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Role of the Immune System in Small Bowel Reperfusion Injury in the Rat.

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ABSTRACT

There is increasing evidence pointing to the role of oxygen-derived free radicals in the pathogenesis of reperfusion injury. However, other substances that also reach ischemic tissue on restoring blood flow may be involved as well. To examine the possible role of immune cells in this process, three different immunologial treatments were tested: cyclophosphamide, whole body irradiation and cyclosporine A. Mortality rate, mean survival time (MST), length of damaged intestine (LDI) and mucosal damage index (MDI) were assessed in an experimental model of intestinal ischemia in the rat. While cyclophosphamide had a lethal effect, whole body irradiation greatly improved mortality and MST. Cyclosporine A, as expected from earlier experiments, increased MST and reduced mortality, LDI and MDI. It thus appears likely that immune cells are involved in the pathogenesis of reperfusion syndrome.

Key words: Acute intestinal ischemia. Reperfusion. Immune system. Pathogenesis. Natural killer cells. Rats. Cyclophosphamide (Cy). Cyclosporine A (CsA). Whole body irradiation (WBI).

INTRODUCTION

Although much attention has centered on ischemic injury of the small bowel, the mechanism involved in its pathogenesis remains uncertain. Of the many hypotheses proposed to explain ischemic injury, the most widely accepted is that hypoxia in itself is the key factor causing the ischemic lesion (1,2). However, current data suggest that, following an ischemic episode, reperfusion of molecular oxygen results in more tissue damage than during a similar period of anoxia (3,4). Thus, oxygen-derived free radicals have been implicated in the pathogenesis of reperfusion syndrome. Moreover, good results have been obtained with superoxide dismutase (5), allopurinol (6) and other free radical scavengers (7).

On the other hand, we previously reported that short-term cyclosporine A (CsA) treatment reduces mucosal damage in an intestinal reperfusion model (8).

The purpose of this study was to determine whether the immune system and/or natural killer cells (NK cells) act as cofactors in the pathogenesis of mucosal lesions during experimental intestinal ischemia, thereby contributing to the cell mortality observed.

MATERIAL AND METHODS

Female Sprague-Dawley rats weighing 200 g were used. The animals received an ordinary pellet diet (Panlab A-04, Spain) and water *ad libitum* prior to the experiments, which began between 9 and 11 am.

Surgical procedures

All animals were anesthetized with sodium pentobarbital (30 mg/kg i.p.). Ether was used to supplement anesthesia when needed. The abdomen was clipped and cleansed with alcohol, and a 2.5 cm midline

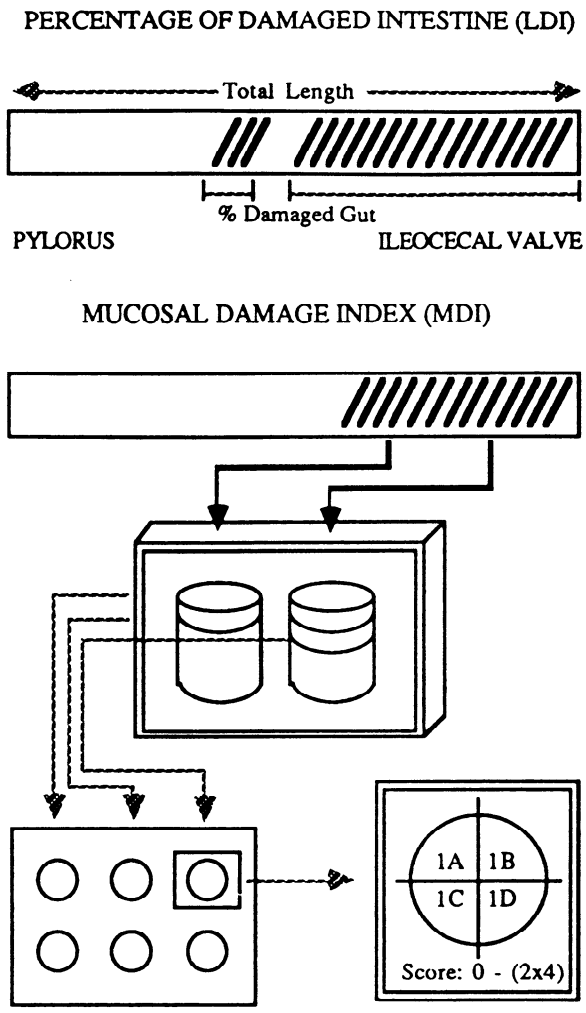

Fig. 1: Preparation of tissue samples for histological quantification of the Mucosal Damage Index.

