Contribution of intercellular adhesion molecule 1 (ICAM-1) to the pathogenesis of splanchnic artery occlusion shock in the rat

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1 It has been suggested that leukocytes play a key role in the pathogenesis of splanchnic artery occlusion shock. Intercellular adhesion molecule 1 (ICAM-1) is an adhesion molecule of crucial importance in the phenomenon of leukocyte accumulation.

2 We investigated the involvement of ICAM-1 in the pathogenesis of splanchnic artery occlusion shock. Splanchnic artery occlusion (SAO) shock was induced in anaesthetized rats by clamping splanchnic arteries for 45 min. Sham-operated animals were used as controls. Survival time, serum tumour necrosis factor- α (TNF- α), white blood cell (WBC) count, mean arterial blood pressure, myeloperoxidase activity (MPO; studied as a quantitative means to assess leukocyte accumulation) and the responsiveness to acetylcholine of aortic rings were investigated. SAO shocked rats had a decreased survival time (90 ± 9.5 min, while sham-shocked rats survived more than 4 h), reduced mean arterial blood pressure, increased serum levels of TNF- α (201 ± 10 u ml⁻¹) and MPO activity in the ileum (0.15 ± 0.03 u $\times 10^{-3}$ per g tissue) and in the lung (1.9 ± 0.8 u $\times 10^{-3}$ per g tissue), leukopenia and reduced responsiveness to acetylcholine (ACh, 10 nM–10 μ M) of aortic rings.

3 Administration of monoclonal antibody raised against rat ICAM-1 significantly increased survival time (225 ± 9 min), reduced leukopenia and MPO activity both in the ileum (0.031 ± 0.003 u $\times 10^{-3}$ per g tissue) and in the lung (0.23 ± 0.03 u $\times 10^{-3}$ per g tissue), improved the cardiovascular changes and restored the responsiveness to ACh of aortic rings.

4 Our findings are consistent with an involvement of adhesion mechanisms *in vivo* in the pathogenesis of SAO shock and suggest that specific adhesion mechanisms, which support leukocyte accumulation, may represent potentially important therapeutic targets in circulatory shock.

Keywords: ICAM-1; splanchnic artery occlusion shock; leukocyte accumulation

Introduction

Splanchnic artery occlusion (SAO) shock is a lethal form of experimental shock that causes animal death within 80–95 min after the occlusive clamps have been released. It is due to prolonged ischaemia of the splanchnic region followed by reperfusion (Sturniolo *et al.*, 1988; Squadrito *et al.*, 1991) and it is also characterized by a profound fall in mean arterial blood pressure (Squadrito *et al.*, 1989), impairment in the activity of the reticuloendothelial system (Sturniolo *et al.*, 1989), enhanced macrophage and plasma levels of thromboxane B₂ (Squadrito *et al.*, 1992b), increased plasma concentration of myocardial depressant factor (MDF) and platelet activating factor (Zingarelli *et al.*, 1992b) and elevated macrophage and serum levels of tumour necrosis factor (TNF- α) (Squadrito *et al.*, 1992a). In addition we have shown that polymorphonuclear leukocytes play a key role in the pathogenesis of SAO shock: indeed leukopenia induced by administering vinblastine protects against the pathological sequelae of this experimental type of circulatory shock (Canale *et al.*, 1993).

Intercellular adherence seems to be important for leukocyte accumulation at sites of inflammation (Osborn, 1990), but recent evidence from our laboratory has suggested that adhesion mechanisms supporting leukocyte adhesion and accumulation are present in ischaemic states (Altavilla *et al.*, 1994).

It has been suggested that in endotoxin shock the administration of monoclonal antibodies against the CD11/CD18 complex reduces neutrophil accumulation (Arfors *et al.*,

1987; Harlan *et al.*, 1991). However, leukocyte accumulation is a complex phenomenon which also involves endothelium-based adhesion molecules.

