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Systemic Toxicity of Diphtheria Toxin-Related Fragments (CRM26, CRM45), a Hormone–Toxin Hybrid Protein (TRH–CRM45), and Ricin A[1] (42234)

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Abstract. As a preliminary step in evaluating the use of thyrotropin releasing hormone (TRH) linked to toxin fragments for systemic treatment of pituitary disease, we have examined the metabolic degradation, tissue distribution, renal excretion, and toxicity in rats of TRH–CRM45 which consists of TRH coupled to CRM45, a diphtheria toxin-related polypeptide. For comparison, we have similarly studied CRM45, CRM26 (a smaller diphtheria toxin-related fragment), and ricin A. All four proteins were found to concentrate in the kidney and liver relative to blood (in comparison to bovine serum albumin), tissue:plasma ratios for the kidney being much higher than those observed for the liver. Radioiodinated CRM45 and ricin A were rapidly cleared from the circulation with similar patterns. Systemic toxicity studies showed that at doses greater than 2 μg/100 g body wt (bw), CRM45 caused a decrease in growth rate and caused renal damage. CRM45 modified with 2-pyridyldithio(propionate) groups as well as TRH–CRM45 was significantly less toxic than CRM45, as was TRH–CRM45. CRM26 had no discernible effect on the growth rate of the animals. Ricin A, at a dose of 50 μg/100 g bw slowed the growth rate of rats, but specific liver or kidney damage could not be detected. These findings define an upper range of doses for possible therapeutic use.

Ligand–toxin hybrid proteins which consist of the specific cell binding properties of hormones (1–3), lectins (4, 5), or antibodies (6–8) coupled to a fragment of either diphtheria or ricin toxin have been reported by many authors. It has been proposed that these hybrid proteins could be targeted selectively to tumor tissues, and therefore may be useful in the treatment of neoplastic disease. We have recently constructed a peptide–toxin conjugate between thyrotropin-releasing hormone (TRH) and a diphtheria toxin-related polypeptide designated cross-reacting material 45 (CRM45) which lacks the cell receptor binding region of native diphtheria toxin. This hybrid protein includes both the enzymatically active region and lipid binding domains of the toxin molecule. It is capable of binding to TRH receptors on pituitary tumor cell membranes and subsequently inhibits protein synthesis in these cells (9).

As a preliminary step in considering the use of this or any other hybrid toxin conjugate for systemic treatment, it is important to determine whether any significant concentration in nontarget tissues occurs and whether toxin taken up by these tissues is damaging. In a study which determined the organ localization of the TRH–CRM45 conjugate after iv injection in the rat, we had previously found that the conjugate, in addition to being bound in a displaceable manner to the pituitary, was taken up by the kidney and liver in a nondisplaceable manner (10). Since uptake into these tissues indicated that TRH–CRM45 might be toxic to them we undertook in this study to determine the pathways of degradation of the complex, the kinetics of clearance from the blood, and the effect of various doses on growth and on renal and hepatic function. To ascertain whether the properties of TRH–CRM45 were attributable to the CRM45 component of the complex, similar studies with CRM45 were also carried out. Further, to determine whether the hydrophobic domain of CRM45 influenced degradation and toxicity, we studied CRM26, another prematurely terminated diphtheria toxin fragment which resembles the diphtheria A chain. Finally we also studied ricin A which consists of the active region and hydrophobic domain of the plant toxin ricin. Both diphtheria A and ricin A are currently being used by other investigators to prepare ligand–toxin complexes. All three of

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these proteins (CRM26, CRM45, and ricin A) are 1000- to 10,000-fold less toxic than whole diphtheria or ricin toxins when tested *in vitro* for inhibition of protein synthesis or *in vivo* for a minimal lethal dose (11–13).

The studies presented here show that all four proteins are concentrated in the kidney and liver and that CRM45 and ricin A have similar patterns of excretion. CRM45 and to a lesser extent TRH–CRM45 were nephrotoxic in high doses. Chemical modification of the CRM45 molecule lessened its nephrotoxicity. In the doses used, neither CRM26 nor ricin A was toxic.