incision was made. The superior mesenteric artery (SMA) was exposed close to its origin from the aorta and occluded for 120 min. with an atraumatic vascular clamp (Yasargil-clip, Aesculap), after which the abdominal incision was closed with continuous 3-0 silk. Once the ischemic period was terminated the clamp was removed, and the blood supply to the intestine reestablished. The SMA pulse was present in all animals following clamp removal. The abdominal incision was then closed using continuous 2-0 silk.

All animals were sacrificed 8 days after surgery. The Draft Convention for the Protection of Animals used for Experimental Purposes was always followed.

Experimental groups

Four groups of 25 rats were used, distributed as follows:

Group 1:
Untreated controls in which the SMA was occluded for 120 min.

Group 2:
A single cyclophosphamide (Cy) dose (20 mg/100 g i.p.) given five days before surgery.

Group 3:
Under sodium pentobarbital anesthesia (30 mg/kg) the animals were placed in a recumbent position and a single total dose of 5 Gy (0.5 Gy/min., distance 117 cm) was administered (ALCYON II.CGR cobalt unit). Each animal bore an individual THERADOS-DPD device to register the radiation received.

Group 4:
Cyclosporine A (CsA)(20 mg/kg i was given 24 and 4 hours before the onset of mesenteric ischemia.

Mortality Rate (MR) and Mean Survival Time (MST)

Mortality Rate (MR) during the first 48 hours was assessed in 20 animals of each oup. Nevertheless, the animals were kept alive in ou. aboratory for a week. Mean Survival Time (MST) for the deceased animals was also recorded.

Lesional studies

Five animals from each group were sacrificed 30 min. after reperfusion, and the gut (from pylorus to ileocecal valve) was rapidly removed and measured. The zones showing macroscopical lesions were evaluated, and the percentage of intestinal length affected by ischemia was calculated. The mean value within each series was termed the Longitudinal Damage Index (LDI).

Two 1-cm fragments of distal ileon were excised and embedded in paraffin. Three histological sections taken at 4 mm intervals were cut and mounted on slides, followed by hematoxylin-eosin staining. The Mucosal Damage Index (MDI) for each animal was calculated as described in Figure 1. Each mounted slide tissue section was divided into four quadrants along its longitudinal and vertical axis. Mucosal damage was then evaluated in each quadrant.

Histological assessment of intestinal injury was carried out using a 0-2 grading scale:

Grade 0 (Fig. 2A):
Indicates minimal tissue damage (good histomorphic preservation with some Gruenhägen subepithelial spaces at the tip of the villi).

Grade 1 (Fig. 2B):
Moderate mucosal damage (subepithelial lifting as well as more advanced lesions).

Grade 2 (Fig. 2C):
Severe mucosal damage (Grades 4 and 5 of Chiu (9)), with completely denuded villi, a disintegrated lamina propia and hemorrhage.

The MDI was obtained adding the grade of mucosal damage for the 24 quadrants evaluated on each slide and dividing by 6 (total no. of sections). Thus, MDI values range 0-8. This procedure was performed consecutively by two independent observers in a blind code fashion. Due to

