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Pharmacological studies of ricin in mice and humans

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Summary. *A highly sensitive enzyme-linked immunosorbent assay (ELISA) for determination of ricin in serum is presented. Using this method it was found that IV-injected ricin disappeared from the plasma of mice and cancer patients according to first-order kinetics.*

DBA mice were found to be more sensitive to ricin than C3H and B6D2 mice. When mice of the different strains were given the same dose of ricin, the concentrations found in liver, spleen, and kidneys were highest in the most sensitive mice. Ricin disappeared most rapidly from serum of the mice with the highest sensitivity. The inverse correlation between the rate of disappearance of ricin from serum and the tissue concentrations reached may be due to the fact that ricin is rapidly and firmly bound to cell surface receptors.

Whole-body autoradiography after IV injection of ^{125}I-labeled ricin showed the highest amount of radioactivity in liver, spleen, and adrenal cortex. Considerable amounts of radioactivity were also present in bone marrow, showing that the lack of myelosuppressive activity of ricin previously found in mice and dogs cannot be accounted for by the failure of ricin to reach the bone marrow.

Part of the ricin in the tissues was present in the form of free chains, the highest fraction being present in the liver.

In this tissue both the free A-chains and those present in whole ricin were found to be modified. However, the modified A-chains had retained their full capacity to inhibit protein synthesis in vitro.

In cancer patients, toxicity appeared at about the same initial serum levels as in the mice, supporting the view that mouse data have a good predictive value for man. At each dose level the individual variations were modest, a finding that is important for eventual clinical use of this potent drug.

Introduction

The plant protein ricin is currently receiving increasing interest due to its unusual biochemical properties. Ricin, which acts by inhibiting protein synthesis, is one of the most toxic proteins known. Furthermore, it inhibits the growth of various murine tumors [2, 6, 16] and of several human tumor xenografts in athymic mice [6, 8, 9, 23].

Like several other toxic proteins (abrin, modeccin, diphtheria toxin), ricin consists of two polypeptide chains, which are joined by a disulfide bond and have different functions [20, 21]. The larger B-chain binds the toxin to cellular receptors, whereas the smaller chain, the A-chain, is responsible for the biological action. The A-chain enters the cytoplasm, where it inactivates the large ribosomal subunit, and evidence has been obtained that the entry of a single A-chain into the cytosol may by sufficient to kill a cell. This is explained by the fact that it acts by a catalytic mechanism (for review see [21]).

Since the mechanism of action of ricin is different from that of most antineoplastic agents in current use, its side-effects are also different [5, 7]; for example it has little or no inhibiting effect on normal bone marrow [2, 7]. In L1210 leukemia in mice, ricin in nontoxic doses was able to potentiate the cytostatic effect of several conventional agents without concurrently increasing the toxicity [3, 4]. These properties suggest that ricin may be a promising candidate for combination therapy. Recently we have carried out a phase I trial of ricin [10].

The extreme toxicity of ricin has prompted several investigators to prepare conjugates of ricin or its A-chain with antibodies directed against tumor-associated antigens in attempts to obtain target specific cytotoxic agents [22, 25, 27]. The possibility exists, however, that in vivo the toxic moiety may be released from the conjugate and give rise to general toxic effects similiar to those seen after the administration of ricin.

Since our previous studies have shown that in mice and dogs there is a narrow range between tht highest ricin dose at which all animals survive and the lowest dose at which all animals die [7], it was considered necessary, before ricin as such or ricin conjugates can be safely used in the treatment of human patients, to be able to monitor the blood levels of ricin after the administration of therapeutic doses. For this purpose we have developed a highly sensitive enzyme-linked immunosorbent assay (ELISA), which is presented here. With the aid of this and other methods we have studied the disappearance of ricin from the plasma of mice and of treated cancer patients, and also the entry of ricin into the tissues of mice and its breakdown there. Unexpectedly, different strains of mice differed significantly in their sensitivity to ricin.

Materials and methods

Mice. B6D2, C3H, and DBA/2 mice (18–22 g) of both sexes were used throughout this study. All animals were obtained from the National Breeding and Research Center, Gl. Bomholdt Gaard, Ry, Denmark.

