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Hepatotoxicity of ricin, saporin or a saporin immunotoxin: xanthine oxidase activity in rat liver and blood serum

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Abstract Male Wistar rats each received an i.p. injection of the ribosome-inactivating proteins ricin or saporin, or a Ber-H2 (anti-CD30)-saporin immunotoxin at a dose corresponding to three times the LD_{50} calculated for mice. Animals were killed 24, 48 or 72 h after treatment. Histological examination showed hepatic necrosis in all treated animals, although the sinusoidal lining was affected only in ricin-poisoned rats. The activities of xanthine dehydrogenase (D-form) and oxidase (O form) were determined spectrophotometrically in liver and serum samples. In ricin-treated animals the liver enzyme was progressively converted from the D- to the O-form, which accounted for more than 60% of total activity after 48 h of poisoning, whilst no change in the xanthine oxidase activity was found in the serum. In the liver of rats treated with free or Ber-H2-conjugated saporin, the D-form was more than 75%, as in normal animals. In the same animals the serum xanthine oxidase activity was up to three-fold control values. The determination of serum xanthine oxidase may prove helpful in the evaluation of liver damage in patients treated with immunotoxins. It may become a diagnostic tool for the differential diagnosis of liver diseases.

Key words Xanthine dehydrogenase · Xanthine oxidase · Ricin · Saporin · Anti-CD30 immunotoxin

Introduction

Ribosome-inactivating proteins (RIPs; reviewed in [5]) are RNA *N*-glycosidases that cleave one or more [4] adenine residues from rRNA. They are single-chain (type 1) or two- or four-chain proteins (type 2), in which an enzymatically active A-chain is linked to a B-chain that is a galactose-specific lectin. The B-chain binds to cells, allowing the entry of the A-chain, which damages ribosomes, thus arresting protein synthesis and killing the cell. Consequently ricin and most other type-2 RIPs are highly potent toxins, with LD_{50}s for mice in the order of few micrograms per kilogram of body weight. Saporin is a type-1 RIP that is functionally similar to the A-chains of ricin and related toxins. Type-1 RIPs enter cells less easily than type-2 RIPs and consequently have only one thousandth the toxicity to animals (LD_{50} for mice in the order of milligrams per kilogram of body weight). Severe liver damage is constantly observed in rats poisoned with lethal doses of ricin [14] or RIPs [5].

Ribosome-inactivating proteins of either type have been used to prepare immunotoxins, conjugates of toxins and antibodies, specifically toxic to the cellullar target of the antibody used (reviewed in [21, 48]). Immunotoxins are currently being studied for their ability to eliminate harmful (often malignant) cells, and some are in clinical trial [21]. The toxin moiety most commonly used to construct immunotoxins is the A-chain of ricin, the toxin of *Ricinus communis*. More recently, however, immunotoxins have been prepared with type-1 RIPs, which are more stable and safer to handle than ricin A-chain, and in some cases have given more active immunotoxins [18, 45].

An immunotoxin containing saporin covalently linked to a Ber-H2 (anti-CD30) monoclonal antibody has been recently described [45]. The Ber-H2 antibody, directed against the CD30 (Ki-1) antigen [38], is present on a small population of normal lymphoblasts as well as on Hodgkin and Reed-Sternberg cells in Hodgkin's disease [42]. The Ber-H2/saporin immunotoxin was used in a phase I clinical trial [16] where patients had liver damage, as shown by the increase in serum transaminases. This is consistent both with the reports of liver lesions observed in mice given lethal doses of saporin or saporin-IgG conjugates, the latter having a higher non-specific toxicity than free saporin [7, 43], and with the hepatotoxicity sometimes reported as a side-effect of the therapeutic use of RIP-containing immunotoxins (reviewed in [21]).

Liver damage often brings about changes in the activity of xanthine oxidase. This enzyme is present in several mammalian tissues, being especially abundant in liver

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and intestine (reviewed in [35]). The natural form of the enzyme is a NAD^+-dependent dehydrogenase (D-form, EC 1.1.1.204), which can be converted into an oxidase (O-form, EC 1.1.3.2.2) either irreversibly, by limited proteolysis [12], or in a reversible manner by chemical [13] or enzymatic [6] oxidation of thiol groups. The reversible conversion also gives rise to an intermediate form, which is able to react with both NAD^+ and oxygen [13, 26]. The enzyme in its O-forms generates superoxide anion and hydrogen peroxide, which in turn may originate hydroxyl radical in the presence of traces of transition metals [27].