Materials and Methods. *Protein purification.* CRM26 and CRM45 were prepared in the laboratory of Dr. John R. Murphy (Harvard Medical School) from culture supernatants of *Corynebacterium diphtheriae* strains C7(β^{tox-2}) and C7(β^{tox-45}), respectively, by methods previously described (14). *N*-Succinimydyl-3-(2-pyridyldithio)propionate (Pierce Chemical) was used to modify amino groups on the CRM45 molecule by replacement with neutral 2-pyridyldithio(propionate) (PDP) groups. Modification was carried out as described before (9) varying the PDP to CRM45 ratio from 2:1 to 6:1.

TRH–CRM45 was prepared as previously described from acetylcystaminyl-TRH and PDP–CRM45 (Bacha *et al.*, 1983a). Ricin A was purchased from Worthington Diagnostics.

Protein iodination. Proteins were iodinated using the technique of Greenwood *et al.* (15), and purified by Sephadex chromatography. The specific activity of these preparations was between 2 and 3 μCi/pmole or approximately 1 atom iodine per protein molecule. Approximately 98–99% of the radioiodine in these preparations was associated with intact protein.

Tissue localization. Tissue:plasma ratios of iodinated test proteins were determined as previously described (10). Approximately 10^7 dpm of iodinated bovine serum albumin (BSA), CRM26, CRM45, TRH–CRM45, or ricin A was injected iv into 150- to 200-g male rats. Animals were decapitated 15 min later and blood, liver, and kidney samples taken; portions were counted immediately and the remainder frozen for subsequent chromatographic analysis. The frozen samples were homogenized and chromatographed on Sephadex G-50 to determine the fraction of radioactivity present in intact test protein which eluted in the void volume. Tissue:plasma ratios reported here were corrected to reflect the portion of total radioactivity that eluted in the void volume.

Clearance experiments. Approximately 10^7 cpm of ^{125}I-protein in 0.1 ml saline were injected into the left external jugular vein of 200- to 250-g male rats (four animals/group) anesthetized with Nembutal. At defined intervals (5, 10, 15, 20, 30, 45, and 60 min) postinjection, 0.2 ml blood was withdrawn from the right external jugular vein and immediately centrifuged. An aliquot of the serum was counted directly. Urinary excretion of the toxin was determined by flushing the bladder at the end of each time interval with 0.5 ml saline through a 23-gauge needle which had been secured in the bladder after the urethra had been ligated. The entire sample wash was counted directly. Serum and urine samples were chromatographed on Sephadex G-50 as described above to determine the percentage of high molecular weight material.

General procedures for toxicity tests. Male rats (150–175 g; Charles River Breeding Labs), four or five animals per group, were given iv injections of varying doses [0.08, 0.4, 2, 10, or 50 μg/100 g body wt (bw)] of CRM45 or TRH–CRM45 diluted in normal saline. Controls received saline alone. Rats were weighed daily for 1 week. In a later experiment animals were injected with 50 μg/100 g bw of CRM26, CRM45, PDP–CRM45, TRH–CRM45, or ricin A diluted in normal saline. Controls received saline alone. Animals were weighed daily for 1 week and then decapitated. Blood was collected; serum was separated and frozen for later analysis.

For long-term studies, groups of animals as designated below were weighed at the intervals shown for 1 month following injection. Blood samples were taken from the external jugular under ether anesthesia at weekly intervals.

Kidney and liver function tests. Serum creatinine, blood urea nitrogen (BUN), alkaline phosphatase, serum glutamic oxaloacetic transaminase (SGOT), and γ-glutaminyl transpeptidase (γ-GTP) measurements were performed on serum samples using kits from Sigma Chemical.

Statistical analysis. Statistical comparisons of the means of groups in all cases reported here were made using Student's *t* test.