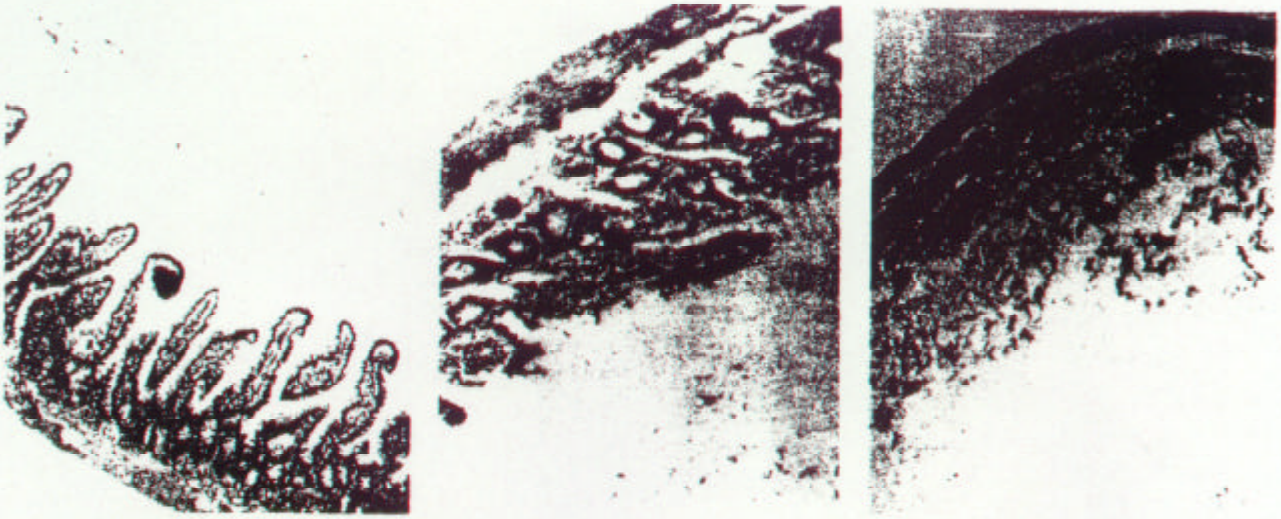

Fig. 2: Intestinal histology. (a) Grade 0: minimal tissue damage; (b) Grade 1: moderate mucosal damage; (c) Grade 2: important structural alterations. HE, x100.

the good correlation observed between both sets of data, their mean was accepted as the definitive value.

Statistical analysis

Comparisons of mean survival rates, LDI and MDI were analyzed using the Kruskal-Wallis and U-Mann Whitney tests, whereas mortality rates were compared using the Fisher Exact Test. Correlation between the measurements made by the two independent observers was performed using Kendall's Concordance test. Values of p under 0.05 were regarded as significant.

RESULTS

Mortality

Rats subjected to intestinal ischemia for two hours showed a 75% mortality and an MST of 11.86 hours (Table I, II). Cyclophosphamide-treated animals died less than 24 hours after the operation, while MST was 7.11 hours. The values for both indices were significantly worse than those calculated for the controls ($p < 0.05$). The mortality rate in WBI animals dropped to 40%, while the MST doubled compared with the controls ($p < 0.001$). However, seven days after apparently complete recovery (normal feces, external appearance and activity), all the animals died. Necropsy was performed in all cases, but failed to reveal any specific cause of death. This phenomenon was observed uniformly in the four sets of animals in our series. CsA treatment induced an effect somewhat similar to that of WBI. The mortality rate in this group was 35% - a value significantly lower than in the controls ($p < 0.001$), and similar to the WBI-treated animals (NS). MST was also increased by CsA (17.42 hours; $p < 0.05$), but without reaching the same levels as in Group 3 ($p < 0.05$).

Longitudinal Damage Index (LDI)

Although the possible variations in intestinal length are not reported in expressing the damage index as a percentage, there were in fact no significant differences.

In the controls, the mean percentage of macroscopically damaged intestine (LDI) was 44% (Table III). In accordance with the poor survival results obtained with Cy, LDI was greatly increased in this group (69%; $p < 0.001$). Nevertheless, despite the improvement in both mortality rate and MST as a result of WBI and CsA, these therapies did not reduce LDI (42 and 41.1%, respectively).

Mucosal Damage Index (MDI)

As previously described, the MDI was calculated separately by two investigators. The two sets of values obtained showed a very good correlation ($r = 0.87$; $w = 0.95$)($p < 0.001$).

Intestinal ischemia, prolonged for two hours, resulted in an MDI of 4.08, 30 min. after reperfusion (Table IV). In agreement with previous results, Cy significantly enhanced this index (5.18; $p < 0.05$). Nevertheless, while WBI did not modify MDI compared with the controls, CsA clearly decreased it (2.9; $p < 0.05$).

DISCUSSION

Tissue hypoxia or anoxia due to ischemia is an obvious cause of cell injury and death. However, it has also been established that reperfusion of ischemic organs increases the damage previously induced by ischemia (1-4). It is thus generally accepted that "something" arriving with the renewed blood flow must be responsible for this effect. Multiple hypotheses have been proposed to date (restoration of blood pressure, hemorrhage, oxygen, etc.), including the production of free radicals in reperfused tissue - an aspect that is currently being widely investigated (5-7). We have previously reported that during SMA occlusion and reperfusion, Peyer's patches become hypertrophic and a significant increase in mononuclear cells is seen in the submucosa (Fig. 3a,b). Accordingly, the immune system was suggested as having a possible role in the genesis of mucosal injury (8), and the effect of three immunomodulatory treatments (splenectomy, steroids and cyclosporine A) on mucosal damage was investigated.