The conversion from D-form to O-form appears to occur in ischaemia [15, 36] and ischaemia/reperfusion or hypoxia/reoxygenation (reviewed in [25]). In these conditions tissue damage may be aggravated by the action of the free oxygen radicals produced by the enzyme in its O-forms (reviewed in [11]). Moreover, in several pathologic conditions the enzyme escapes from damaged cells, passing into blood plasma. These conditions include liver damage in experimental carbon tetrachloride poisoning [1], human hepatitis [22, 30, 37, 39], halothane anaesthesia both in rat [23] and in man [24], tourniquet-related ischaemia and reperfusion in man [20] or after experimental haemorrhagic shock in rat [44], infection by influenza virus in rat [33] and rheumatic inflammatory diseases in man [31]. It is noteworthy that the leakage of xanthine oxidase appears to precede that of other enzyme markers of liver damage [37]. In the plasma the enzyme is rapidly converted to the O-form [8], which, through the production of free radicals, can cause distant damage, especially to endothelial cells [50].

The present experiments were performed to ascertain whether modifications of the xanthine oxidase activity in rat liver and blood plasma occur as a consequence of the hepatotoxic damage induced by ricin, saporin or a saporin immunotoxin.

Materials and methods

Materials

Ricin was prepared as previously described [32]. Saporin (saporin-S6, according to the nomenclature proposed in [5]) was prepared as described elsewhere [3]. The Ber-H2 monoclonal antibody (IgG1 subclass [38]) was a generous gift from Dr. B. Falini, Istituto di Ematologia, Policlinico Monteluce, Perugia, Italy.

Immunotoxin

Ber-H2/saporin immunotoxin was prepared as described elsewhere [45] with sulfhydryl-modified antibody and saporin, the latter trace-labelled with $Na^{125}I$ (Amersham International, Bucks., UK) by the method of Fraker and Speck [19] with the Iodogen reagent (Pierce, Chester, UK). The conjugate was separated from unreacted components and from high M_r conjugates by gel filtration through a Sephacryl S-200 high resolution (Pharmacia LKB Biotech, Uppsala, Sweden) column. The saporin-to-antibody ratio in the conjugate was estimated from the radioactivity content and from the protein concentration measured from the A_{280}. RIP activity was assayed on a rabbit reticulocyte lysate [2] as described previously [34]. The immunotoxin was reduced for 30 min at 37°C in the presence of 20 mM 2-mercaptoethanol prior to addition to the assay mixture.

Animals

Male rats of the Wistar strain, average body weight 200 g, were used. The animals had free access to food and water, and the national guidelines for the care and use of laboratory animals were followed. To induce liver damage, the rats received Ber-H2/saporin immunotoxin (3 mg/kg i.p., as saporin, corresponding to $3\times LD_{50}$ for mice), or saporin (6 or 12 mg/kg i.p., i.e. 1.5× and $3\times LD_{50}$, respectively), or ricin (10 μg/kg i.p., i.e. $3\times LD_{50}$) dissolved in phosphate-buffered saline (0.15 M NaCl containing 5 mM Na-phosphate buffer, pH 7.4). For each experimental group normal rats were killed at the same time as controls.

Determination of xanthine dehydrogenase/oxidase activity

At the appropriate time after poisoning, rats were killed by guillotining, the blood was collected, and the liver was quickly excised, cooled in ice-cold 50 mM Na-phosphate buffer, pH 7.4, containing 1 mM EDTA, and homogenised in the same buffer (5 ml/g of liver). The homogenate was centrifuged at 30,000 *g* for 15 min at 4°C, and the supernatant was centrifuged again at 100,000 *g* for 60 min at 4°C. The final supernatant and blood serum were dialysed extensively against the homogenising buffer at 4°C and used for xanthine oxidase and dehydrogenase determinations as described elsewhere for liver supernatant [12] and for blood serum [8]. Xanthine dehydrogenase activity was determined spectrophotometrically at 28°C by measuring the formation of NADH at 340 nm. The oxidase and total (oxidase+dehydrogenase) activities of the enzyme were determined by measuring the formation of uric acid at 292 nm in the absence (oxidase activity) or in the presence (total activity) of NAD^+.