In fact, endothelial adhesion molecules are considered to play a pivotal role in the localization and development of an inflammatory reaction (Butcher, 1990). Intercellular adhesion molecule 1 (ICAM-1) is an adhesion molecule normally expressed at a low basal level on endothelial cells, but its expression can be enhanced by various inflammatory mediators such as interleukin-1 (IL-1) and TNF- α (Wetheimer *et al.*, 1992). Structurally ICAM-1 is a member of the Ig supergene family with five extracellular Ig-like domains, a single transmembrane region and a short cytoplasmic tail (Simmons *et al.*, 1988). It is a ligand for at least two members of the CD 18 family of leukocyte adhesion molecules: LFA-1 or CD11a/CD18 and Mac-1 CD11b/CD18 (Marlin & Springer, 1987; Diamond *et al.*, 1990).

In the present study we investigated the involvement of ICAM-1 in the pathogenesis of SAO shock. Administration of monoclonal antibody raised against rat ICAM-1 was found to increase the resistance of rats to experimental shock, to reduce leukocyte accumulation and finally to restore impaired vascular reactivity.

Methods

Surgical procedures

Male Sprague-Dawley rats weighing 250–300 g were permitted access to food and water *ad libitum*. The rats were anaesthetized with urethane (1.3 g kg⁻¹, i.v.). After anaes-

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thetia, catheters were placed in the carotid artery and jugular vein. For monitoring blood pressure a cannula (PE 50) was inserted into the left common carotid artery as described elsewhere (Caputi *et al.*, 1980). The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiometer, and arterial blood pressure was displayed on a polygraph. Arterial blood pressure is reported as mean arterial pressure (MAP) in mmHg. After midline laparotomy, the coeliac and superior mesenteric arteries were isolated near their aortic origins. During this procedure, the intestinal tract was maintained at 37°C by placing it between gauze pads soaked with warmed 0.9% NaCl solution.

Rats were given heparin (1000 u kg⁻¹, i.v.) and were observed for a 30 min stabilization period prior to either splanchnic ischaemia or sham ischaemia. SAO shock was induced by clamping both the superior mesenteric artery and the coeliac trunk resulting in total occlusion of these arteries for 45 min. After this period of occlusion the clamps were removed. Following reperfusion the rats were observed for 240 min. Sham shocked rats were subjected to all the same surgical procedures as SAO shocked rats except that arteries were not occluded.

Survival evaluation

Three hours before the splanchnic artery occlusion shock procedures, treated rats received intravenously anti-rat ICAM-1 monoclonal antibody (1 mg kg⁻¹) dissolved in 0.3 ml of a phosphate-buffered solution at pH 7.4 and control rats received the carrier vehicle. Survival was evaluated for 4 h.

Biological assay for tumour necrosis factor- α activity

Killing of L929 mouse tumour cells was used to measure TNF- α levels in serum on the basis of a standard assay (Ruff & Gifford, 1980). L929 cells in RPMI 1640 medium containing 5% foetal calf serum were seeded at 3×10^4 cells per well in 96-well microdilution plates and incubated overnight at 37°C in an atmosphere of 5% CO₂ in air. Serial dilutions of serum (drawn at different time intervals) were made in a medium containing actinomycin D 1 μ g ml⁻¹ and 100 μ l of each dilution was added to the wells. On the next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% methanol) and 0.1 ml of 1% sodium dodecyl sulphate was added to each well to solubilize the stained cells. The absorbance of each well was read at 490 nm with a model BT-100 Microelisa Autoreader. The percentage of cytotoxicity was calculated as $[1 - (A_{490} \text{ of sample} / A_{490} \text{ of control})] \times 100$. One unit of TNF- α was defined as the amount producing 50% cell cytotoxicity. TNF- α content in the sample was calculated by comparison with a calibration curve performed with recombinant murine TNF- α (Nuclear Laser Medicine, Italy). To verify if the cytotoxicity tested was due to the presence of TNF- α or to other factors, we preincubated our samples for 2 h at 37°C with an excess of rabbit antirecombinant murine TNF- α polyclonal antibody (Nuclear Laser Medicine, Milan, Italy) or with control rabbit serum. Our results showed that cytotoxicity against L929 cells was completely neutralized by rabbit antirecombinant TNF- α polyclonal antibody but not by control rabbit serum.

