22 (9.69)	245.97 (9.37)	15.31 (2.35)
AG	2570.64 (106.74)	246.71 (10.24)	16.02 (3.10)
BG	2585.08 (69.48)	248.22 (6.66)	15.12 (1.92)

The values are expressed as means, with the standard deviations in brackets. CG: control, AG: Treated with SS-14 from the start, BG: Treated with SS-14 after 8 hours.

RESULTS

The samples have been shown to be homogenous both with regard to the number of individuals as a function of weight, the volumes of serum administered during the entire experimental procedure, as to the mean duration of the two surgical interventions **(Table 1)**.

Condition of the Intestinal Wall

In the second intervention, all the animals that were not treated with SS, showed surgical signs of intestinal obstruction, with distension, thickening of the wall, and dullness of the serous layer, cephalic to the second ligature, while in treated animals the signs were minimal or did not exist.

The viability of the intestinal wall of the animals treated with Somatostatin was compared with that of the animals which were not treated, by means of a microscopic study at the closed loop **(Figure 1)**. The samples of intestinal wall distal to the closed loop in all cases did not show histological alterations, with the mucous layer even having a normal villous and mucous secreting cells distribution. In the closed loop of the untreated cases there was a decrease in the villous length, shortening of the crypts, decrease of the mucous producing cells, and even epithelial desquamation. In the submucous layer there was evident edema, areas of hemorrhagic infarction, and an abundant inflammatory infiltrate. Edema and infiltration were also seen in the muscular layer and in the serous layer. There was a significant reduction of the lesions of the intestinal wall in the treated groups, in which we saw an adequate villous and crypt length, good epithelial distribution, conservation of the mucous producing cells, and less edema and infiltration than in the untreated group. Neither the muscular layer nor the serous layer were affected. These differences were quantifiable by means of the application of the described semiquantitative procedure [CG: 16.11 (1.83); AG: 3.9 (0.86); BG: 3.33 (1.5)].

Considering all the analyzed categories and doing the ANOVA test, the observed differences show statistical significance ($F=219.18$, $p<0.01$), confirmed with

