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A link between extracellular reactive oxygen and endotoxin-induced release of tumour necrosis factor α *in vivo*

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Abstract—Pretreatment with the reactive oxygen species scavengers superoxide dismutase (SOD) and catalase or with the xanthine oxidase inhibitor allopurinol protected mice against hepatitis induced by the combined administration of lipopolysaccharide (endotoxin) and D-galactosamine. In the sera of protected animals no tumor necrosis factor (TNFα) was detectable in contrast to abundant amounts in the sera of injured control animals. A similar protection by the suppression of systemic TNFα was observed following the pretreatment of mice with polystyrene-coupled SOD prior to endotoxic challenge. Both pretreatments were ineffective when hepatitis was evoked by administration of the mediator TNFα instead of endotoxin. These findings indicate that the formation of extracellular reactive oxygen species is a condition needed to induce the release of TNFα and thus to mediate endotoxin-induced toxicity.

Administration of various bacterial endotoxins, i.e. lipopolysaccharides, to mammals leads to shock and death caused by multi-organ failure (for review see Refs 1 and 2). Treatment of mice with D-galactosamine (GalN*) sensitizes the liver of these animals towards subtoxic doses of endotoxin resulting in a fulminant hepatitis [3].

In contrast to liver injuries caused via lipid peroxidation, e.g. inducible by paracetamol or carbon tetrachloride, GalN/endotoxin-hepatitis does not implicate direct oxidative tissue degradation. It rather depends on the release of potent mediators such as TNFα [4]. Based on earlier findings [5–7], we assume the following sequence of mediators leading to hepatitis upon endotoxin challenge of GalN-sensitized mice: endotoxin $\rightarrow$ LTD_4 $\rightarrow$ROS $\rightarrow$ TNFα $\rightarrow$ hepatitis. Accordingly, direct intravenous administration of either LTD_4 [5] of TNFα [6] to GalN-sensitized mice induced a liver injury which was indistinguishable from the one seen after administration of endotoxin. We reported in a previous study that administration of SOD, catalase or allopurinol protected against endotoxin-toxicity in this model [8]. Since we considered it unlikely that ROS have a direct effect on cytokine release, we interpreted our findings in terms of a reversible and transient ischemia/reflow episode caused by the local vasoconstrictor LTD_4 as a source of enzymatically generated ROS during reflow [9].

We report now that either removal of ROS or block of their enzymatic production suppresses endotoxin-induced secretion of TNFα into the circulation. Previous *in vivo* findings in our laboratory indicated that the sequestration of TNFα upon endotoxin-stimulus depends on the activity of serine-protease sensitivity to inhibition by α_1-PI [10]. On this basis we propose a mechanism for the proteolytic processing of TNFα which is triggered by the oxidative inactivation of an antiprotease.

Materials and Methods

Animals and experimental design. In male NMRI mice (30 g) liver injury was induced in two alternative models by administration of endotoxin or TNFα. Details are given in the legends to the Tables and in Refs 6 and 8.

Reagents. GalN · HCl was purchased from Serva (Heidelberg, F.R.G.), lipopolysaccharide *Salmonella abortus equi* from Sebak (Aidenbach, F.R.G.), catalase (suspension from beef liver) from Boehringer Mannheim (F.R.G.) and allopurinol from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Bovine recombinant SOD was from Grünenthal (Aachen, F.R.G.). Polystyrene-coupled SOD was synthesized as described previously [11]. Murine recombinant TNFα was a generous gift by Dr Müller-Neumann, Knoll AG (Ludwigshafen, F.R.G.).

Assays. TNF in the serum of mice was determined as described in Ref. 2 using a bioassay with a TNF-sensitive fibrosarcoma cell line (WEHI 164 clone 13, kindly provided by Dr T. Espevik, Trondheim, Norway). Recombinant murine TNFα was used as a standard. Specificity of the TNFα signal was checked by neutralization of cytotoxic activity with an anti-murine TNFα antiserum. Plasma ALT activities were determined according to Ref. 3.

Statistics. The results were analysed according to Student's *t*-test and are given as mean values ± SEM. $P \leq 0.05$ was considered to be significant.

