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CROSS-REACTIVITY OF METALLOTHIONEINS FROM DIFFERENT ORIGINS WITH RABBIT ANTI-RAT HEPATIC METALLOTHIONEIN ANTIBODY

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SUMMARY. Rat hepatic Cd-metallothionein was purified and isolated into its two components, metallothionein 1 and 2, by disc electrophoresis. Antibodies to metallothionein 2 were generated in rabbits. The antiserum reacted with the protein and formed a single precipitin band on a double diffusion plate. By ammonium sulfate precipitations, it was found that the antiserum cross-reacted with rat hepatic metallothionein 1. Cross-reactivity of the antiserum was also observed for components of rat renal Cd-metallothionein, rabbit hepatic Cd-metallothionein and human renal metallothionein.

A low molecular weight Cd-binding protein was first isolated from equine kidney (1) and designated as metallothionein (2). The protein does not contain aromatic amino acids and one-third of its amino acids are cysteine (2). MT is an intracellular protein and is induced in animals upon exposure to Cd, Zn or Cu (3-5). In Cd poisoned animals MT-like protein also appears in plasma (6-8) and urine (8-10).

Methods to quantitate MT in tissues have been described (11,12). The protein is measured indirectly by either estimating the Hg- or Cd-binding capacity. Since Hg and Cd ions may bind to sulfhydryl groups other than those of MT, the methods lack specificity. A radioimmunoassay for MT would offer a great advantage over these methods in terms of specificity, sensitivity and precision.

Recently it was reported that antibodies to low molecular weight Cu-binding protein, purified only by Sephadex G-75 chromatography, cross-reacted with MT of similar purity (13). MTs separated by anion-exchange chromatography

Abbreviations: MT(s), metallothionein(s); FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant.

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also produced the specific antibodies in rabbits (14). Studies carried out in our laboratory show that the proteins isolated by anion-exchange chromatography can be further purified by disc electrophoresis. This report describes the specific antibody formation in rabbits against rat hepatic MT 2 and demonstrates its cross-reactivity with MTs isolated from different sources.

MATERIALS AND METHODS. In order to induce MTs in animals, male Wistar rats were injected daily with a dose of 5 μmoles $CdCl_2$ (3 μCi ^{109}Cd)/kg for 20 days, while a dose of 10 μmoles $CdCl_2$ (0.4 μCi ^{109}Cd)/kg was injected in male albino rabbits for 15 days. All injections were made subcutaneously. Human kidneys were obtained at autopsies from males older than 60 years.

The purification of MTs was carried out essentially by the method of Shaikh and Lucis (3). Rat liver and kidney, rabbit liver and human renal cortex were used as starting materials. Each sample was homogenized (20% w/v) in 5 mM Tris-HCl, pH 8.6. The homogenate was centrifuged at 105,000 x g for 90 min. The supernatant was applied on a Sephadex G-75 column (5 x 80 cm) equilibrated with 5 mM Tris, pH 8.6. The fractions exhibiting high 250/280 absorbance ratio and/or high radioactivity were combined and lyophilized. The dried sample was reconstituted in deionized water to achieve Tris concentration of 50 mM and NaCl concentration of 10 mM. This solution was passed through a DEAE Sephadex A-25 column (1.6 x 30.5 cm) equilibrated with 50 mM Tris, pH 8.6, containing 10 mM NaCl. The column was eluted with a linear gradient of 10-100 mM NaCl in the Tris buffer.

Disc electrophoresis was carried out at pH 9.4 (15). The protein bands which corresponded to MT 1 and 2 were cut out and homogenized using deionized water. After centrifugation the supernatant was lyophilized, desalted on a Sephadex G-25 column and re-lyophilized. The purified proteins were stored under vacuum at -20°. The MTs from various origins were isolated and labeled with ^{109}Cd in vitro. About 200-700 μg MT was mixed with 50 μl (18 μCi) carrier free $^{109}CdCl_2$ in 50 mM HCl at 4°. After 10 min, 0.3 ml aliquote of 125 mM borate-NaOH, pH 8.3, containing 75 mM NaCl was added and the mixture was further incubated at 4° for 20 min. Free ^{109}Cd and salts were removed by Sephadex G-25 column chromatography and the protein was lyophilized. The dry weight of each protein was determined by a Cahn electrobalance.

The preimmune serum was obtained before injection with MT. To generate antibodies, rabbits were injected subcutaneously and intradermaly with an emulsion of polyacrylamide gel slices containing 0.5 to 1.0 mg of rat hepatic MT 2, 1 ml of 0.9% NaCl and 1 ml of FIA or FCA. The first two injections were given at one week interval and contained FIA. Two weeks later, the protein was injected along with FCA. This was again followed one week later by an injection with FIA. The fifth injection after 10 days consisted of protein emulsified with 30-fold diluted FCA in FIA. For subsequent monthly injections no adjuvant was used. The animals were bled 5 days after each injection and the serum was incubated at 56° for 30 min and stored at -20°.