The reversibility of xanthine oxidase from the O-form to the D-form was determined by measuring the enzyme activity after incubation of the samples with 20 mM dithiothreitol for 20 min at 37°C [13].

Protein determination

Protein was determined by the method of Lowry et al. [28], with bovine serum albumin (Sigma, St Louis, Mo.) as a standard.

Histological techniques

Liver specimens were fixed in 3.7% formaldehyde, dehydrated through graded ethanols, embedded in paraffin and cut in 4 μm-thick sections, which were stained with haematoxylin/eosin for histological examination.

Statistical analysis

Results are given as means±SEM. Student's *t*-test was used for statistical comparisons. At $P<0.05$ the differences were considered statistically significant.

Results

Immunotoxin

The Ber-H2/saporin immunotoxin contained an average of 1.63 mol saporin/mol antibody. Saporin inhibited by 50% protein synthesis by a rabbit reticulocyte lysate at the concentration (IC_{50}) 0.41×10^{-10} M before and 1.87×10^{-10} M after the conjugation to Ber-H2 monoclonal antibody.

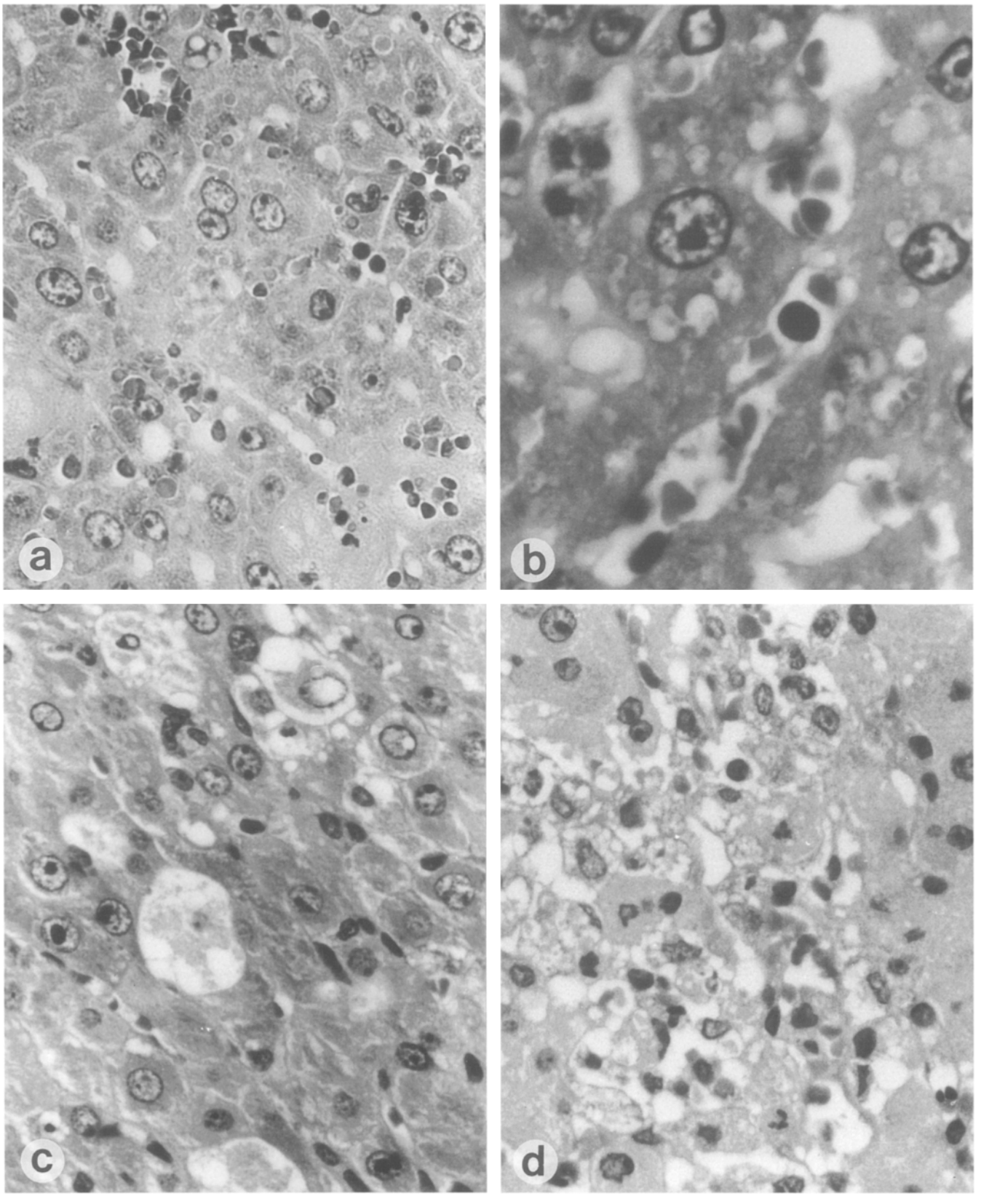

Fig. 1 Histological liver alterations in rats poisoned 48 h before being killed with **a** ricin (10 µg/kg body weight): some capillaries packed with erythrocytes and small areas of necrosis; **b** the same: damage to the sinusoidal lining; **c** saporin (6 mg/kg body weight): some isolated necrotic parenchymal cells; **d** Ber-H2/saporin (3 mg/kg body weight as saporin): an area of necrosis. HE, **a** ×600, **b** ×1500, **c** ×600, **d** ×600

Pathology and histopathology

Rats were poisoned with ricin prepared as in previous research [14] to ensure reproducibility of effects, and the lesions observed in rats used in the present experiments were indeed identical to those described previously. Macroscopically, the intestine was hyperaemic, there was ascites, and the liver appeared enlarged, congested and very dark in colour, indicating defective oxygenation. These gross changes were consistent with the stasis of blood suggested by the swelling of sinusoids observed in histological sections (Fig. 1a). Furthermore, the histological examination showed patches of necrosis in parenchymal cells, extravasation of some erythrocytes (Fig. 1a) and damage to the sinusoidal cell lining (Fig. 1b).