Results and Discussion

Administration of endotoxin to GalN-sensitized mice led to a severe liver injury as assessed by measurement of the plasma activities of the liver-specific enzyme ALT which reached massive levels after 8 hr before lethality started. Shock doses of endotoxin (i.e. 100 μg/animal) are known to result in the release of TNFα into the circulation [14]. This TNFα release also occurred in GalN-sensitized mice which had received as little as 150 ng endotoxin. The serum TNF concentrations increased from $\leq$50 pg/mL 45 min after endotoxin administration to reach a peak of 7000 pg/mL 90 min after endotoxin administration. Subsequently, the TNF concentration decreased to undetectable amounts within the following 3 hr. Administration of TNFα instead of endotoxin to GalN-sensitized mice caused similar liver damage and induced lethality [6] in our animals. In comparison to the endotoxin model, the time-course of enzyme release was shifted towards earlier times by about 1 hr (data not shown).

We reported previously [7] that intravenous administration of either SOD or catalase protected GalN-sensitized mice against endotoxin-induced hepatitis. However, both pretreatments were ineffective when hepatitis was induced by the administration of TNFα to GalN-sensitized mice. These observations indicate that the interaction point of ROS in the pathomechanism of this liver injury is localized subsequent to endotoxin and prior to TNFα action sites.

Therefore, we investigated whether antioxidant pretreatment would affect the systemic concentrations of TNFα upon endotoxin challenge. The results in Table 1 show that administration of any of these compounds suppressed completely endotoxin-induced TNFα-secretion (right-hand column) and protected against liver injury (left-hand column). A similar protection pattern was obtained when allopurinol had been administered before challenge. Regardless of an assumed xanthine-oxidase inhibition [9] by this compound or a direct ROS scavenging potency [15] of the drug, these findings demonstrate that the antioxidant treatment protects against endotoxin but not against TNFα. In view of the known cross-tolerance of endotoxin and TNF, a possible protection due to endotoxin contamination in the injected agents can be excluded by the lack of protection against TNF. The conclusion is, then, that reactive oxygen species are needed to provide bioactive TNFα which is responsible for the organ injury. This interpretation extends our previous one, i.e. that successful intervention prevented endotoxin-induced, LTD_4-mediated [9] ischemia/reperfusion and concomitant generation of ROS by linking the processing of TNFα to the generation of ROS.

In order to localize the compartment of ROS activity enabling synthesis or secretion of bioactive TNFα, we carried out analogous experiments using site-directed, polystyrene-coupled SOD [11] administered intravenously prior to endotoxic challenge. As shown in Table 2, pretreatment of mice with polystyrene-coupled SOD protected against GalN/endotoxin-induced hepatitis by suppression of systemic TNFα. Since intravenously injected polystyrene-coupled SOD circulates primarily as an albumin-bound adduct with a markedly prolonged plasma half-life of 6 hr [11], we conclude that extracellular ROS contribute to the mechanism providing bioactive TNFα.

The question remains as to by which mechanism ROS might have led to TNF formation. Since we showed previously that leukocyte activation is an epiphenomenon rather than a causal event in our model [16], a simple block of leukocyte activation cannot explain our results. A rationale is possible if the following facts are taken into account: (1) A 26-kDa transmembrane TNFα exists on the surface of stimulated mononuclear cells known to secrete 17-kDa TNFα into the circulation [17]. (2) Endotoxin-induced TNFα secretion requires *in vivo* the activity of a α_1-PI-sensitive protease [10]. (3) Under inflammatory conditions, i.e. stimulated and phagocytosing leukocytes, i.e. during oxidative burst [9], this antiprotease becomes oxidative inactivated [15]. (4) Low molecular weight serine protease inhibitors blocked the *in vitro* TNF release from

* Abbreviations: α_1-PI, α_1-proteinase inhibitor; ALT, alanine amino transferase (EC 2.6.1.1); GalN, D-galactosamine; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF, tumor necrosis factor; LTD_4, leukotriene D_4.

Table 1. Serum TNF levels in mice protected against GalN/endotoxin-induced hepatitis by pretreatment with allopurinol, catalase or SOD

Pretreatment	ALT (U/L)	TNF (pg/mL)	M/N
None	4320 ± 640	4940 ± 620	3/6
Allopurinol (100 mg/kg)	180 ± 40*	≤50	0/6
Catalase (10^6 U/kg)	95 ± 45*	≤50	0/6
SOD (3.3×10^4 U/kg)	75 ± 20*	≤50	0/6

* $P \leq 0.05$ vs hepatic injury control (no pretreatment prior to administration of 700 mg/kg GalN plus 5 μg/kg endotoxin). Allopurinol was administered intraperitoneally. Catalase or SOD was given intravenously 1 hr prior to induction of liver injury. Plasma ALT was determined 8 hr after challenge, serum TNF concentrations 90 min after GalN/endotoxin administration. Data: means ± SE; N = number of animals, M = number of animals which died within 8 hr.

