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The role of polymorphonuclear leukocytes and oxygen-derived free radicals in experimental acute pancreatitis: mediators of local destruction and activators of inflammation

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Abstract Using a retrograde infusion sodium taurocholate pancreatitis model in the rat treatment with oxygen radical scavengers or monoclonal anti-ICAM-1 antibody decreased tissue damage and polymorphonuclear leukocytes (PMN) infiltration. Scavengers or anti-ICAM-1 treatment attenuated the activating capacity of blood PMNs following zymosan stimulation. The local production of oxygen free radicals in the pancreas by systemic infusion of hypoxanthine and regional infusion of xanthine oxidase did not induce acute pancreatitis, although an increase of infiltrating PMNs was observed. Our data suggest that oxygen free radicals and infiltrating PMNs aggravate acute pancreatitis and that both are important mediators of local destruction and systemic activation of PMNs.

Key words: Oxygen radical; Pancreatitis; Polymorphonuclear leukocyte

1. Introduction

Many previous reports using experimental animal models of pancreatitis have suggested that oxygen free radicals play an important part in the initiation and development of pancreatitis.

The local release of oxygen-derived free radicals and the sequestration of inflammatory cells, particularly neutrophils, within the pancreas is generally believed to be an early and important event in the evolution of both pancreatitis and pancreatitis-associated organ complications. In recent studies, various chemo-attractant substances responsible for polymorphonuclear (PMN) accumulation within the pancreas during acute pancreatitis have been investigated [1–4].

One of the molecules which is often thought to play an important role in the generation and enhancement of acute pancreatitis are oxygen-derived free radicals [5–9]. Oxygen radicals can be generated by different systems in acute pancreatitis. One source is the inflamed hypo-oxygenated pancreatic tissue, the other source are the PMNs, which infiltrate the inflamed pancreatic tissue at very early time points of acute necrotizing pancreatitis.

The aim of our study was to evaluate the role of oxygen-derived free radicals on the one hand and the influence of PMN leukocytes in acute necrotizing experimental pancreatitis on the other. We therefore induced necrotizing pancreatitis in rats by retrograde infusion of sodium taurocholate. In order to investigate the effects of oxygen radicals, the scavengers catalase (CAT) and superoxide dismutase (SOD) were administered prior and in parallel to taurocholate infusion. The effects of PMN leukocytes were evaluated by infusion of anti-ICAM-1 monoclonal antibody (mAb).

2. Material and methods

2.1. Experimental procedures

Male Wistar rats (285 ± 54 g) were acclimated for at least 1 week before use. Studies were performed in accordance with the national guidelines for the use and care of laboratory animals and approved by the local animal care and use committee. Rats were anesthetized with halothane (Fluothane, Zeneca, Plankstadt, Germany) after receiving 0.15 mg/kg body weight buprenorphine subcutaneously (Temgesic, Grünethal, Aachen, Germany). A 26 gauge polyethylene catheter (Abbocath-T, Abbott, Wiesbaden, Germany) was inserted in the abdominal aorta and advanced to the origin of the celiac artery for continuous regional arterial (CRA) perfusion of the pancreas. In some rats, selective perfusion of the pancreas was confirmed by fluorescein injection. For CRA perfusion longer than 20 min, the abdomen was partially closed. After completion of the CRA perfusion, the catheter was removed. For intravenous application of hypoxanthine (HX), an additional 24 gauge polyethylene catheter (Insyte, Beckton Dickinson, Heidelberg, Germany) was inserted into the inferior vena cava. Rats killed at 3 h or later were allowed to recover from anesthesia.

Acute necrotizing pancreatitis was induced by a standardized retrograde infusion of 0.1 ml/100 g body weight of a freshly prepared 3% sodium taurocholate solution (Sigma-Aldrich Chemie, Steinheim, Germany) into the biliopancreatic duct as previously described [5].

2.2. Experimental groups

Rats were randomly assigned to one of the following experimental groups. After observation periods of 30 min, 3, 6, 12 and 24 h following induction of pancreatitis, blood was drawn for functional assays, serum was asservated and the pancreatic tissues were snap frozen or fixed in formalin. Each group consisted of five rats.

2.3. Pancreatitis group

Fifteen minutes prior to induction of acute necrotizing pancreatitis, rats received CRA perfusion with isotonic saline at a flow rate of 1 ml/h/100 g body weight, which was continued for a maximum of 60 min after pancreatitis induction.