Myeloperoxidase activity

Leukocyte accumulation was investigated using the activity of myeloperoxidase (MPO). MPO activity was determined in intestinal mucosa and in the left lung, as previously described (Squadrito *et al.*, 1993). The samples were obtained at 0 and 45 min before reperfusion (release of the arterial clamp) and at 80 min after reperfusion. The samples were first homogenized in a solution containing 20 mM potassium phosphate buffer (pH 7.4), 0.01 M EDTA, 50 u ml⁻¹ of a protease inhibitor (aprotinin) in proportions of 1:10 (W:v)

and then centrifuged for 30 min at 20,000 g at 4°C. The supernatant of each sample was then discarded and the pellet was immediately frozen on dry ice; freezing was continued for 14 h before sonication. After thawing, the resulting pellet was added to a buffer solution consisting of 0.5% hexacyltrimethylammonium bromide (Sigma Chemical Co., St. Louis, MO, U.S.A.) dissolved in 50 mM potassium phosphate buffered solution (pH 6) containing 30 u ml⁻¹ of protease inhibitor. Each sample was then sonicated for 1 min at intensity 2 and at a temperature of 4°C. After the sonication the samples were allowed to chill on ice for approximately 30 min, and then centrifuged for 30 min at 40,000 g at 4°C. An aliquot of the supernatant was then allowed to react with 0.167 mg ml⁻¹ δ -dianisidine dihydrochloride (Sigma Chemical Co.) and 0.001% H₂O₂, and the rate of change in absorbance was measured at 405 nm in a microtitre plate reader. MPO activity was defined as the quantity of enzyme degrading 1 μ mol of peroxide min⁻¹ at 25°C and was expressed in milliunits per gram weight ($u \times 10^{-3}$) of tissue.

Leukocyte count

Tail vein blood samples for the leukocyte count (Squadrito *et al.*, 1992a) were taken at different time intervals (0 and 45 min before occlusion, and 80 min after reperfusion). The number of leukocytes (WBC $\times 10^3$ /mm³) is shown as mean $\pm$ s.d.

Isolated aortic rings

Animals were killed 80 min after the start of reperfusion. Thoracic aortae were removed and placed in cold Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 25.0 and glucose 11.7. Then aortae were cleaned of adherent connective and fat tissue and cut into rings of approximately 2 mm in length. Rings were then placed under 1 g of tension in an organ bath containing 10 ml Krebs solution at 37°C and bubbled with 95% O₂ and 5% CO₂ (pH 7.4). All experiments were carried out in the presence of indomethacin (10 μ M) in order to exclude the involvement of eicosanoids and their metabolites. Developed tension was measured with an isometric force transducer and recorded on a polygraph (Ugo Basile, Torino, Italy). After an equilibration period of 60 min during which time the rings were washed with fresh Krebs solution at 15–20 min intervals and basal tension was readjusted to 1 g, the tissue was exposed to phenylephrine (PE, 100 nM). When the contraction was stable, the functional integrity of endothelium was assessed by a relaxant response to acetylcholine (ACh, 100 nM). The tissues were then washed occasionally for 30 min.

Endothelium-dependent relaxation was evaluated with cumulative concentrations of ACh (10 nM–1 μ M) in aortic rings precontracted with PE (100 nM). Relaxation of the rings was calculated as percentage decrease of contractile force.

Drugs

Acetylcholine chloride, phenylephrine hydrochloride and indomethacin were obtained from Sigma.

Monoclonal mouse antibody raised against rat ICAM-1 (clone IA 29, subclass: IgG₁) was purchased from British Biotechnology Products Ltd (Abingdon, UK). This antibody was found to inhibit rat neutrophil adherence to rat ICAM-1. Control rats were injected with a non-immune mouse serum dissolved in the carrier vehicle (0.3 ml of phosphate-buffered saline solution at pH 7.4). Testing for the presence of endotoxin (chromogenic *Limulus* amoebocyte lysate assay) revealed <1 pg ml⁻¹ endotoxin in both the immune and non-immune serum.