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Ricin. Ricin was isolated and purified as described earlier [19]. Traces of agglutinin were removed by additional chromatography on Sephacryl S-200 (Pharmacia, Uppsala, Sweden). The purified toxin was free of agglutinin, as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Formalin-treated ricin for immunization was prepared as described earlier [12]. Ricin was iodinated with ^{125}I according to the method of Fraker and Speck [11], as described earlier [12].

Whole-body autoradiography. Whole animals were mounted in a 1% (w/v) gel of carboxymethyl cellulose and frozen in a bath of *n*-hexane, cooled with solid CO_2 to about −75° C. Further processing was performed according to Ullberg [26]. The freeze-dried sections were pressed against x-ray film (Kodirex, Kodak) and stored at −20° C. The film were developed after 8 weeks' exposure.

Anti-ricin. Antisera against ricin were obtained from immunized rabbits [12], from humans (cancer patients having received several courses of treatment with ricin), or from immunized mice. Formalin-treated ricin was mixed with an equal volume of Freund's incomplete adjuvant and sonicated for 30 s. B6D2 mice received the preparation SC at 2-week intervals, three times with 5 µg/mouse and once with 10 µg/mouse. Eight days after the last injection the mice were bled to death. $F(ab')_2$ fragments were prepared from rabbit IgG, essentially by the methods of Nisonoff and Rivers [17] as described by others [14], except that the pepsin digest was separated on a Sephacryl S-200 column (1.5 × 95 cm).

Assay buffers. The assay buffers used in this study were: 7 m*M* Na phosphate, pH 7.4, containing 0.14 *M* NaCl and 0.02% Na azide, hereafter referred to as PBS, and PBS containing 0.1 *M* lactose and 0.05% (v/v) Tween 20 (Baker Chemicals, Deventer, Holland), hereafter referred to as ELISA buffer.

Alkaline phosphatase conjugates. Alkaline phosphatase (ALP) was conjugated to antibodies essentially as described by Engvall and Perlmann [1], with some modificatons [13].

The assay. Anti-ricin $F(ab')_2$ fragments were coated onto the wells of a Dynatech Microelisa plate (M129 B) by adding 0.5−1 µg $F(ab')_2$ fragments in 200 µg PBS to each well and incubating at +4° C for 2−3 days. All incubations were carried out in moist chambers.

After washing the wells with ELISA buffer, the human or mice sera to be analyzed (diluted 1 : 10 with buffer) were added. The plate was incubated at 37° C for 4 h, after which it was washed in ELISA buffer and a diluted antibody against ricin was added. The plate was then incubated for 3 h at 37° C. After washing with ELISA buffer, ALP-conjugated second antibody was added to each well and the plate was incubated overnight at room temperature. The plate was then washed in assay buffer and in PBS, followed by the addition of *p*-nitrophenol phosphate (1 mg/ml) in 10% (v/v) diethanolamine buffer, pH 9.8, containing 1 m*M* $MgCl_2$ and 0.02% Na azide. After 6−7 h at 37° C, 200 µl from each well was mixed with 800 µl water and the absorbance at 400 nm was measured.

Determination of ricin in mouse serum. The animals received increasing doses of ricin IV, and after different periods of time two mice were bled to death and their sera were pooled and assayed for toxin, as described above. The ricin content was calculated from a standard curve (Fig. 1A). The standards were prepared by dissolving known amounts of ricin in normal human or mouse serum (diluted 1 : 10 in buffer). Both standards and unknowns were analyzed in triplicate. The assay permitted the determination of ricin in concentrations of 25 pg/ml of plasma.