Though splenectomy and steroids showed little effect, cyclosporine A significantly reduced histological damage in intestinal lesions (10). Because of the early nature of the response and the results obtained

Groups	Alive	Dead	% Mort.	X^2	p
Controls	5	15	75	-	-
Cy	0	20	100	5.7	0.02
WBI	12	8	40	5	0.026
CsA	13	7	35	6.4	0.012

Table I: Mortality rate (48 h). Cy: Cyclosphosphamide, WBI: Whole Body Irradiation, CsA: Cyclosporine A.

Groups	N	Mean	SD	U	p
Controls	15	11.86	4.70	-	-
Cy	20	7.11	4.88	71	0.008
WBI	8	23.75	9.23	14	0.001
CsA	7	17.42	4.27	20	0.021

Table II: Mean Survival Time. Cy: Cyclosphosphamide, WBI: Whole Body Irradiation, CsA: Cyclosporine A.

	Mean	SD	U	p
Controls	44.4	3.7	-	-
Cy	69	7.17	0	0.007
WBI	42	3.12	7.5	NS
CsA	41.4	5.77	8.5	NS

Table III: Length of Damaged Intestine (Percentage). Cy: Cyclosphosphamide, WBI: Whole Body Irradiation, CsA: Cyclosporine A.

Groups	Mean	SD	U	p
Controls	4.08	0.50	-	-
Cy	5.18	0.35	0	0.007
WBI	3.82	0.76	10	NS
CsA	2.92	0.60	1.5	0.031

Table IV: Mucosal Damage Index. Cy: Cyclosphosphamide, WBI: Whole Body Irradiation, CsA: Cyclosporine A.

with cyclosporine A, Natural Killer cells were suggested as being involved in the pathogenesis of intestinal reperfusion injury.

In order to evaluate this hypothesis, cyclosporin treatment was compared with two other therapies that also inhibit NK activity. Cy was selected because of its well-known inhibiting effect on NK cells (11). It also suppresses T cytotoxic lymphocytes and decreases the Th/Ts ratio (12). As no selective suppressor of NK cells is available, WBI was used to assess the effectiveness of the treatment tested. In searching for a more comprehensive analysis of the results, three other parameters were also recorded. Mortality rate is closely related to the systemic disorders induced by intestinal lesions. It may be assumed that a decrease in local intestinal damage will be followed by a certain improvement in survival rate, or at least death will be delayed (and so mean survival time in a sense gives mortality rate greater accuracy).

LDI can be considered to be of little value since the length of damaged intestine depends mainly on the vessel occluded during ischemia, which was constant in our experiment. However, collateral blood flow does provide zones of low-flow in which treatments protecting against ischemia may prevent the appearance of mucosal damage. From this point of view, it is not surprising that LDI paralleled MDI.

As expected from our previous experiment (10), CsA exerted a beneficial effect on intestinal reperfusion syndrome. In addition to reducing mucosal injury, it reduced mortality rate and increased mean survival time. The only exception was LDI, which remained unchanged. It seems likely that the lack of sensitivity of this index depends more on the time employed in our study (30 min. after reperfusion) than on the particular CsA mechanism of action involved (13).

WBI operated contradictorily. It improved both survival indices without modifying either of the damage indices. This defies simple explanation. The beneficial effect we expected from WBI is based on its potential ability to reduce mucosal damage through generic immunosuppression (14,15). Moreover, the apparent increase in survival rate had a deleterious effect, as all animals died suddenly between the 6th and 8th day. Probably, the moment of the morphological study was not the most opportune, as it failed to show the amelioration induced by treatment, whereas mortality rate (a much later index) was able to provide such information. This hypothesis agrees with the reported effects of Cy concerning polymorphonuclear and macrophage depletion (12); both types of cell have been shown to be involved in ischemic injury because of their ability to produce free radicals. However, a beneficial effect was also expected according to our initial hypothesis suggesting an active role of the immune system in reperfusion lesions.