2.4. Pancreatitis scavenger group

Fifteen minutes prior to induction of acute necrotizing pancreatitis, rats were CRA-perfused with SOD (bovine SOD, Boehringer Mannheim, Mannheim, Germany) at a dosage of 100 000 U/kg body weight/h and a flow rate of 1 ml/h/100 g body weight, which was again continued for a maximum of 60 min following pancreatitis induction. Animals observed for 3 h or longer received a CRA bolus injection of 200 000 U CAT (bovine CAT, Boehringer Mannheim, Mannheim, Germany) both at the beginning and the end of the CRA perfusion period. Rats killed at 30 min received only one bolus injection of 200 000 U at the beginning of the CRA perfusion.

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2.5. Oxidative stress group

Xanthinoxidase (XOD) (bovine XOD, Boehringer Mannheim, Mannheim, Germany) was applied at a dosage of 4 U/h and a flow rate of 0.5 ml/h/100 g body weight over the CRA catheter. After 5 min of XOD perfusion, continuous intravenous infusion of 10 mM HX (Sigma-Aldrich Chemie, Steinheim, Germany) was started with the same flow rate.

2.6. Sham-operated group

(SHX group, $n=40$): Animals of this group received retrograde infusion of isotonic saline instead of 3% sodium taurocholate into the biliopancreatic duct after 15 min of CRA perfusion with isotonic saline.

2.7. Anti-ICAM group

Anti-ICAM-1 mAb 1 mg/kg/h (1A29, Serotec, Oxford, UK) treatment was applied by continuous regional perfusion of the upper mesenteric artery, through a 26 G Abbocath catheter (Abbot, Sligo, Ireland) placed in the aortic artery. The infusion was given for 1 h. The onset was 15 min before the induction of the acute necrotizing pancreatitis.

2.8. Measurements

Oxidized glutathione (GSSG) concentrations were determined using a kinetic method described by Brehe and Burch [10], modified by Griffith [11]. Malondialdehyde (MDA) was determined according to the method of Ohkawa et al. [12]. Myeloperoxidase (MPO) was measured as described by Bradley et al. [13]. Tissue concentrations of the respective parameters were related to the protein content in the pancreatic samples.

2.9. Light microscopy

Tissue samples comprising about half of both the duodenal and splenic part of the pancreas were fixed in 4% phosphate-buffered formalin for 24 h and embedded in paraffin. Five μm thick sections were stained with hematoxylin/eosin and examined and graded in a blinded fashion as described below.

2.10. Assessment of cell damage and inflammatory infiltrate

Cell damage was defined as zymogen degranulation, cytoplasmic vacuolization or shrinkage, loss of the basal basophilic/apical acidophilic staining of the cytoplasm and pyknosis of acinar cell nuclei. At least 1000 random acinar cells per each histological section were assessed at a magnification of 160×. The frequency of cell damage is given as percentage.

Inflammatory cells were quantified by analyzing at least 10 random fields containing inter- or intralobular blood vessels at a magnification of 400×. The numbers of inflammatory cells (PMN leukocytes, lymphocytes and monocytes) within the vascular lumen, marginated/adherent to the endothelium, and in the perivascular pancreatic tissue were counted in each high power field. The inflammatory infiltrate is presented as the mean number of inflammatory cells per high power field counted separately for each of the three compartments.

2.11. Zymosan stimulation

For analysis, the volume of 50 μl cell suspension (5×10^5 cells) was added to 150 μl of RPMI 1640 without phenol red (Life Technologies, Renfrewshire, UK). Signal amplification was achieved by the addition of 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol, Sigma Chemicals) in a concentration of 20 μM. Two wells of PMN granulocytes were stimulated with Zymosan (Sigma Chemicals) in the final concentration of 1 mg/ml and at least another two wells were stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma Chemicals) in the final concentration of 1 μM/ml. The autoluminescence of luminol at the time of the achieved peak values in the wells with stimulated PMN granulocytes was subtracted and the obtained values were compared.

2.12. Statistics

All values are presented as means and S.E.M. In all instances, P values <0.05 at an $\alpha<0.05$ were considered significant. Statistical calculations were done using the GraphPad Prism software package Version 3.0 by GraphPad Software (San Diego).