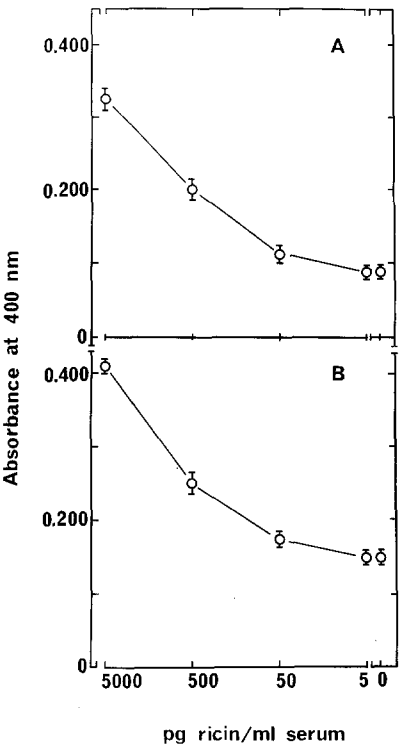

Fig. 1A, B. Typical standard curves for the determination of ricin in mouse serum (**A**) and human serum (**B**). Each *point* represents the mean of triplicate samples. The SD is given by the *vertical bars*

Determination of ricin in tissues. Tissues containing ^{125}I-ricin were homogenized for 10 s in 5 ml ice-cold 1% (v/v) Triton X-100 in 0.1 *M* lactose, using a blender. The homogenate was sonicated for 30 s at 0° C, and centrifuged for 100,000 *g* for 60 min. Usually, 60%−80% of the radioactivity originally present was recovered in the supernatant. The amount of protein-bound radioactivity was determined by precipitation in 10% (w/v) trichloroacetic acid (TCA).

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out as described by Laemmli [15], using a 3% concentrating gel and a 10% separating gel. Fractions to be analyzed were mixed with staphylococci charged with antibody against ricin, prepared as described elsewhere [18]. The mixture was rotated for 2 h at room temperature and centrifuged, and the pellet was washed once in buffer and once in water. Finally, it was boiled for 10 min in 10−20 µl sample buffer containing 2% SDS and centrifuged; the supernatant was then applied onto the gel. After electrophoresis the gel was fixed overnight in acetic acid : methanol : water (1 : 6 : 14), dried, and autoradiographed.

Results

Strain differences in sensitivity to ricin

In experiments where mice were used for the biological standardization of ricin, indications had been obtained that different strains of mice may differ in their sensitivity to this substance. Since this is a question of both theoretical and practical interest, the toxicity of ricin in three different strains of mice was estimated.

The dose-lethality curve for ricin is very steep [5, 7]. A reliable LD_{50} value is therefore difficult to establish and the minimum lethal dose (MLD) is a more meaningful parameter. The MLD was determined graphically from curves of the survival time plotted versus the dose, as previously described [7]. The MLD doses derived from the curves were 1.95 μg/kg for the DBA mice, 2.35 μg/kg for the C3H mice, and 2.40 μg/kg for the B6D2 mice. That the DBA mice were significantly more sensitive than the other mouse strains tested follows from the fact that the DBA mice started to die at a dose of 1.75 μg/kg and all of them that received a dose of 2.25 μg/kg died, whereas none of the other mice died until doses of 2.5 μg/kg were reached.

Disappearance of ricin from mouse plasma

To elucidate the mechanism responsible for the inter-strain differences in sensitivity to ricin, the plasma and tissue levels of ricin were measured in the three strains of mice after IV injection of increasing doses. In the case of the plasma studies it was necessary to use different mice for the different time points and to pool the sera of two mice for each time point. In view of this and the analytical difficulties, it is gratifying that when the plasma levels of ricin were plotted against time in a semilogarithmic plot (Fig. 2), in all cases straight lines were obtained. The results show that ricin disappears from plasma according to first-order kinetics.

Inspection of the curves in Fig. 2 shows that the rate of elimination of ricin from plasma differed with the type of mice used. Interestingly, ricin disappeared most rapidly from plasma of the DBA mice, which showed the highest sensitivity to ricin. This is evident from the data in the inset, where each point represents the rate constant of one disappearance curve. At all six ricin doses tested the rate constant for the elimination of ricin was clearly higher for the DBA mice than for the other two strains of mice.