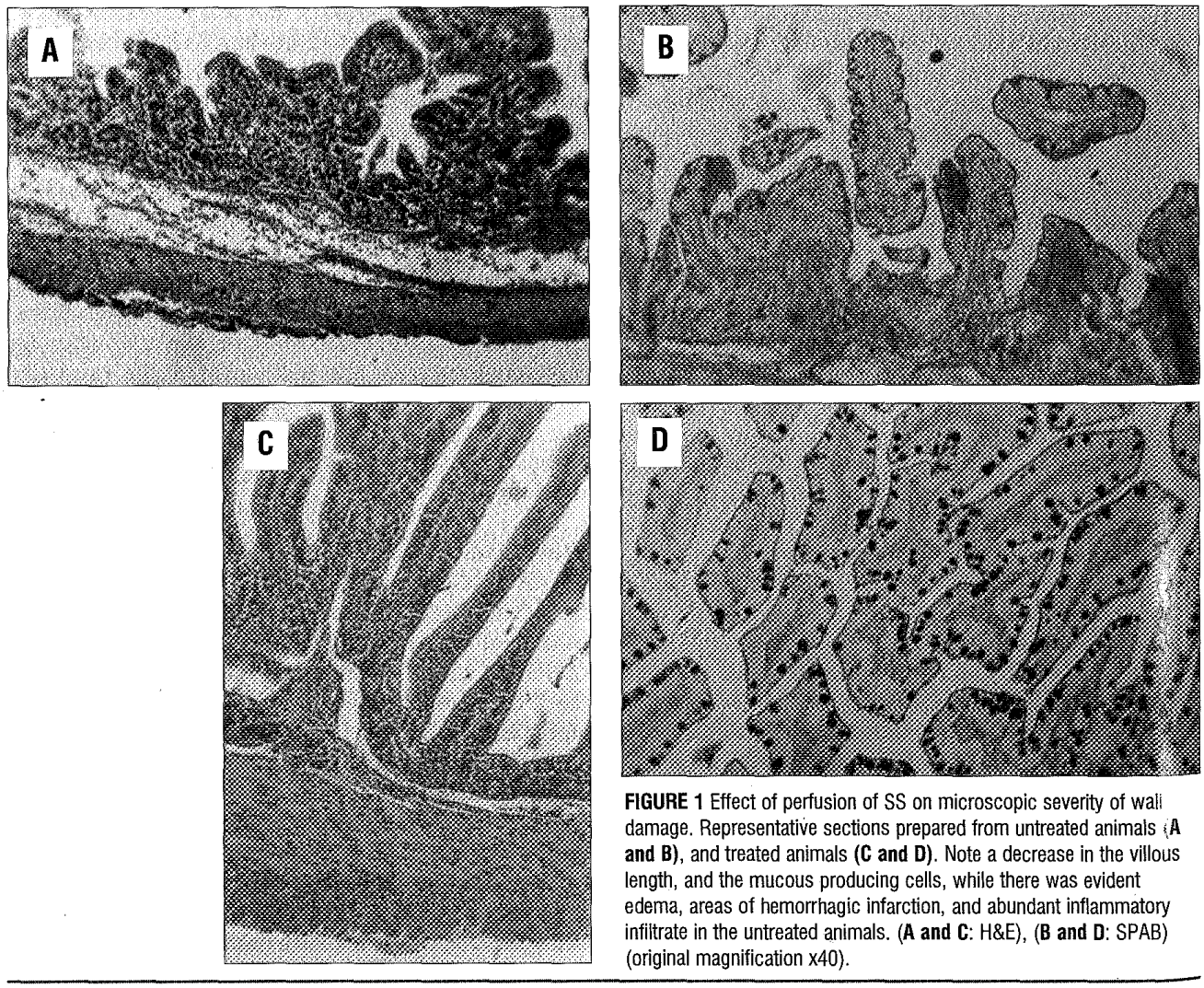

FIGURE 1 Effect of perfusion of SS on microscopic severity of wall damage. Representative sections prepared from untreated animals (**A and B**), and treated animals (**C and D**). Note a decrease in the villous length, and the mucous producing cells, while there was evident edema, areas of hemorrhagic infarction, and abundant inflammatory infiltrate in the untreated animals. (**A and C**: H&E), (**B and D**: SPAB) (original magnification x40).

TABLE 2 TNF, IL-2, IL-6 and Serotonin levels in the Intestinal Liquid (ELISA)

Group	TNF (pg/mL)	IL-2 (pg/mL)	IL-6 (pg/mL)	Serotonin (pg/mL)	Vol (mL)
CG	236.67 (71.87)	207.73 (52.13)	23.19 (18.54)	27.55 (12.84)	2.90 (2.19)
AG	129.92 (40.04)	14.56 (12.16)	18.84 (10.46)	90.56 (45.20)	1.95 (0.92)
BG	142.27 (82.40)	8.21 (3.32)	19.61 (13.82)	91.04 (51.18)	2.53 (1.57)

The values are expressed as means, with the standard deviations in brackets.
CG: control, AG: Treated with SS-14 from the start, BG: Treated with SS-14 after 8 hours.
Volumes of intestinal liquid in the closed loop.

the NK Test with AG and BG with respect to CG, and there not being any statistical significance between AG and BG.

Volumes of the Intestinal Liquid

Contrary to the measured levels of intestinal liquid in the simple ileus cephalic to the second ligature, in the closed loop there was no difference between the treated and untreated groups **(Table 2)**.

TNF Levels in the Intestinal Liquid

There was a difference of approximately 100pg/mL between the control animals and those perfused with SS **(Table 2)**, differences which showed statistical significance with the ANOVA test ($F=6.78$, $p<0.01$), the NK Test confirmed the significance of these differences between the treated and untreated groups, but not between the treated groups. There was, therefore, a reduction of the TNF levels in the intestinal liquid, with a parallel decrease in histological lesions in the treated rabbits. When comparing the two groups of results, there was a positive correlation ($r=0.6$) with a sufficient degree of significance ($p<0.01$) **(Figure 2A)**.