TABLE I. TISSUE:PLASMA RATIOS OF RADIOIODINATED BSA, CRM26, CRM45, TRH-CRM45, AND RICIN A 15 min AFTER iv INJECTION, CORRECTED FOR THE UNDEGRADED FRACTION IN THE LIVER AND KIDNEY

	Kidney	Liver
BSA[a]	0.085 ± 0.019	0.088 ± 0.005
CRM26	12.671 ± 1.219	0.378 ± 0.020
CRM45[a]	1.935 ± 0.158	0.151 ± 0.011
TRH-CRM45[a]	1.752 ± 0.116	0.152 ± 0.004
Ricin A	0.581 ± 0.017	0.555 ± 0.045

Note. Values are mean ± SEM. $P < 0.005$ versus BSA for all values.

[a] Data previously reported (Endocrinology **113**:1072, 1983).

Results. *Tissue concentrations of BSA and the toxin-related proteins.* The tissue:plasma ratios in the kidney and liver for BSA, CRM26, CRM45, TRH-CRM45, and ricin A are shown in Table I. These data have been corrected for the portion of radioactivity associated with intact protein as described under Materials and Methods. BSA was used in this experiment as a control protein which would not be specifically concentrated in any tissue. The 15-min accumulation of each of the toxin-related proteins was significantly higher than that of BSA in both the liver and the kidney indicating that the toxins are concentrated by these organs. For example, there is approximately 150 times more CRM26 found in kidney relative to serum than BSA during this time period. The accumulation of CRM45 and TRH–CRM45 in the kidney was less (approximately 25 times that of the BSA control) while ricin A had a value only 5 times greater than BSA. Liver concentrations of the four toxins relative to BSA were much less than those in the kidney. They ranged from 2 times greater for CRM45 to 5 times greater for ricin A.

Clearance of CRM45 and ricin A from the circulation. The disappearance of radioiodinated CRM45 and ricin A from blood consisted of an initial rapid phase due to dilution of the label into the plasma and tissue fluids followed by a later slow phase indicative of slow degradation (Fig. 1). Chromatographic studies of serum samples revealed that the amount of radioactive material present in the form of intact CRM45 or ricin A initially decreased with time and then leveled off so that by 20 min only 30% of the total radioactivity measured in both cases was intact protein. The raw data were corrected by subtracting the low molecular weight radioactive material from the slow component and then subtracting the extrapolated slow component from the fast component (Fig. 1). These corrections gave $t_{\frac{1}{2}}$ values for the fast components of the CRM45 and ricin A curves of 5.5 and 7.5 min, respec-

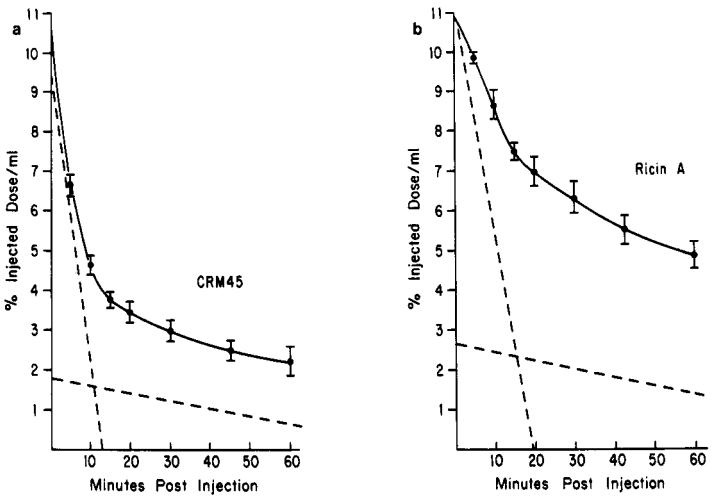

FIG. 1. Clearance rates of [^{125}I]CRM45 (a) and [^{125}I]ricin A (b) from blood over the first hour post-iv injection. Radioactivity remaining in the circulation is expressed as a percentage of the total injected dose/ml serum. Values are the means ± SEM (four animals per group). Solid lines represent raw data. The dashed lines are the corrected data.