It follows from the above results that when the different strains of mice are compared the area under the plasma concentration × time curve was not proportional to the observed toxicity. Thus, for all ricin doses tested the area under the curve was smallest for the DBA mice (Table 1). Clearly, these results are in contrast to the situation for most substances, where the toxicity is proportional to the area under the serum concentration × time curve.

The initial amount of ricin in plasma was calculated by extrapolating the plasma curves back to time zero. The values found (Table 2) correspond to about half the dose administered. This is as expected since we have established that about half the ricin in the blood is present in the plasma, while the rest is bound to the cells (data not shown). The results support the validity of the experimental procedure.

The inset in Fig. 2 shows that in the DBA mice the rate constant initially increased with increasing dose and then leveled off, whereas the rate constants for B6D2 and C3H were almost independent of the dose. The possible significance of this finding will be discussed below.

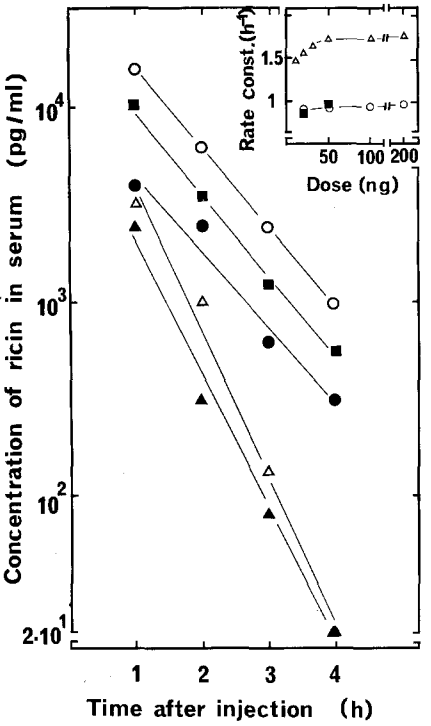

Fig. 2. Typical serum profiles of ricin in different strains of mice after IV injection. Each *point* represents the average of two mice (see text). Regression lines were obtained by the method of least squares ($R \geq 0.98$). ▲ 20 ng, DBA; △, 50 ng, DBA; ●, 20 ng, B6D2; ■, 50 ng, C3H; ○, 100 ng, B6D2. *Insert:* rate constant plotted against the dose administered. △, DBA; ○, B6D2; ■, C3H

Table 1. Pharmacokinetic parameters for ricin in mice

Strain	Dose (ng/mouse)	Amount of ricin in plasma at time zero[a] (ng)	Area[b] (ng/ml × h)
DBA	10	5	3
B6D2	20	11	12
DBA	20	10	6
C3H	20	12	14
DBA	30	15	9
B6D2	50	24	26
DBA	50	23	13
C3H	50	25	26
B6D2	100	41	44
DBA	100	68	39
B6D2	200	95	98
DBA	200	110	62

[a] Calculated by extrapolation of the serum curves, assuming that the total plasma volume of a 20 g mouse is 1 ml

[b] Area under plasma curve

Tissue distribution of ricin

Whole-body autoradiography. The gross distribution of ricin in mice was studied by whole-body autoradiography 10 min and

3, 8, and 24 h after IV adminstration of ^{125}I-labeled ricin. The results obtained after 10 min and 3 h are shown in Fig. 3. After 10 min (upper panel) the highest amount of radioactivity was found in liver and spleen, confirming and extending our earlier results [5] obtained with chemical methods. High activity was also found in the adrenal cortex (not shown in the picture presented), whereas only moderate activity was present in kidneys, lungs, salivary gland, blood, and gastric mucosa. Considerable activity was found in the bone marrow (not clearly evident in the picture shown). Hence, the lack of myelosuppressive activity of ricin in mice and dogs [7] cannot be accounted for by failure of ricin to reach the bone marrow. No activity was found in the myocardium. Also, no activity was detected in the brain, which is consistent with our experience that animals given toxic doses of ricin do not exhibit symptoms clearly attributable to the central nervous system [5, 7].