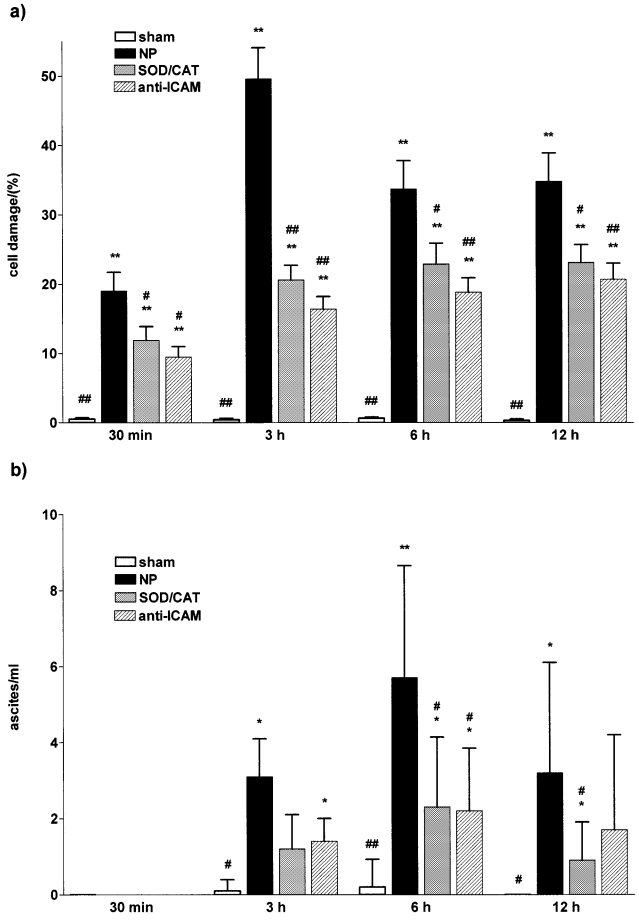

Fig. 1. Relative amount of death cells in the pancreatic tissue (a) and the produced intraperitoneal ascites (b) 30 min, 3, 6 and 12 h after the induction of necrotizing pancreatitis. The pancreatitis group (NP) is compared to sham-operated rats (sham) and rats treated with scavengers (SOD/CAT) or anti-ICAM (anti-ICAM). *: $P<0.05$ and **: $P<0.01$ vs. sham. #: $P<0.05$ and ##: $P<0.01$ vs. NP.

3. Results

3.1. Anti-ICAM-1 infusion and administration of scavengers reduce local damage in acute necrotizing pancreatitis

In comparison to tissue damage in acute necrotizing pancreatitis, the number of dead cells was significantly reduced when anti-ICAM-1 or scavengers were added (Fig. 1a). Thirty minutes, 3, 6 and 12 h following induction of acute necrotizing pancreatitis, these differences were statistically significant. In the anti-ICAM-1 as well as the scavenger group 3 h after induction, the tissue damage was reduced to half (Fig. 1a). Twenty-four hours following induction of acute pancreatitis, the cell damage was nearly the same in all three groups. Similar to the tissue damage, the ascites amount was significantly reduced in these two groups as compared to the sodium taurocholate infusion alone (Fig. 1b).

The addition of scavengers did also reduce the amount of MDA (Fig. 2). Interestingly, also treatment with anti-ICAM-1 antibody exerted these effects, pointing to a partial liberation of oxygen-derived free radicals in acute pancreatitis by infiltrating PMN leukocytes.

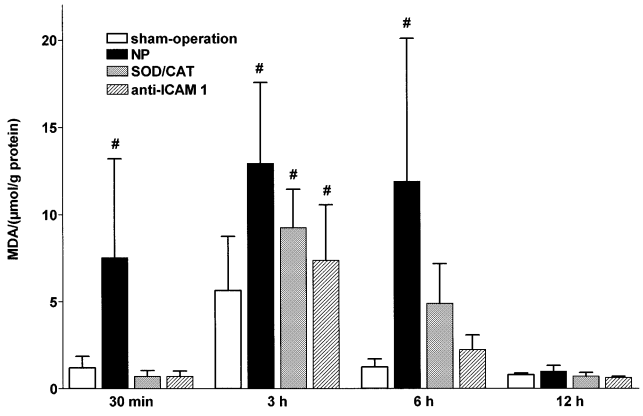

Fig. 2. Concentration of MDA in the pancreatic tissue 30 min, 3, 6 and 12 h after the induction of necrotizing pancreatitis. The pancreatitis group (NP) is compared to sham-operated rats (sham) and rats treated with scavengers (SOD/CAT) or anti-ICAM (anti-ICAM). #: $P < 0.05$ vs. NP.