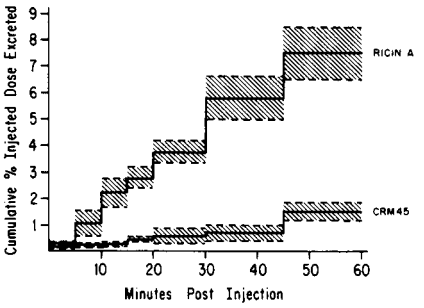

FIG. 2. The excretion of radioactivity in the urine over the first hour after iv injection of [^{125}I]CRM45 and [^{125}I]ricin A. Values are expressed as a percentage of the total injected dose excreted in the urine. Cumulative values shown are the means ± SEM (four animals per group).

tively, which is comparable to the initial half-lives in other systems (16–18). The $t_{\frac{1}{2}}$ values for the slow components were 48 and 60 min, respectively. During the 60-min time period of the study, very little radioactive material was excreted in the urine (1.5% of the total injected dose for CRM45, 8% for ricin) (Fig. 2). In contrast to the material in the blood the radioactive material in the urine at all time points consisted entirely of very small fragments.

Systemic toxicity of the toxin-related proteins. The effect of varying doses of CRM45 and TRH–CRM45 on the growth rate of rats was evaluated in short-term (1 week) and long-term (4 week) experiments. Weight gain in animals treated with doses greater than approximately 2 μg/100 g bw was significantly suppressed 1 week post-iv injection of the toxin in comparison to control animals (Fig. 3). The highest dose of CRM45 and TRH–CRM45 was further tested by following weight gain, as well as liver and kidney function with specific blood tests. CRM26 and ricin A were also included in this study. Data from these experiments are included in Table II. Control animals had an average weight gain of 65 ± 8 g and creatinine and BUN levels of 0.48 ± 0.07 and 19.6 ± 0.6 mg/100 ml, respectively. CRM26 appeared to have no effect on growth rate or liver and kidney function, even at a dose of 50 g/100 g bw. In contrast, CRM45 at this dose caused rapid weight loss (−29 ± 7) and markedly elevated creatinine (1.21 ± 0.14) and BUN (56.4 ± 8.5) levels indicative of severe renal damage. Liver function tests of treated animals showed normal alkaline phosphatase and −GTP levels while SGOT was slightly elevated. A lower dose of CRM45 (10 μg/100 g bw) appeared to have caused less but still significant damage while renal toxicity with high dose TRH–CRM45 was moderate with only slightly elevated creatinine (0.81 ± 0.18) and BUN (26.7 ± 2.8) levels in conjugate-treated animals. Animals treated with 50 μg/100 g bw ricin A had normal blood studies but there was some evidence of systemic toxicity since they did not gain as much weight during the experimental period as did the controls.

Microscopic examination of sections of kidneys stained with hematoxylin and eosin from CRM45-treated animals showed damage primarily in the cortical region with some infiltration seen. The proximal tubules were

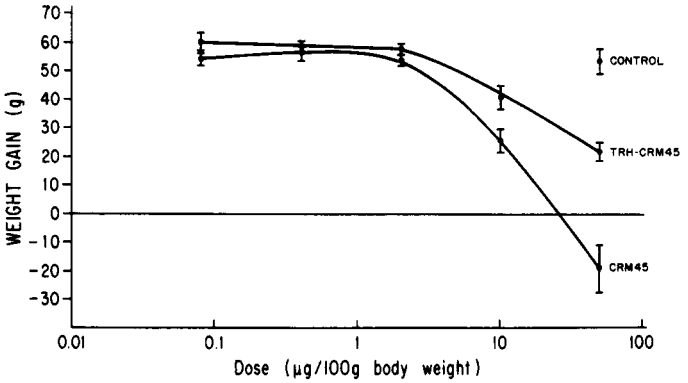

FIG. 3. Weight gain of rats 1 week after iv injection of CRM45 or TRH–CRM45 at the indicated doses. Values shown are the means ± SEM (four animals per group).