After 3 h the radiolabeling of the liver was markedly reduced, whereas the radioactivity in lungs, kidneys, bone marrow, and adrenals remained at about the same level as that observed at 10 min (Fig. 3, lower panel). The radiolabeling in the salivary gland and the gastric mucosa had increased strongly up to 3 h and abundant amounts of radiolabeled material were present in the urinary bladder at his time. After 8 h only traces of radioactivity were detected in the liver, while radioactivity could still be detected in spleen, kidneys, adrenals, and salivary gland. After 24 h no radioactivity could be detected in any organ by this method.

Table 2. Rate constants for disappearance of ricin from serum of humans

Dose level ($\mu g/m^2$)	Rate constant[a] (h^{-1})
14	0.238 ± 0.005
16	0.26 ± 0.02
18	0.273 ± 0.006
20	0.273 ± 0.003
22	0.276 ± 0.006
23	0.275 ± 0.007

[a] Mean values (± SD) for three to five patients per dose

Chemical studies. To determine the uptake of ricin in specific organs, the tissue radioactivity after injection of ^{125}I-labeled ricin was measured. Since ricin is not excreted as such [5], and since separate experiments showed that ricin was not decomposed in serum, the disappearance of ricin from plasma reflects solely the tissue uptake of ricin.

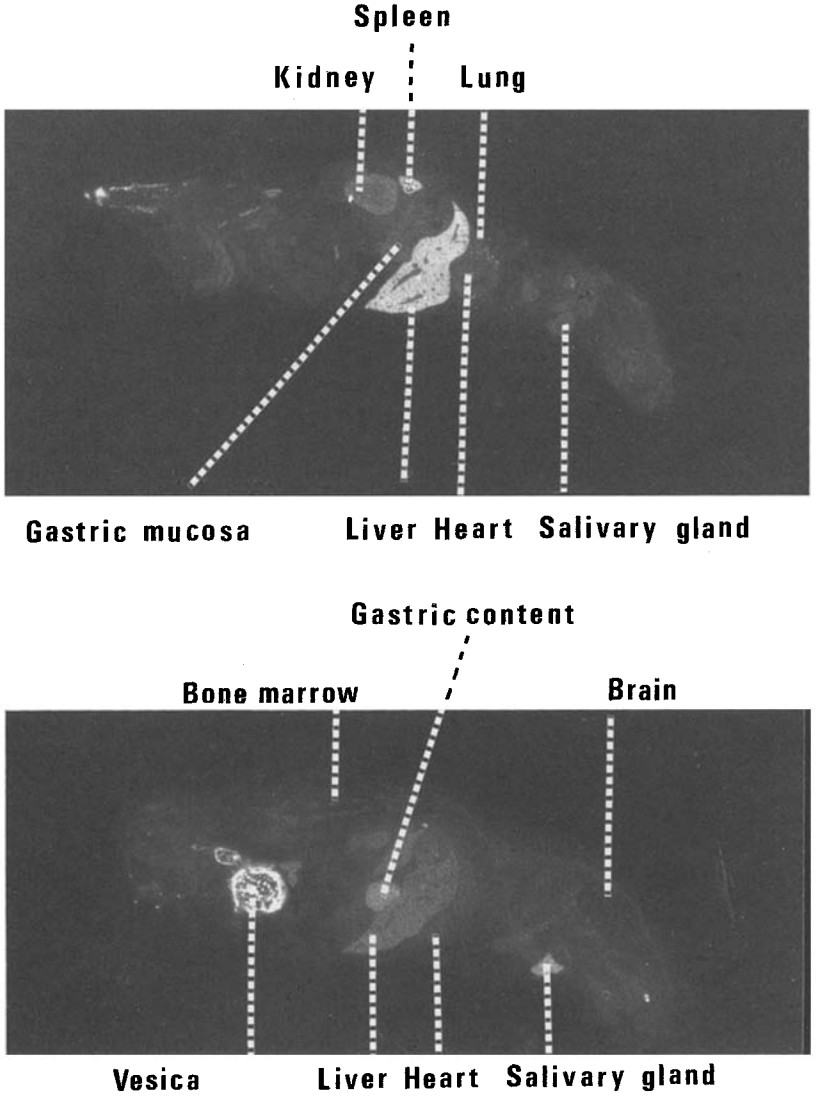

Fig. 3. Whole-body autoradiography (representative sections) of B6D6 mice 10 min (*upper panel*) and 3 h (*lower panel*) after IV injections of 200 ng ^{125}I-labeled ricin (0.01 μCi/ng)