Table 2. Plasma ALT and serum TNF concentrations in mice protected against GalN/endotoxin-induced hepatitis by pretreatment with polystyrene-coupled SOD

Pretreatment	ALT (U/L)	TNF (pg mL)	M/N
None	3150 ± 520	2320 ± 1010	4/6
Immob. SOD 5 mg/kg	5310 ± 1560	1870 ± 850	1/6
Immob. SOD 10 mg/kg	180 ± 40*	≤50	0/6
Immob. SOD 20 mg/kg	210 ± 30*	≤50	0/6
Carrier of Immob. SOD	3210 ± 1530	2670 ± 880	3/6

* $P \leq 0.05$ vs injury. SOD (linked to poly(styrene-co-maleic acid) butylester) (Immob. SOD) was administered intravenously as a suspension in phosphate-buffered saline 1 hr prior to GalN/endotoxin. Mice of the carrier control group received an equivalent amount of poly(styrene-co-maleic acid) butyl-ester. Blood for determination of serum TNF was withdrawn by puncture of the retro-orbital plexus 90 min after GalN/endotoxin administration. Liver injury was assessed by measurement of plasma ALT 8 hr after challenge. Data: means ± SE, N = number of animals, M = number of animals which died within 8 hr.

mononuclear blood cells stimulated with phorbol ester [19]. With these presumptions the protection by antioxidant treatment as observed in this study can be explained by the assumption that upon neutralization of ROS, α_1-PI remains in an active state and thus prevents proteolytic processing of the 26-kDa TNFα. These findings open an alternative perspective as to the involvement of ROS in inflammatory processes by linking the extracellular oxidant/antioxidant ratio to the protease/antiprotease balance which in turn controls the activation of potent inflammatory mediators such as TNFα.

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Modulation of melphalan uptake in murine L5178Y lymphoblasts *in vitro* by changes in ionic environment

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Abstract—The alkylating agent melphalan is actively transported in mammalian cells by two amino acid transport carriers: the sodium-dependent carrier with substrate preference for alanine-serine-cysteine (system ASC), and a sodium-independent carrier with preference for leucine (system L). The effect of altering the ionic environment of murine L5178Y lymphoblasts was investigated in order to determine not only the direct effects of hydrogen and calcium ions on these transport systems, but also the indirect effects of agents or modulators known to alter intracellular calcium. Melphalan transport followed a bell-shaped distribution curve over a pH range from 3 to 9 with a pH optimum of 4.3 and 4.6 for transport by systems ASC and L, respectively. Those agents that could cause a decrease in cytosolic calcium such as the calcium channel blockers verapamil, diltiazem and nitrendipine, the calcium chelator (ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) and reduction of pH were found to augment melphalan uptake, whereas conditions that would elevate intracellular calcium such as the calcium ionophore A23187, the calcium channel agonist (−) Bay K 8644, elevation of extracellular calcium and the calcium pump inhibitor trifluoperazine were all found to decrease melphalan uptake. These findings suggest that modification of ionic environment directly or indirectly by agents known to alter intracellular calcium can modulate melphalan uptake.

Melphalan influx is an active process mediated by two separate neutral amino acid transport carriers [1, 2], whereas melphalan efflux apparently occurs by passive diffusion [3]. One amino acid transport carrier (system ASC*) is sodium dependent, whereas the other (system L) is sodium independent. However, the effect of altering the concentration of other extracellular ions on melphalan uptake has not been investigated. In addition, the calcium channel blocker verapamil has been reported to enhance the cytotoxicity of melphalan against murine bone marrow and fibrosarcomas, and this appeared to be due, at least in part, to increased melphalan uptake [4]. Accordingly, the following investigation was undertaken to evaluate the direct effects of manipulation of the ionic environment, as well as the indirect effects of agents or modulators of intracellular calcium, on melphalan uptake by murine L5178Y lymphoblasts.

* Abbreviations: ASC, alanine-serine-cysteine; L, leucine; EGTA, ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; BCH, 2 aminobicyclo-[2.2.1]heptane-2-carboxylic acid; and BSS, balanced salt solution.

Materials and Methods

Drugs and chemicals. Melphalan [L-*p*-(di-2-chloro[^{14}C]-ethylamino)phenylalanine], specific activity 9.9 or 14.2 mCi/mmol, was synthesized by M. Leaffer of the Stanford Research Institute, Menlo Park, CA; the radiochemical