Lethal doses of saporin and saporin immunotoxins caused lesions in the livers of rats, and these were comparable to those observed in mice [7, 43]. Macroscopically, the liver appeared enlarged, soft and creamy in colour. Consistently, histological examination (Fig. 1c, d) showed swelling and vacuoles reminiscent of fatty changes in parenchymal cells. Moreover, necrosis of isolated parenchymal cells with condensation and margination of the chromatin was observed, whereas the sinusoids were not affected. The necrosis in the liver was more severe for immunotoxin-treated than for saporin-treated rats; in the former larger numbers of cells were involved, sometimes grouped in small areas (Fig. 1d).

Xanthine dehydrogenase/oxidase activity

In the liver of rats poisoned with ricin a modest and not statistically significant increase of total xanthine oxidase activity was observed. The enzyme was progressively converted from the D-form to the O-form, which increased from an initial 9% to account for more than 60% of total activity after 48 h of poisoning (Table 1). In all cases this conversion was fully reversible after treatment of the samples with dithiothreitol (results not shown). No significant changes were observed in the enzyme activity in blood serum of ricin-treated rats.

In rats poisoned with either 6 or 12 mg saporin per kg the serum xanthine oxidase activity was up to three-fold control values as early as 24 h after poisoning. Only a modest increase in the percentage of the O-form was observed in the liver of the same rats. This change was not statistically significant and was not dose- or time-related. No significant changes were observed in the total activity of the liver enzyme (Table 2).

A similar increase in xanthine oxidase activity was present in the blood serum of rats 48 h after receiving le-

Table 1 Xanthine oxidase activity in the liver and serum of ricin-treated rats. To determine the oxidase activity, the reaction mixture contained 50 mM Tris-HCl buffer, pH 8.1, 60 μM xanthine (Sigma, St Louis, Mo.) and the appropriate amount of liver supernatant or blood serum. Xanthine oxidase in serum was present only in the O-form. Total and dehydrogenase activities were determined in the presence of 0.67 mM NAD^+ (Boehringer Mannheim Italia, Milan, Italy). The percentages of the oxidase and dehydrogenase activities were calculated, assuming as 100% the total activity of each sample. The sum of the O- and the D-forms may exceed 100% when part of the enzyme is in the intermediate form. A unit of enzyme activity is defined as the formation of 1 μmol of NADH or uric acid per min at 28°C. Values given are means±SEM of the indicated number of animals

Treatment	Time (h)	Rats (*n*)	Liver			Serum
			Total activity (mU/mg proteins)	O-form (%)	D-form (%)	Enzyme activity (mU/100 μl)
None (control)		4	2.69±0.28	9±3	90±4	0.81±0.04
Ricin (μg/kg)						
10	24	6	3.02±0.32	32±4*	71±8	0.60±0.05
10	48	5	3.35±0.14	62±8*	51±10*	0.92±0.07