To see how the observed inter-strain differences in the rate of disappearance of ricin from plasma were reflected in the ricin concentrations in the organs, 100 ng ^{125}I-ricin was injected IV into mice of the different strains. After increasing periods of time, mice were killed and the radioactivity present in different organs was measured and expressed as the percentage of the injected dose per gram of tissue.

In Fig. 4 the results, corrected for non-TCA-precipitable radioactivity, are plotted against time. It is seen that in liver, spleen and, particularly, kidneys, the concentrations of ricin were higher in the DBA mice than in the B6D2 and C3H mice. Thus the more rapid elimination of ricin from plasma in the DBA mice was associated with a higher concentration in several vital tissues, a finding which may account for the higher sensitivity of the DBA mice. The ricin concentration in the lungs showed no corrrelation with the strain differences in sensitivity (Table 1), suggesting that the lungs do not play an important role in the toxicity of ricin.

During the first 5–6 h, about 80%–90% of the total radioactivity extracted from lungs, kidneys, spleen, and liver was precipitable by TCA. In contrast, in the mucosa of stomach and duodenum only 30% of the radioactivity was precipitable and only a small fraction of this appeared to represent ricin, as judged by gel filtration and by polyacrylamide gel electrophoresis. Thus, the increasing radioactivity with time in these tissues (Fig. 3) apparently represents mainly breakdown products of ricin, in agreement with the finding that gastrointestinal damage is not prominent in mice and dogs given corresponding doses of ricin [7].

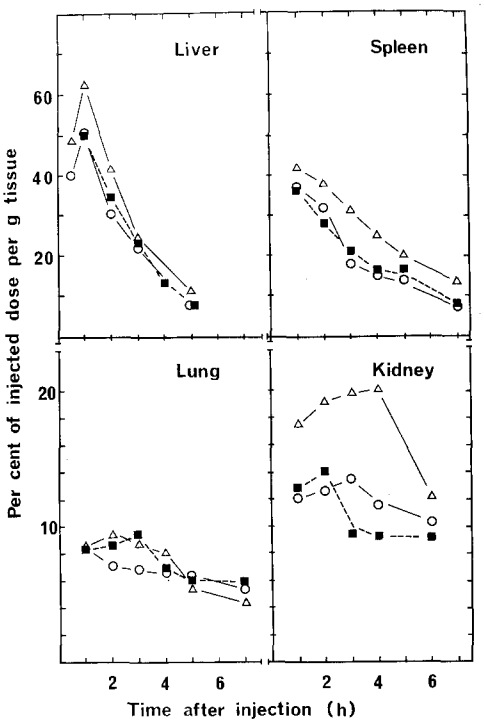

Fig. 4. Concentration of ricin in different organs after IV injection of ^{125}I-ricin into B6D2 (○), C3H (■), and DBA (△) mice. The results were corrected for non-TCA-precipitable radioactivity as described in the text

Structure of labeled substances

Little is known about the degradation of ricin in tissues. In attempts to elucidate the structure of the labeled substances present after the injection of labeled ricin, the extract from a liver removed 4 h after injection of ricin was subjected to gel filtration. Figure 5 A shows the elution profile of the radioactivity. It is seen that the radioactivity was eluted in two main peaks. The smaller peak contained degradation products of molecular weight less than about 5,000. The high-molecular-weight substances were eluted as a large peak (a) with a small shoulder (c). In addition, a small peak (d) is seen. The size of c differed only slightly in the various mouse strains tested. In tissue removed 1 h after injection such a shoulder was hardly detectable (not shown). When ^{125}I-ricin was added in vitro to liver tissue (Fig. 5 B) the ricin-containing peak showed no shoulder.