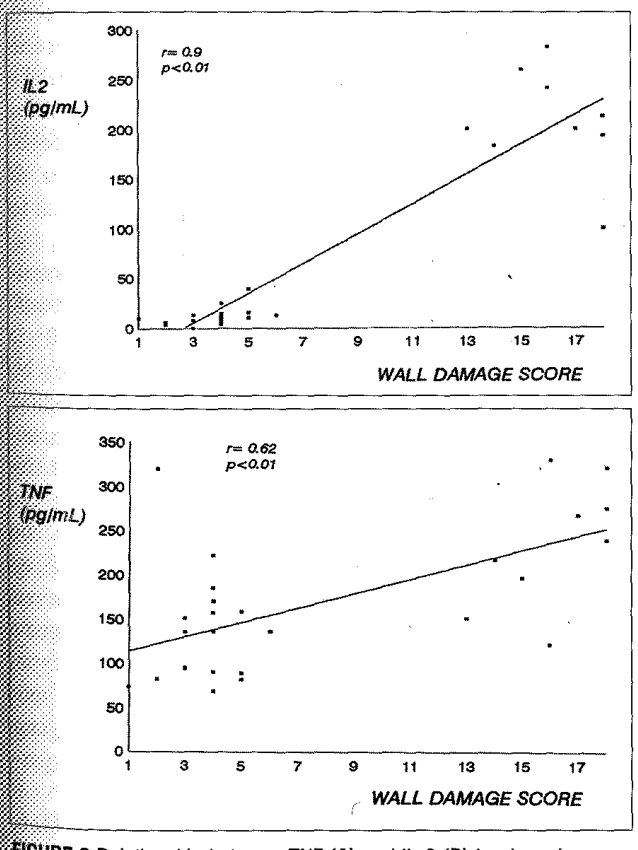

FIGURE 2 Relationship between TNF **(A)** and IL-2 **(B)** levels and intestinal wall damage score. The score are quantified by means of a semiquantitative method in 4 degrees for 6 categories previously established.

Levels of IL2 in the Intestinal Liquid

The differences observed between the treated and untreated groups were in the same direction as those observed with TNF, but much more marked (close to 200pg/mL) **(Table 2)**, showing at the same time, a statistical significance with the ANOVA test ($F=120.70$, $p<0.01$). The IL-2 showed higher levels in the untreated group, as did the histological damage score, and the correlation between both variables ($r=0.91$) was much more pronounced than that observed with TNF, and with equal significance ($p<0.01$). Both variables among them also showed positive correlation ($r=0.51$, $p<0.01$) **(Figure 2B)**.

Levels of IL6 in the Intestinal Liquid

Contrary to IL-2 and TNF, there were barely any differences between the treated and untreated groups in the IL-6 levels measured in the intestinal liquid **(Table 2)**, and they had no statistical significance. As a whole, IL-6 did not appear to show any response to the infusion of SS in the closed loop obstruction model.

Levels of Serotonin in the Intestinal Liquid

The serotonin produced in the enterochromaffin cells of the intestinal wall, was present in a lower concentration in the intestinal liquid of the untreated rabbits **(Table 2)**, and it showed statistical significance ($p<0.01$). The analysis of the correlation with the histological lesions, showed a negative slope with sufficient significance ($r= -0.64$; $p<0.01$).

DISCUSSION

The present state of knowledge of the intestinal anatomy and physiology, and the new experimental evidence of the levels and functions of the different cytokines in the intestinal wall, imply the coexistence of two systems with regulatory functions (epithelial hormone and neuropeptidergic) and two with effector functions (the nutritional and the immune), which are influenced not only by the first two, but also among them, in what has come to be called "a physiologic state of inflammation" of the intestinal mucous layer (11), where the regulatory systems prevent the uncontrolled expansion of the epithelial damage (12).