3.2. *Anti-ICAM-1 infusion and administration of scavengers reduce PMN leukocyte infiltration in acute necrotizing pancreatitis*

Determination of the number of infiltrating cells revealed that the number of PMN leukocytes was significantly reduced when the animals were treated with anti-ICAM-1 or scavengers. These differences were especially visible 6, 12 and 24 h following the induction of acute pancreatitis (Fig. 3a). These data were confirmed by the evaluation of MPO concentrations of the tissues, which have been shown to be an accurate marker for the infiltration of PMN leukocytes (Fig. 3b).

3.3. *The pre-activation of blood PMN leukocytes in acute necrotizing pancreatitis is attenuated by anti-ICAM-1 and scavenger treatment*

In order to evaluate the influence of anti-ICAM-1 and scavenger treatment to the activation status of peripheral blood PMN leukocytes, we determined at 6 and 24 h following induction of acute necrotizing pancreatitis the response of peripheral blood to the stimulation with zymosan. The responsiveness following zymosan stimulation of peripheral blood after 24 h was the highest in acute necrotizing pancreatitis (244 ± 16 U) and significantly diminished when the animals were treated with anti-ICAM-1 (162 ± 28 U) or scavengers (156 ± 20 U).

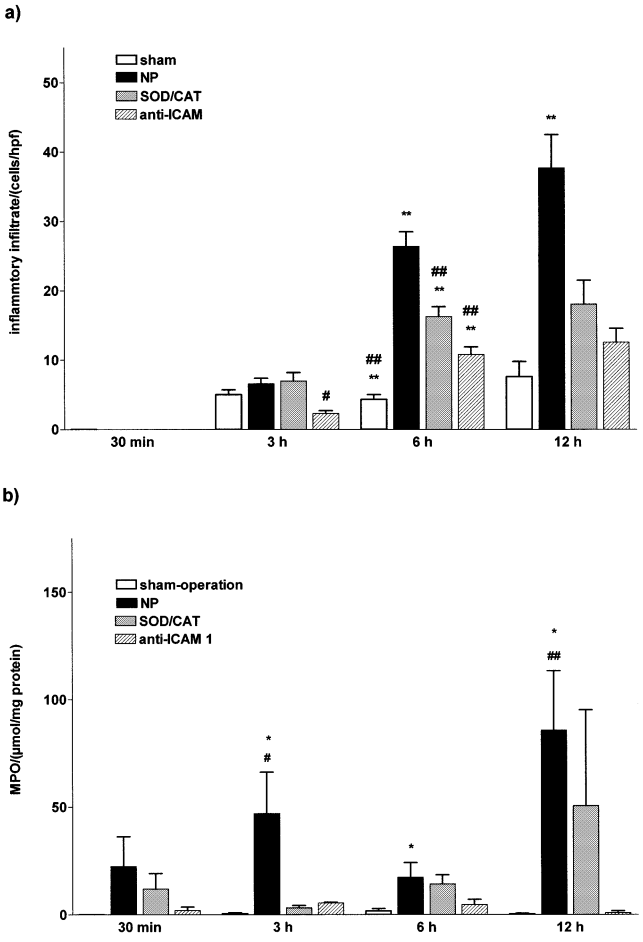

Fig. 3. Amount of inflammatory infiltrating cells per high power field (hpf) (a) and the concentration of MPO in the pancreatic tissue (b) 30 min, 3, 6 and 12 h after the induction of necrotizing pancreatitis. The pancreatitis group (NP) is compared to sham-operated rats (sham) and rats treated with scavengers (SOD/CAT) or anti-ICAM (anti-ICAM). *: $P < 0.05$ vs. initial values. #: $P < 0.05$ and ##: $P < 0.01$ vs. NP.