To characterize the high-molecular-weight substance (s), fractions from the column were treated with staphylococci charged with antibodies to ricin, and submitted to polyacrylamide gel electrophoresis as described in *Materials and methods*. It was found (Fig. 6) that, whereas the main peak (fractions a and b) consisted of ricin with no demonstrable free chains, the shoulder (fraction c) seemed to contain only free A- and B-chains. The small peak (d) contained degradation products having molecular weights between those of the free chains and the tracking dye.

When the fractions were treated with 2-mercaptoethanol, the A-chains, both the free ones in fraction c and those liberated from fractions a and b, migrated slightly faster than those obtained by reduction of a control sample of ricin. The B-chains migrated a the normal rate. The results indicate that the A-chains were slightly modified and that this modification had occurred while the A-chain was still part of the intact

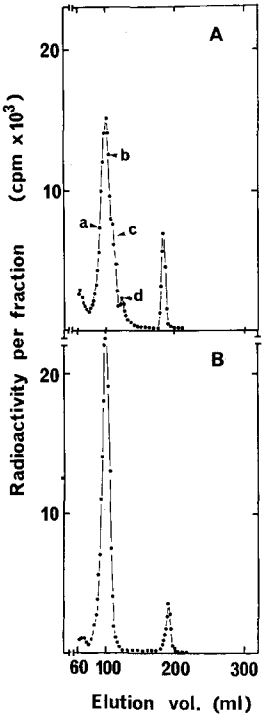

Fig. 5 A, B. Gel filtration on Sephacryl S-200 (1.6 × 95 cm) of extracts from liver tissue. The column was equilibrated with 0.1 *M* Tris-HCl in 0.2 *M* NaCl and 0.1 *M* lactose, pH 8.0, and run at a rate of 30 ml/h. **A** Liver removed 4 h after IV injection of labeled ricin; **B** normal liver homogenized in the presence of labeled ricin. Fractions indicated by *arrows* were submitted to SDS electrophoresis as described in the text

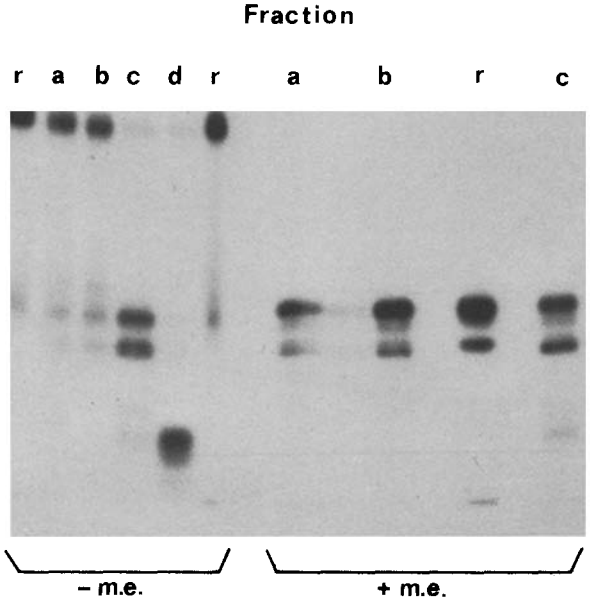

Fig. 6. SDS electrophoresis of fractions from the S-200 column (Fig. 5) with (+ *m.e.*) or without (−*m.e.*) the addition of 2-mercaptoethanol. *r*, whole ricin; the other *letters* refer to the fractions in Fig. 5A

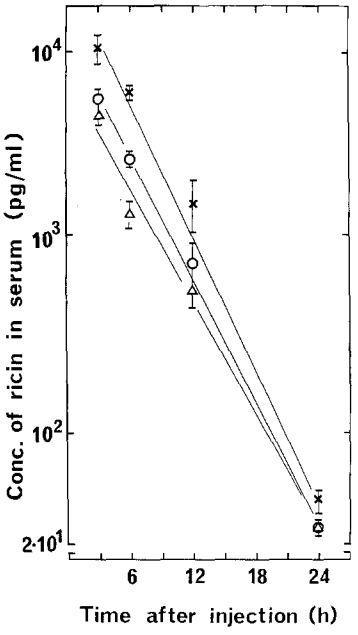

Fig. 7. Serum level of ricin in humans after IV injection of various doses. Each *point* represents the mean (± SD) or three to five patients. The regression lines were obtained by the method of least squares ($R \geq 0.99$). △, 14 μg/m²; ○, 16 μg/m²; ×, 23 μg/m²

molecule. In experiments not shown here it was found that the modified ricin and its A-chain had retained full biological activity, as measured by the ability to inhibit protein synthesis.