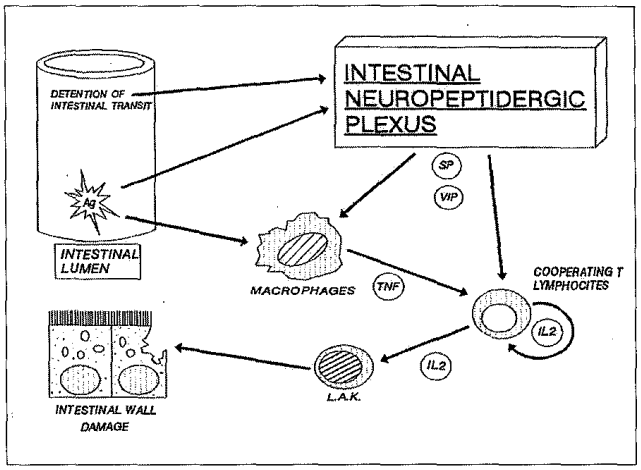

FIGURE 3 Schematic diagram of suggested model, showing the local changes during simple mechanical intestinal obstruction, which relates the most recent experimental evidence (Ag: antigenic stimulation; SP: substance P; VIP: vasoactive intestinal peptide).

This implies that there is room for an increasing number of experimental evidence in the phenomena related to intestinal obstruction, which implies a disregulation of the effector systems secondary to intestinal distension, and the bacterial overgrowth, which is probably of neurohormonal and immune origin, given the increase in measured specific peptidergic factors (5) and inflammatory mediators (7). The consequences of which are an abnormal functioning of the wall with the increase of excretion, the decrease of absorption, the inversion of the ion flow, and the increase of the motility, which ensure that there is not a reverse, a continuous distension with the increase of pressure and the stopping of the parietal blood flow, as can be deduced from the first experiments with animals (4).

SS is a tetradecapeptide produced in the central nervous system and at the intestinal level, which has been shown to have many actions which are contrary to those described in intestinal obstruction (8), and it is used experimentally on this process, leading to a decrease of the intestinal secretion, the redirection of the ion flow, and the decrease of intestinal motility (9), but at the same time it promotes a better condition of the wall which cannot be explained only, as was being done, by the direct mechanical distension by the endoluminal pressure (10). Besides this, there is a clear state of inflammatory infiltration which at least suggests the co-responsibility of the immune system, which could be countermanded by the use of SS, which has been shown to have immunomodulatory functions (13,14).

In the present descriptive study, we investigated the local levels of inflammatory mediators during intestinal obstruction while SS, like an intestinal peptide with immunomodulatory functions, is perfused, and related to the histological changes observed in the intestinal wall. This could achieve experimental evidence of the participation of the intestinal immune system in the changes observed in SMIO.

In agreement with previous reports there is a lower level of the intraluminal liquid at the closed loop (9,10), but in our study there is no difference between the treated and untreated groups. This discrepancy could be the result of differences in intestinal site of the closed loop (jejunum in our model), or the absence of intestinal vessels damage which cannot be guaranteed in mesenteric ileo-cecum in rabbits (9,10). Although our findings are not consistent with the hypothesis that increased intraluminal volume is the only cause of intestinal ischemia, and suggest that abnormalities in the condition of the intestinal wall has another origin.

TNF is a cytokine produced by macrophages, which modulates various functions of the cells involved in inflammation, immunity, and tissue repair, and it participates in the inflammatory infiltration of the intestinal wall (15). At the level of the intestine there is a constant production (16), and it has been related to the damage of the wall in inflammatory bowel disease and in shock models (17,18). Recently its participation in epithelial ionic secretion through prostaglandins, has been recognized (19). In the model we made, the levels of TNF in the intestinal liquid, as a reflection of the local production during the obstruction, are lower with the perfusion of SS, either from the beginning of the obstruction or after a few hours, and exits a correlation with the destruction of the wall. It would explain the discrepancy between the intraluminal volumes, like origin of the distension and intraluminal pressure in the closed loop, and the necrosis of the wall which is seen in the group not treated with SS **(Table 2)** and in similar models (10), probably by the prevention of the development of the inflammation cascade. Nevertheless, there is not an adjusted proportion between the levels of TNF and the degree of tissue damage, as can be deduced from the analysis of the correlation between the two variables **(Figure 2)**. Therefore, just as the induction of intestinal secretion by TNF needs the participation of prostaglandins (19), the necrosis of the wall seems to need another factor.