Statistical analysis

The difference between the means of two groups was evaluated with an ANOVA followed by Bonferroni's test and was considered significant when $P < 0.05$.

Results

Survival

Sham-shocked rats, treated either with the carrier vehicle or with anti ICAM-1 antibody, survived the entire 4 h observation period. In contrast, in rats treated with vehicle, SAO shock produced a profound shock state characterized by a high lethality and no rats survived at 2 h (survival time = 90 ± 9.5 min; $n = 8$). Administration of monoclonal antibody raised against ICAM-1 significantly protected rats from the lethality induced by splanchnic artery occlusion shock: in fact survival time in these animals was 225 ± 9 min ($n = 8$; $P < 0.001$).

Arterial blood pressure

Occlusion of the splanchnic arteries produced a marked increase in mean arterial blood pressure. Subsequently pressure decreased upon release of the occlusion (Figure 1). The administration of monoclonal antibody raised against ICAM-1 significantly blunted the reduction in mean arterial blood pressure (Figure 1).

Serum TNF- α

Serum levels of TNF- α were undetectable in sham-shocked rats treated either with the immune or non-immune serum. TNF- α was also undetectable during the occlusion period. In contrast, serum TNF- α was significantly enhanced at the end of the reperfusion period in SAO shocked rats (201 ± 10 u ml⁻¹). Treatment with anti ICAM-1 antibody did not modify TNF- α levels during reperfusion (209 ± 19 u ml⁻¹).

Myeloperoxidase activity

The kinetics of ileal and pulmonary leukocyte infiltration in SAO-shocked rats were determined by measurement of the myeloperoxidase (MPO) activity content in rats at different times: 0 and 45 min after occlusion and 80 min post superfusion. MPO levels were significantly increased in the ileum

Figure 1 Effects of anti ICAM-1 antibody (1 mg kg/i.v.) or non immune serum administration (0.3 ml/i.v. dissolved in a phosphate-buffered saline solution at pH 7.4) on mean arterial blood pressure (MAP) in rats subjected to splanchnic artery occlusion (SAO) shock or sham operated. Open circles = sham operated animals + non immune serum; closed circles = sham operated animals + anti ICAM-1 antibody; open squares = SAO shocked rats + non immune serum; closed squares = SAO shocked rats + anti ICAM-1 antibody. Each point represents the mean $\pm$ s.d. of eight experiments. * $P < 0.001$ vs SAO + non immune serum.

(0.15 ± 0.03 u $\times 10^{-3}$ per g tissue) and in the lung (1.9 ± 0.8 u $\times 10^{-3}$ per g tissue) at 80 min after reperfusion (Table 1) in shocked rats pretreated with the non-immune serum.

Administration of monoclonal antibody raised against ICAM-1 significantly lowered the increase in ileal and pulmonary MPO activity (Table 1).

Leukocyte count

The administration of either the immune or non-immune serum did not modify the leukocyte count in sham-shocked rats. In contrast, SAO shock produced a marked leukopenia. Our data show that the leukocyte count was markedly decreased at the end (80 min) of reperfusion. The administration of monoclonal anti ICAM-1 antibody significantly ameliorated leukopenia (Table 1).

Table 1 Effects of anti ICAM-1 or non immune serum on myeloperoxidase (MPO) activity of ileum and lung and on white blood cell count (WBC) of rats subjected to splanchnic artery occlusion (SAO) shock