As to the late deaths among WBI animals after bowel ischemia, no references could be found. It seems reasonable that death was related to anemia and/or infection which often takes place after ischemic accidents of the gut (16), but worsened in this case by medular aplasia.

The results obtained with Cy were rather disappointing. The drug was chosen because it has been shown to inhibit NK activity at the doses used in this experiment (11). However, all parameters considered were significantly worse following Cy treatment - even LDI, which was not modified by other therapies. Obviously, there may be one or several explanations for these results. Cy depresses B lymphocytes more selectively, thus inducing antibody depletion (17) that could cause acute infection and sepsis. In fact, toxin absorption and bacterial

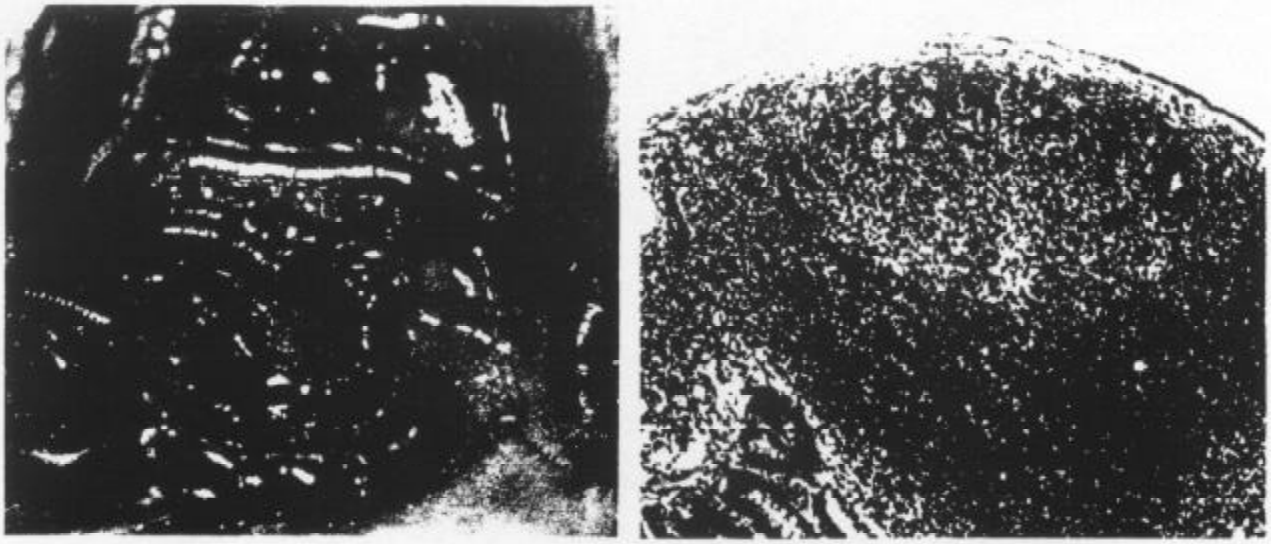

Fig. 3: Hypertrophic Peyer's patch following short-term reperfusion of the bowel (a). A mononuclear infiltrate is seen in the submucosa (b). HE, x100.

proliferation have been involved in ischemic damage of the bowel (18). On the other hand, Cy causes thrombopenia that, in association with coagulation disorders induced by ischemia, could increase the hemorrhagic trend developed during reperfusion. Unfortunately, we found no reference concerning Cy in experimental ischemia.

Considering all the above-mentioned findings and observations, we feel a modification of our original hypothesis should be proposed. It was asserted that the precocity of the cellular response observed during ischemia and reperfusion discarded T lymphocyte intervention, because these cells require an activating period (19); NK cells were thus proposed. However, results obtained with Cy require us to explore other possibilities. If we assume the free radical hypothesis (5-7), ischemic tissues generate highly reactive radicals that, via lipid peroxides [illegible] cell membrane; however, it is also proposed that this mechanism occurs in ageing (20). Thus, membrane antigen modification by lipid peroxides is a common event in the organism, and lymphocytes already activated against such modified antigens are usually available. If this is the case, then T lymphocytes should be the last effectors of ischemic/reperfusion injury and CsA should be useful in invalidating this step in pathogenesis. Moreover, assuming that free radicals and immune attack take place sequentially, free radical scavengers and CsA should act synergically. Accordingly, we are carrying out new investigations involving both types of treatment to verify this modified hypothesis.

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