Just like TNF, IL-2 is a protein with immunomodulatory actions, produced by epithelial and the intestinal propria lamina cooperating T lymphocytes (20), it is directly involved in the destruction of epithelium by means of the activation of lymphokine activated killer (LAK) (21), and *in vitro* it responds to the immunomodulatory action of SS (22). Our results support the hypothesis that the action of IL-2 is involved in the epithelial lesion. Like TNF the levels of IL-2 in the intestinal liquid are lower with the perfusion of SS, as the levels are better adjusted to the changes observed in the wall destruction than do those of TNF **(Figure 2B)**, whether this be mediated by LAK or other cytotoxic cellular effectors. Once again, SS is shown here as a cytoprotector of the intestinal wall, and this time is associated with a decrease of the liberation or production of IL-2 **(Table 2)**.

Another mediator of inflammation, such as IL-6, which is associated with humoral immune response, has not been related, in our model, to the local changes in the physiopathology of intestinal obstruction, despite it being a cytokine which is also produced at

the intestinal level (23), and is related to abdominal injury (24), which could be interpreted as a lack of response to SS, or to the scant influence of humoral immunity in the histological damage observed in intestinal obstruction.

SS produces an improvement in the histological condition of the wall, and a better preservation of its functions is to be expected, in this way a good level of preservation of the absorptive epithelium is evident, but also of the mucous-producing cells and the production of serotonin by epithelial and myenteric plexus cells with hormonal capacity. Serotonin plays an important part in the intestinal secretion of water and electrolytes, influences the mesenteric flow, and participates in the control of the intestinal migratory complex (25). The levels present in the intestinal liquid 24 hours after initiating the obstruction, given the short half-life of this substance, and associated with a better preservation of the wall, indicates that this keeps its regulatory and control functions through the products of the intestinal enterochromaffin cells, and among these, the hormones and neuropeptides.

These results corroborate the anatomical and functional protection of the intestinal wall by SS, like other previous models (9,10), besides this, in our model they are associated to a lower intestinal levels of IL-2 and TNF, but do not elucidate whether this relation is causal or indirect. And at the same time create new questions, such as the possible reduction of the intestinal SS secretion by the epithelial hormonal cells during the obstruction, or the relative deficiency due to the exaggerated production of other peptides with opposing actions, such as vasoactive intestinal poly-peptide (5), or the mechanism of interaction of peptides and cytokines, increased in the intestinal wall during obstruction (26).

As well as this, it may contribute to a new pathophysiological hypotheses of the local changes during SMIO, which relates the mechanical obstruction with the hypothetical immune parietal damage, although more experiments have been initiated to confirm the different aspects, such as the direct inhibition with antibodies specific for IL-2 and TNF, to draw a direct link between the cytokines measured and the clinic pathologic changes associated with mechanical obstruction, or the levels of SS produced in the intestinal wall during SMIO. These new hypotheses related the detention of intestinal transit and the antigenic stimulation during intestinal obstruction with the production of neuropeptides by the submucous layer and myenteric plexus (5), as well as the degranulation of the macrophage with the liberation of TNF (7). Both the action of these peptides with immune-facilitating actions (26-28) and the TNF, increase the production of IL-2 by the cooperating lymphocytes, and these in turn increase the activation of cytotoxic cells (21), with the latter being responsible for the parietal damage **(Figure 3)**. All of this could be interpreted as an uncontrolled amplification of the physiological and immune condition of the intestinal wall, which in turn is a reflection of the crushing, uncontrolled, indiscriminate and self-destructive behavior of the host towards the antigenic stimulus of the intestinal bacteria.

In conclusion the most significant finding of our study is the association of an evident impairment of the anatomical and functional condition of the intestinal wall during mechanical obstruction together with a marked increase of the intraluminal content of IL-2 and TNF, known as the cytokines that promotes the development of the inflammation cascade.

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