*$P<0.05$ (difference from controls)

Table 2 Xanthine oxidase activity in the liver and serum of saporin-treated rats (experimental conditions and calculations as in Table 1). Xanthine oxidase was present in serum only in the O-form. Values given are means±SEM of the indicated number of animals

Treatment	Time (h)	Rats (*n*)	Liver			Serum
			Total activity (mU/mg proteins)	O-form (%)	D-form (%)	Enzyme activity (mU /100 μ l)
None (control)		7	2.67±0.30	14±3	89±3	0.61±0.07
Saporin (mg/kg)						
6.0	24	4	2.78±0.18	22±4	85±2	1.59±0.22*
6.0	48	4	2.41±0.20	28±7	75±10	1.62±0.18*
6.0	72	5	2.89±0.15	20±2	89±4	1.25±0.23*
12.0	24	6	2.75±0.16	22±3	77±4	1.69±0.39*
12.0	72	2	2.96±0.30	25±6	83±8	1.70±0.09*

*$P<0.05$ (difference from controls)

Table 3 Xanthine oxidase activity in the liver and serum of Ber-H2/saporin-treated rats. The dose of immunotoxin was expressed as saporin (experimental conditions and calculations as in Table 1). Xanthine oxidase was present in serum only in the O-form. Values given are mean±SEM of the indicated number of animals

Treatment	Time (h)	Rats (*n*)	Liver			Serum
			Total activity (mU/mg proteins)	O-form (%)	D-form (%)	Enzyme activity (mU/100 µl)
None (control)		6	2.29±0.25	17±2	82±4	0.58±0.08
Ber-H2/saporin (mg/kg)						
3	24	4	2.21±0.11	15±1	78±3	0.95±0.14*
3	48	6	2.57±0.10	16±1	86±2	1.62±0.23*
3	72	3	1.96±0.13	24±10	80±7	1.17±0.26*

*$P<0.05$ (difference from controls)

thal doses of Ber-H2/saporin, whereas the enzyme activity in liver was unchanged (Table 3).

Discussion

We ran parallel studies in rats poisoned with ricin, saporin and Ber-H2/saporin immunotoxin at lethal doses, which all induced liver necrosis. The effect of free Ber-H2 was not tested, since this antibody did not cause any detectable damage when injected into patients [17]. In keeping with previous observations (reviewed in [5]), there were differences between the histological lesions brought about by ricin and those brought about by free or conjugated saporin. The reasons for these differences in the effects of proteins with the same enzymatic activity are not yet understood. Presumably, the presence of the B-chain in ricin and of the antibody in the immunotoxin may affect the uptake by different cell types and the intracellular routing of the molecule. Indeed, the total in vivo uptake of ricin [40, 41] by rat non-parenchymal cells was higher than that by parenchymal cells, whereas the uptake of Ber-H2/saporin [9] by both cell types was almost the same. However, the in vitro protein synthesis was inhibited more strongly in non-parenchymal than in parenchymal cells, both by ricin [40] and by Ber-H2/saporin [9].

The presence of vacuoles reminiscent of fatty changes in the livers of rats treated with free or conjugated saporin may be due to the inhibition of protein synthesis. The weaker evidence for this type of damage in the liver of ricin-treated rats could be due to the high uptake of ricin by non-parenchymal cells [29, 40], which appeared to have been killed 24 h before parenchymal cells, suggesting that the necrosis of the latter cells was a consequence of circulatory alterations consequent to the damage to the sinusoidal lining [14].

It is noteworthy that the pathological and histological changes observed in the liver of rats treated with both saporin or Ber-H2/saporin are qualitatively similar, although the alterations induced by the immunotoxin are more severe than those produced by free saporin. This result is consistent with the higher non-specific toxicity of immunotoxins than of free saporin [7, 43].

Both type-1 and type-2 RIPs affected xanthine oxidase activity, although in different ways. Ricin poisoning brought about some conversion of the xanthine oxidase from the D-form to the O-form in the liver, whereas in the animals treated with saporin or immunotoxin no significant conversion was observed. Presumably this difference was associated with the diversity in liver damage observed in the two conditions, namely a distribution of the necrosis affecting only parenchymal cells with free or conjugated saporin, or both parenchymal cells and sinusoids with ricin. The subsequent blood stasis in the case of ricin-treated rats produced further changes.