TABLE II. BODY WEIGHT AND KIDNEY AND LIVER FUNCTION TESTS 1 WEEK POST-TREATMENT WITH 50 μg/100 g BODY WEIGHT CRM26, CRM45, TRH-CRM45, AND RICIN A

	Weight gain (g)	Serum creatinine (mg/100 ml)	BUN (mg/100 ml)	Alkaline phosphatase (u/l)	SGOT (u/l)	γ-GTP (u/l)
Saline	65 ± 8	0.48 ± 0.07	19.6 ± 0.6	390 ± 49	99 ± 10	4 ± 1
CRM26	58 ± 1	0.43 ± 0.02	18.0 ± 0.8	381 ± 31	101 ± 11	5 ± 1
CRM45[d]	4 ± 6[a]	0.70 ± 0.03[c]	24.6 ± 0.5[c]	356 ± 22	111 ± 9	3 ± 1
CRM45	−29 ± 7[a]	1.21 ± 0.14[a]	56.4 ± 8.5[b]	332 ± 23	169 ± 21[b]	5 ± 2
TRH-CRM45	28 ± 7[a]	0.81 ± 0.18[c]	26.7 ± 2.8[c]	391 ± 33	138 ± 6[b]	3 ± 1
Ricin A	48 ± 1[c]	0.54 ± 0.05	18.5 ± 0.8	403 ± 46	125 ± 14	2 ± 1

Note. Values are mean ± SEM.
[a] $P < 0.005$ versus saline.
[b] $P < 0.01$ versus saline.
[c] $P < 0.05$ versus saline.
[d] Dose is 10 μg/100 g body weight.

damaged while glomeruli and distal tubules remained intact. When treated animals were followed closely for one month we observed a rapid weight loss and elevated kidney function tests over the first week postexposure to the toxin followed by slow improvement over the next few weeks. The growth rate returned to normal by the second week of the experiment though both the creatinine and BUN levels remained slightly elevated 4 weeks after the injection of CRM45 (Fig. 4).

Structural determinants of nephrotoxicity of CRM45-related toxins. From our *in vivo* studies with TRH–CRM45 shown in Fig. 1 and Table II, it was clear that this modified molecule of CRM45 was not as nephrotoxic as CRM45 alone although its organ distribution was identical. In the synthesis of TRH-CRM45, a single PDP group is added to the CRM molecule so that a disulfide crosslink can be constructed between CRM45 and a modified TRH molecule that contains a free sulfhydryl group. The PDP group replaces an amino group on the protein, thereby substituting a bulky neutral moiety for a positively charged one. We examined the potential toxicity of a series of PDP-modified CRM45 molecules (Table III). Adding increasing numbers of PDP groups to CRM45 slightly lowered its intrinsic enzymatic activity as measured by *in vitro* techniques previously described (19, 20) but the nephrotoxicity of the modified compounds was dramatically reduced. Treatment of animals with a PDP–CRM45 molecule with only two PDP groups which contained 90% of the activity of the native molecule did not cause a significant increase in the animals' creatinine and BUN levels.

Discussion. We have previously shown that the hormone–toxin complex TRH–CRM45 is bound specifically to the pituitary but is also localized in the liver and kidney following systemic injection (10). In this study we determined whether this localization led to tissue damage. Several toxin fragments were also evaluated; CRM45 to determine the role of the TRH link in potentially causing systemic toxicity; CRM26 (a diphtheria toxin fragment that lacks both the binding and the hydrophobic domain), and ricin A, a plant toxin used by many workers to construct ligand–toxin hybrid.

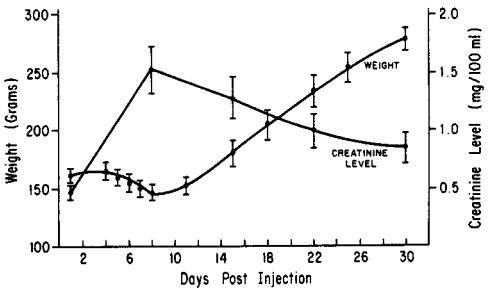

FIG. 4. Body weight and serum creatinine levels of rats were measured at indicated intervals for 1 month after iv injection of CRM45 at a dose of 50 μg/100 g body wt. Values shown are the means ± SEM (five animals per group).