3.4. *Oxygen-derived free radicals do not induce acute necrotizing pancreatitis but recruit PMN leukocytes in acute necrotizing pancreatitis*

In order to evaluate whether oxygen radicals generated in the pancreas induce an acute pancreatitis, we infused systemically HX and administered locally xanthine oxidase into the celiac trunc. The oxidation-related changes as expressed in the

Table 1
GSSG, MDA and cell damage expressed by the relative amount of death cells in the pancreatic tissue, intraperitoneal ascites and the reaction of leukocytes to zymosan stimulation 30 min, 6 and 24 h after induction of necrotizing pancreatitis

	30 min		6 h		24 h	
	NP	HX/XO	NP	HX/XO	NP	HX/XO
GSSG (µmol/g protein)	030 ± 0.1	1.1 ± 0.07[a]	0.25 ± 0.1	0.17 ± 0.03	0.48 ± 0.05	0.19 ± 0.03[b]
MDA (µmol/g protein)	7.5 ± 5.7	2.7 ± 2.3	11.9 ± 8.9	1.8 ± 0.5	9.6 ± 7.3	0.9 ± 0.6
Cell damage (%)	19 ± 2.7	0.0 ± 0.0[b]	33.7 ± 4.1	0.0 ± 0.0[b]	27.5 ± 5.0	0.0 ± 0.0[b]
Ascites (ml)	0.0 ± 0.0	0.0 ± 0.0	5.7 ± 1.0	0.0 ± 0.0[b]	1.6 ± 2.5	0.0 ± 0.0[a]
Zymosan stimulation (U)	148 ± 19	114 ± 12	214 ± 21	209 ± 11	244 ± 16	133 ± 15[b]

The necrotizing pancreatitis group (NP) is compared to the oxidative stress group (HX/XO), induced by local generation of free radicals within the pancreatic tissue.
[a] $P < 0.05$ HX/XO vs. NP.
[b] $P < 0.01$ HX/XO vs. NP.

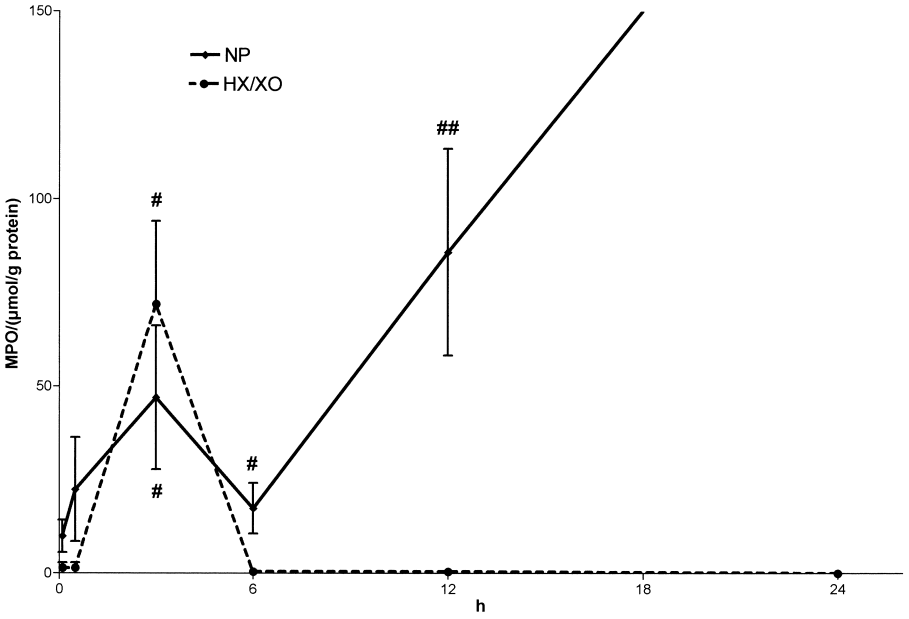

Fig. 4. Course of MPO in the pancreatic tissue within 24 h after induction of necrotizing pancreatitis. The pancreatitis group (NP) is compared to rats with pancreas exposed locally to free radicals (HX/XO). #: $P < 0.05$ and ##: $P < 0.01$ vs. initial values.

amount of MDA and GSSG were comparable to those produced in acute necrotizing pancreatitis (Table 1). However, we did not observe any tissue damage or ascites formation. Interestingly, 3 h following administration of HX/xanthine oxidase, an accumulation of PMN leukocytes was observed in the pancreatic tissue. This PMN leukocyte infiltration was confirmed by a significantly increased amount of MPO (Fig. 4). Six hours following stimulation, the amount of infiltrating PMN leukocytes returned to normal values, indicating that oxygen radicals generated in the pancreas in similar concentrations as they are produced during acute necrotizing pancreatitis act as PMN leukocyte attractants but are not able to induce acute necrotizing pancreatitis themselves. This is underlined by the responsiveness of PMNs following zymosan stimulation of peripheral blood after 24 h, which was unchanged in the xanthine oxidase group.