	Basal 0	Time (min) Ischaemia 45	Reperfusion 80
MPO activity in ileum (U $\times 10^{-3}$ g ⁻¹ tissue)			
Sham + non-immune serum	0.018 ± 0.03	0.02 ± 0.05	0.02 ± 0.04
Sham + Ab anti-ICAM-1 (1 mg kg ⁻¹ i.v.)	0.021 ± 0.02	0.023 ± 0.05	0.023 ± 0.06
SAO + non-immune serum	0.035 ± 0.01	0.05 ± 0.02	0.15 ± 0.03
SAO + Ab anti-ICAM-1 (1 mg kg ⁻¹ i.v.)	0.033 ± 0.02	0.047 ± 0.01	$0.031 \pm 0.03^*$
MPO activity in lung (U $\times 10^{-3}$ g ⁻¹ tissue)			
Sham + non-immune serum	0.22 ± 0.7	0.2 ± 0.8	0.2 ± 0.5
Sham + Ab anti-ICAM-1 (1 mg kg ⁻¹ i.v.)	0.27 ± 0.5	0.25 ± 0.9	0.28 ± 0.8
SAO + non-immune serum	0.36 ± 0.4	0.65 ± 0.5	1.9 ± 0.8
SAO + Ab anti-ICAM-1 (1 mg kg ⁻¹ i.v.)	0.32 ± 0.3	0.5 ± 0.6	$0.23 \pm 0.3^*$
Circulating white cells ($\times 10^{-3}$ mm ⁻³)			
Sham + non-immune serum	11.2 ± 1.2	11.5 ± 1.0	11.0 ± 1.0
Sham + Ab anti-ICAM-1 (1 mg kg ⁻¹ i.v.)	11.1 ± 1.1	11.3 ± 1.2	11.1 ± 1.0
SAO + non-immune serum	11.4 ± 1.0	8.0 ± 0.5	3.5 ± 0.6
SAO + Ab anti-ICAM-1 (1 mg kg ⁻¹ i.v.)	11.0 ± 0.4	7.8 ± 0.3	$8.3 \pm 0.4^*$

Each point represents the mean $\pm$ s.d. of seven experiments. * $P < 0.001$ vs SAO + non immune serum.

Relaxant response to acetylcholine

Addition of PE (100 nM) to the organ bath contracted intact aortic rings (80–90% of the maximum response). These rings were relaxed in a concentration-dependent manner by ACh (10 nM–1 μ M). The maximum relaxation by 1 μ M ACh in aortic rings from shocked rats was significantly lower (Figure 2; $38 \pm 3.2\%$) in SAO shocked rats than in sham-shocked rats (Figure 2; $80 \pm 4\%$). Administration of monoclonal antibody raised against ICAM-1 significantly improved responsiveness to ACh (Figure 2; $90 \pm 5\%$ after 1 μ M ACh).

Discussion

It has been shown that leukocytes as well as endothelium express adhesion molecules and their expression is considered to play a pivotal role in the localization and development of an inflammatory reaction (Mackay & Imhof, 1993). The first step in leukocyte-endothelium interaction is the 'rolling' of the leukocytes, a signalling event that, by altering the conformation of the extracellular binding domain of these molecules, produces the activation of other adhesion molecules belonging to the integrin family and the immunoglobulin superfamily (Butcher, 1990; Mackay & Imhof, 1993). This second interaction results in the strong adhesion of leukocytes to the endothelium and provokes the transendothelial migration of leukocytes. This second event in leukocyte-endothelium interaction seems to be mediated by the adhesion molecule ICAM-1.

ICAM-1 has a molecular weight of 95 kD (Marlin & Springer, 1987). The binding molecules for ICAM-1 in neutrophils and monocytes are the integrin ligands LFA-1 and Mac-1 (Marlin & Springer, 1987; Diamond *et al.*, 1990).

De novo expression of ICAM-1 has been described on different cell types in inflammatory lesions, during rejection and on melanoma cells (Vejlsgaard *et al.*, 1989; Johnson *et al.*, 1989; Adams *et al.*, 1989). Experimental studies have also shown that antibodies against ICAM-1 inhibit leukocyte adhesion to endothelial cells, granulocyte migration through endothelium and mixed lymphocyte reactions *in vitro* (Dougherty *et al.*, 1987; Boyd *et al.*, 1988; Merluzzi *et al.*, 1989). In addition, *in vivo* administration of antibodies raised against ICAM-1 reduces neutrophil infiltration into inflamed lungs in the rabbit, non-human primate kidney and heart allograft rejection and it blunts Ag-induced airway eosinophil

influx and airway hyperresponsiveness (Barton *et al.*, 1989; Cosimi *et al.*, 1990; Flavin *et al.*, 1990; Wegner *et al.*, 1990).