TABLE III. WEIGHT GAIN AND KIDNEY FUNCTION TESTS 1 WEEK POST-TREATMENT WITH 50 μg/100 g BODY WEIGHT CRM45 AND PDP-CRM45 MODIFIED DERIVATIVES

	PDP group (No.)	Enzyme activity (%)	Weight gain (g)	Serum creatinine (mg/100 ml)	BUN (mg/100 ml)
CRM45	0	100	-29 ± 7^a	1.21 ± 0.14^a	56.4 ± 8.5^b
PDP-CRM45	2	90	43 ± 4^c	0.65 ± 0.02	21.4 ± 1.0
PDP-CRM45	4	70	54 ± 1	0.58 ± 0.04	20.6 ± 2.8
PDP-CRM45	6	58	62 ± 3	0.67 ± 0.02	16.7 ± 1.1
Saline	—	—	65 ± 8	0.48 ± 0.07	19.6 ± 0.6

Note. Values are mean ± SEM.
[a] $P < 0.005$ versus saline.
[b] $P < 0.01$ versus saline.
[c] $P < 0.05$ versus saline.

Our study showed that all four proteins are concentrated in the liver and kidney to varying degrees; TRH–CRM45 and CRM45 show identical patterns. In general renal processing of the various proteins is dependent on factors such as size and charge of the molecules. The fact that the toxin-related proteins studied here are all smaller than the size excluded by the glomeruli, while BSA is greater than this size may explain the increased kidney:plasma ratios of the toxins versus that of BSA. The size difference between CRM26 (26,000 mol wt) and CRM45 (45,000 mol wt) could potentially account for the greater accumulation of CRM26 in the kidney due to increased filtration and reabsorption of the smaller protein.

In toxicity experiments presented in this report relatively high doses (1000–5000 times the minimal lethal dose of diphtheria toxin in sensitive species) of CRM45 and TRH–CRM45 exhibited nephrotoxicity. The clinical response which we observed with CRM45 treatment was indicative of severe renal damage with elevated creatinine and BUN levels. Hepatic function in these animals was normal except for a slight increase in SGOT. Kidney tissue does contain some SGOT activity therefore the slight increase in this enzyme level in the treated animals may be due to the renal damage. While a review of the literature shows no mention of elevated SGOT levels in acute renal failure, there have been reports of its elevation in renal infarction and necrosis (21).

In light of these findings, tissue:plasma ratios of various toxin-related proteins and conjugates cannot be used to predict the occurrence of nonspecific toxicity. In addition studies on the clearance of these proteins from the circulation give little indication of the differences in their systemic toxicity. Both CRM45 and ricin A were rapidly eliminated from the blood in a biphasic manner. Therefore other explanations for the striking discrepancy in toxicity among the proteins studied must be sought.

The differences in nephrotoxicity between CRM26 and CRM45 may be accounted for by the fact that the latter retains the hydrophobic region of the native toxin molecule. This region has been postulated to be important in the toxin entry process into the eucaryotic cell by facilitating the molecule's passage through the cell membrane and is responsible for the ability of CRM45 to insert itself into lipid bilayers (22, 23).

To provide a possible explanation for the differences in toxicity for the kidney between CRM45 and ricin A, an examination of the mechanisms by which diphtheria and ricin toxins enter the eucaryotic cell is important. Diphtheria toxin requires an acidic transit vesicle for translocation into the cytosol while ricin toxin appears to enter via the more common intracellular endocytic vesicles (24–28). If proteins reabsorbed by the kidney tubule are taken up into vesicles with low pH then it is possible that CRM45 and not ricin A may be able to pass through the vesicular membrane and intoxicate the cell.

Protein charge can affect tubular reabsorption and therefore potentially effect its nephrotoxicity. Solling (29, 30) and Mogensen and Solling (31) have shown that infusions with

positively charged molecules such as lysine greatly reduce the reabsorption of several small proteins such as β-microglobulin and immunoglobulin light chains. Although attempts to alter the clearance of CRM45 by administering lysine failed to influence uptake (Bacha *et al.*, unpublished), the CRM45 modification experiments support the idea that a charge-dependent reuptake mechanism may be involved, since replacement of positively charged amino groups on the CRM45 molecule with bulky uncharged PDP groups drastically lowered its nephrotoxicity. Even TRH–CRM45 which has only one modified amino group followed this trend. Other substituents may prove to be even more effective.

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