These differences suggest that the conversion from xanthine dehydrogenase to oxidase observed in the congested livers of ricin-intoxicated rats is not a consequence of necrosis, which is present in all poisoned animals, but rather a consequence of the poor oxygen supply due to the clotted plasma and platelet thrombi described in the liver sinusoids of rats 48 h after ricin treatment [14]. This conversion of the enzyme is consistent with the effects of ischaemia [15] and of hypoxia [10] on rat liver, and of hypoxia on isolated hepatocytes [8]. Moreover, the conversion induced by ricin in the liver enzyme was fully reversible, and thus must have been due to oxidation of enzymatic thiol groups and not to proteolysis. The blood stasis could also be responsible for the slight enhancement of total xanthine oxidase activity observed in the livers of rats treated with ricin. Indeed, an increased xanthine oxidase activity at low oxygen tension has been reported [47].

Present experiments demonstrate that the administration of lethal doses of saporin or immunotoxin induces a marked enhancement of xanthine oxidase in rat blood plasma. This is another difference between the effects of ricin on the one hand and saporin and the immunotoxin on the other. The possibility should be considered that in ricin-poisoned rats the enzyme was released, but did not pass into the general circulation owing to liver stasis and consequent decreased blood circulation.

The increase of serum xanthine oxidase was comparable in animals poisoned with immunotoxin or with an amount of free saporin higher than that present in the immunotoxin, but equivalent in terms of toxicity. The correlation between the elevation of serum enzyme and the

liver lesions suggests (1) that this increment was due to leakage of xanthine oxidase out of liver cells and (2) that the changes are due to the saporin, which is consistent with the lack of effects of the free Ber-H2 antibody on hepatocytes [9].

The elevation of serum xanthine oxidase without the conversion of liver xanthine dehydrogenase after the poisoning with free or conjugated saporin suggests that in these cases the enzyme can leak out from necrotic cells in the D-form, the conversion occurring in plasma anyway [8]. The increment in serum xanthine oxidase activity without a measurable decrease in total liver activity is compatible with the high concentration of the enzyme in the liver and consistent with the extension of necrosis observed in the hepatocytes. Similarly, the elevation of xanthine oxidase activity in rat serum following haemorragic shock was not associated with any significant change of the enzyme activity in liver, intestine, kidney, or lung [44].

The possibility was considered that the increased xanthine oxidase activity found in the serum of rats intoxicated with saporin or with immunotoxin could be due to the enzyme coming from the intestine, which is the organ richest in xanthine oxidase [35]. Although ricin causes severe intestinal lesions [49], the xanthine oxidase activity was not increased in the serum of ricin-poisoned rats. Thus, it seems unlikely that the excess of xanthine oxidase in the serum of rats treated with free or conjugated saporin came from the intestine, which in these animals did not appear to be damaged, which is consistent with previous observations [7].

Although xanthine oxidase is normally present in rat serum, and no tissue injury is apparently caused by its activity, it is possible that the antioxidant defences of plasma are overwhelmed by the elevation in oxygen active species produced by the xanthine oxidase leaked out from hepatocytes. As a consequence, a direct injury to vascular endothelium may follow, and tissue damage could be mediated and/or amplified by neutrophil activation (reviewed in [25]). In keeping with this, lung damage was associated with the increase of circulating enzyme level after intestinal ischaemia-reperfusion [46].

A leakage of xanthine oxidase will probably occur as a consequence of the liver damage observed in patients treated with immunotoxins containing saporin [16] and possibly with other immunotoxins. Were the enzyme present in plasma, it might cause endothelial damage [50] and could contribute to the pathogenesis of the capillary leak syndrome, a major side-effect of immunotoxins [48]. Also, the determination of serum xanthine oxidase may be useful to detect and assess liver damage in patients treated with immunotoxins containing saporin and presumably other toxins.

The fact that the xanthine oxidase activity is increased in the serum of rats poisoned with saporin or saporin immunotoxin, but not of rats poisoned with ricin, could mean that the enzyme is released from hepatocytes only in certain types of liver damage. If this is so, then the determination of plasma xanthine oxidase could be applied as a diagnostic tool in the differential diagnosis of liver diseases; this would be particularly useful and sensitive in human medicine, since human serum is normally devoid of xanthine oxidase [35].

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