Analysis of extracts from kidneys, lungs, and spleen showed a main peak containing ricin (not shown) and a small shoulder, consisting of free A- and B-chains. The fraction of ricin dissociated into free chains was appreciably smaller than in the liver. Also, in contrast to the situation in liver, the A-chains, both the free ones and those obtained by reduction of ricin, migrated at normal rates (not shown).

Plasma levels of ricin in humans

In a phase-I clinical trial reported in detail elsewhere [10], cancer patients were given doses of ricin ranging from 4.5 μg/m² to 23 μg/m². Blood samples were collected 3, 6, 12, and 24 h after injection, and the plasma samples were assayed for toxin. The concentration of ricin was calculated from a standard curve (Fig. 1B) obtained as outlined in *Materials and methods*. As was the case in mice, ricin disappeared from plasma according to first-order rate kinetics (Fig. 7) and the rate constant depended on the dose (Table 2), although to a much lesser degree than in the DBA mice. At each dose level the individual variations in the rate constants were slight.

By extrapolation of the curves to time zero, the initial plasma concentrations were calculated to be 24–26 ng/ml plasma after injection of doses in the range 20–23 μg/m² (Table 2). Thus, at these dose levels, at which the clinical side-effects became significant [10], the initial plasma concentrations corresponded well to those obtained in B6D2 and C3H mice given an MLD (approx. 50 ng/mouse).

Discussion

The results presented here document that the ELISA used in this study is sufficiently sensitive and reproducible to permit the monitoring of the plasma ricin levels in humans given therapeutic ricin doses. The finding that at each dose level the individual variations in the pharmacokinetics of ricin were modest, both within each mouse strain and in the patients, is important for the eventual clinical use of this potent drug. It is of practical interest that in the patients, toxicity appeared at about the same initial plasma levels of ricin as in mice. This is in agreement with the general experience that toxicity studies in mice are useful in predicting toxicity in man.

The finding that different strains of mice showed different sensitivities to ricin permitted us to make some interesting observations on the relationship between plasma and tissue concentrations of ricin. As might be expected, the inter-strain differences in sensitivity to ricin correlated with strain differences in the ricin concentrations reached in important tissues. Thus, when mice of the different strains were given the same dose of ricin, the concentrations found in liver, spleen, and kidneys, both of ricin and of free chains, were highest in the most sensitive mice, i.e., those belonging to the DBA strain.

However, the higher tissue concentrations found in the DBA mice were associated with a more rapid disappearance of ricin from the blood plasma. Consequently, the tissue concentrations reached, and the concomitant biological activity, were not proportional to the area under the plasma concentration × time curve, in contrast to the situation with most substances. The unusual pharmacokinetics of ricin may be due to the fact that ricin is rapidly and firmly bound to cell surface receptors [24].

The inter-strain differences in sensitivity and pharmacokinetics may conceivably reflect differences in the number and/or affinity of cell surface receptors for ricin. The observation that, both in DBA mice and in humans, the rate constants for the elimination of ricin increased with the dose until a plateau was reached may possibly reflect the existence of several types of receptors with different affinities for the toxin. In this event, after increasing doses of ricin it will be bound, not only to high-affinity receptors, but to an increasing extent also to the low-affinity receptors.

Whatever the underlying reason, the observed strain differences in sensitivity to ricin are of considerable practical interest, as the biological activity of ricin to be used in humans must be assayed in animals [5, 7]. If different laboratories use different mouse strains for this purpose, the results may not be comparable.

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