Besides inflammation, recent evidence has suggested that the phenomenon of leukocyte adhesion and accumulation is involved in the mediation of myocardial reperfusion injury (Altavilla *et al.*, 1994), thus suggesting that adhesion molecules may also be important in ischaemic and/or low-flow states.

Circulatory shock is a low-flow state where leukocyte-endothelium interaction might play a key role. In fact we have previously suggested that leukocytes are deeply involved in the pathogenesis of SAO shock. Leukopenia, induced by administering vinblastine, dramatically increased the resistance of experimental animals to SAO shock (Canale *et al.*, 1993).

We therefore investigated whether ICAM-1 might be involved *in vivo* in mediating leukocyte accumulation in SAO shock. Our results shown that leukocytes significantly accumulate in the ileum and in the lung of SAO-shocked rats at the end of reperfusion. The kinetics of leukopenia and TNF- α appearance in the serum demonstrated that both pathological events occur at the end of reperfusion. Indeed a good relationship was noted between peak leukopenia, peak levels of TNF- α and maximum increase in leukocyte accumulation. Furthermore SAO shocked rats had high mortality.

Administration of monoclonal antibody raised against ICAM-1 increased the resistance of rats to the experimental procedures of SAO shock. Survival time in the group of rats given the non-immune serum was 90 ± 9.5 min, whereas rats given monoclonal anti ICAM-1 antibody showed enhanced survival (225 ± 9 min). Furthermore, the passive immunization with specific anti ICAM-1 antibodies reduced leukopenia and leukocyte accumulation in the ileum and in the lung. These data, taken together, strongly suggest that ICAM-1 is involved in the pathogenesis of SAO shock and, moreover, that this adhesion molecule significantly contributes, *in vivo*, to the mechanisms underlying leukocyte accumulation.

The mechanisms underlying the irreversible circulatory failure observed in shock are not yet well known. The involvement of the L-arginine/nitric oxide (NO) pathway in the vascular dysfunction that occurs in experimental shock has been proposed (Zingarelli *et al.*, 1992a; Nava *et al.*, 1992). As far as SAO shock is concerned, experimental pieces of evidence have pointed out that NO donors exert beneficial effects in feline SAO shock (Aoki *et al.*, 1990), thus suggesting that a dysfunction in the L-arginine/nitric oxide pathway (i.e. a decrease in NO production) might also be present in this type of experimental shock. In accordance with this hypothesis our present data show that aortic rings from SAO shocked rats had a markedly reduced responsiveness to vasorelaxant effects of ACh. Indeed this result indicates that NO generated by the endothelial constitutive NO synthase is blunted in SAO shocked rats. Adherence of leukocytes to the endothelium, by releasing cytokines such as TNF- α or oxygen-derived free radicals, may impair the release of NO from endothelial cells (Aoki *et al.*, 1989). Therefore therapeutic approaches that reduce leukocyte adherence to the endothelial cells are expected to restore the impairment in NO dysfunction. In agreement with this hypothesis, administration of monoclonal antibody raised against ICAM-1 markedly improved the responsiveness to ACh of aortic rings from SAO shocked rats by inhibiting leukocyte-endothelium interaction.

In conclusion, our study would suggest that the mechanism supporting the strong adhesion of leukocytes to endothelium (which is ICAM-1 dependent) is likely to be involved in the pathogenesis of SAO shock.

Figure 2 Relaxant effect of acetylcholine in aortic rings (contracted with phenylephrine, 100 nM) of sham operated rats (open squares), or splanchnic artery occlusion (SAO) shocked rats given either specific anti ICAM-1 antibody (1 mg/kg, i.v.) (open circles) or a non immune serum (0.3 ml/i.v. dissolved in a phosphate-buffered saline solution at pH 7.4) (closed circles). Bar heights represent the mean $\pm$ s.d. from seven experiments. * $P < 0.001$ vs SAO + non immune serum.

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