4. Discussion

The role of oxygen-derived free radicals in experimental pancreatitis has been intensively studied during the past years. Several groups provided evidence that during the initiation and manifestation of experimental acute pancreatitis, large amounts of oxygen radicals are produced [6,7,9,14,15]. With regard to the prevention of local damage in acute pancreatitis, several experimental animal systems have been investigated. Steer and colleagues showed that in cerulein pancreatitis, the local edema can be prevented by scavenging therapies [15] and our group has also recently shown that lipid peroxidation, zymogen degranulation and tissue necrosis can be sufficiently inhibited by the infusion of the radical scavengers SOD and CAT in cerulein-induced pancreatitis of the rat [16]. Also in experimental acute necrotizing pancreatitis, represented in the retrograde infusion of sodium taurocholate, oxygen free radical-mediated damage was shown [14]. Possible reasons for this local overproduction seem to be an increase of endogenous enzymes producing oxygen radicals like xanthine oxidase [7], together with a downregulation of endogenous scavenging systems like SOD or CAT [17]. Further reasons for an increase in oxygen radical production are thought to be ischemia reperfusion processes [18] and the release of oxygen free radicals by infiltrating PMN leukocytes.

Besides the local toxic effects, oxygen radicals seem to be involved in a concerted role of the release of platelet activating factor (PAF) and leukotriene B4 (LTB4), which results in PMN leukocyte accumulation. In blocking experiments, Hotter and colleagues have recently shown that scavenging oxygen free radicals in acute experimental pancreatitis reduces tissue damage as well as the release of the leucotactic substances LTB4 and PAF results in decreased infiltration of PMN leukocytes [19].

In our study, we have shown that scavenging therapy using SOD and CAT can prevent local tissue damage to a certain extent as shown by reduced tissue edema, ascites and tissue necrosis. Since sufficient prevention of PMN infiltration into the inflamed pancreatic tissue by anti-ICAM-1 treatment also reduced these changes and the concentrations of lipid peroxidation products, it can be assumed that a substantial portion of free radicals in acute pancreatitis is due to the release by PMN leukocytes. Interestingly, treatment with scavengers or anti-ICAM-1 antibody also attenuated the release of oxygen radicals by peripheral blood PMNs following zymosan stimulation 24 h after initiation of acute pancreatitis, suggesting that the local reduction of tissue damage by scavenger or anti-ICAM-1 treatment attenuated the systemic activation of peripheral blood PMNs. This hypothesis is also supported by the observations of Inoue and colleagues who found an attenuated release of superoxide anion production in peritoneal PMMs in rats with acute pancreatitis following anti-ICAM-1 treatment [20], Folch and colleagues who found that scavenger treatment reduced systemic complications in acute pancreatitis in rats like the infiltration of PMNs into the lungs [21], Frossard and colleagues who found that ICAM-1 plays an important, neutrophil-mediated, pro-inflammatory role in

experimental pancreatitis and pancreatitis-associated lung injury [22] and finally by the observation of Tsuji and colleagues that peripheral blood PMNs produce higher amounts of oxygen radicals following PMA stimulation in human necrotizing pancreatitis [23].

In order to test the hypothesis whether oxygen radicals themselves are able to induce acute pancreatitis in rats, we chose a combination between locally administered xanthine oxidase and parallel systemic infusion of HX. Using this system, we observed a local oxygen radical production in the pancreas comparable to that seen in sodium taurocholate pancreatitis, as determined by the concentrations of lipid peroxidation products and especially of GSSG. Using this system, we observed no local changes in the pancreas. However, 3 h following infusion of HX and xanthine oxidase, we observed an infiltration of PMNs in the pancreas as determined by light microscopy and determination of MPO concentrations. Interestingly, this PMN accumulation did not lead to local damage, which implies that besides the leucotactic effects of oxygen radicals, further locally released stimuli are required for the activation of the infiltrating PMN leukocytes.

In conclusion, we have shown that scavenger as well as anti-ICAM-1 treatment reduce the local tissue damage in experimental acute pancreatitis and that these treatments also reduce the activation of peripheral blood PMNs. Furthermore, we showed that oxygen radicals produced during acute pancreatitis do not lead themselves to acute pancreatitis but increase the local tissue damage and act as PMN attractants in acute pancreatitis.

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