Double diffusion was performed with 1% agar on a glass plate at room temperature. The antigenic reactivities of various ^{109}Cd-MTs and the potency of various rabbit antisera were determined essentially by the method of Farr (16). All dilutions were carried out with 125 mM borate-NaOH, pH 8.3, containing 75 mM NaCl and 1% preimmune rabbit serum. A series of tubes were assayed in duplicate with 0.1 ml of 10-fold diluted antisera or preimmune sera and 0.1 ml of various ^{109}Cd-MTs. After incubation at 4° for 5 hr, 0.2 ml aliquote of saturated ammonium sulfate (pH 8.6) was added and mixed immed-

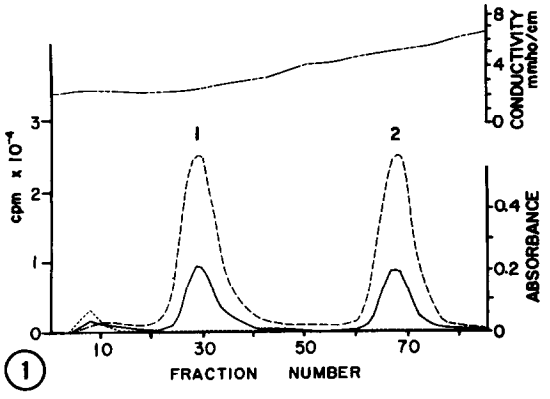
iately. After 30 min at 4° the samples were centrifuged at 15,000 x g for 4 min. The precipitate was washed with 50% ammonium sulfate, and the radioactivity was determined in a Packard scintillation counter.

RESULTS. Typical results of rat hepatic MT isolated by DEAE Sephadex A-25 chromatography are depicted in Fig. 1. The MT fraction of Sephadex G-75 was resolved into two main protein peaks, designated as rat hepatic MT peak 1 and 2, respectively. The absorbance of 280 nm was negligible in both peaks. The first peak was eluted at 2.4 mmho/cm and the second at 4.8 mmho/cm. Of the ^{109}Cd applied to the column 46 and 41% was recovered in MT peak 1 (fraction 22-44) and peak 2 (fraction 59-80), respectively. The two proteins were further purified by disc electrophoresis. The migration profiles of the protein isolated by Sephadex G-75 or DEAE Sephadex A-25 chromatography are shown in Fig. 2. Several protein bands were separated from the G-75 preparation and two major bands each from DEAE preparations. Protein bands having mobility ratios to bromophenol blue of 0.5 and 0.7 and containing about 70% of the ^{109}Cd were designated as rat hepatic MT 1 and 2, respectively.

Fig. 3 illustrates the formation of a single precipitin line when the antiserum, produced after the sixth injection of rat hepatic MT 2, was diffused against rat hepatic MT peak 1 and 2. No precipitin lines were observed when the preimmune serum was used (not shown). The titration curve of a 10-fold diluted rabbit anti-rat hepatic MT 2 serum with increasing concentration of rat hepatic ^{109}Cd-MT 2 is shown in Fig. 4. A maximum of 40% of the ^{109}Cd in the reaction mixture was precipitated.

The reaction of MTs from rat liver and kidney, rabbit liver and human kidney with rabbit anti-rat hepatic MT 2 serum is shown in Table 1. About 10-34% of the radioactivity was precipitated when the MTs of various origins were incubated with the antiserum. This indicated that MTs from different mammalian sources shared similar immunogenic characteristics.

DISCUSSION. The results of the double diffusion experiment suggested that rat hepatic MT 1 and 2 shared a similar immunogenic molecular structure. The precipitin line, however, might have been produced by aggregated forms of



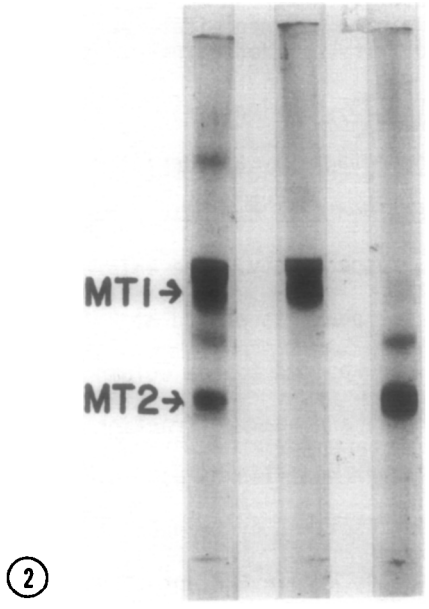


Fig. 1. Ion-exchange chromatography of rat hepatic crude Cd-MT on a DEAE Sephadex A-25 column (1.6 x 30.5 cm). (----) Radioactivity of the ^{109}Cd, (——) absorbance at 250 and (-------) 280 nm, (—·—·—) conductivity of the eluate. The flow rate was 31.1 ml/hr and 5.7 ml fractions were collected.

Fig. 2 Disc electrophoresis of rat hepatic MTs. (a) crude protein (60 μg) from Sephadex G-75 step. (b) MT peak 1 (40 μg) and (c) peak 2 (40 μg) from DEAE Sephadex step. The electrophoresis was performed for 2 hr at constant current (5 mA/tube). The proteins were stained with Amido Black 10 B.

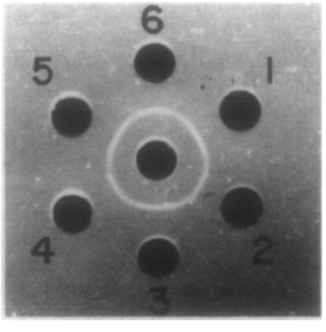


Fig. 3. Double diffusion between fresh rabbit anti-rat hepatic MT 2 serum (center well), and rat hepatic MT peak 1 (well 1, 3 and 5) and peak 2 (well 2, 4 and 6).

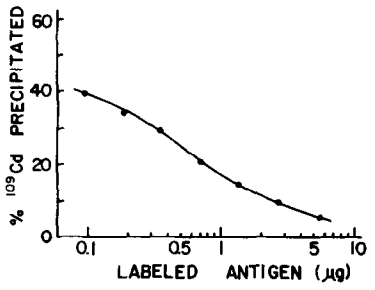


Fig. 4. Titration curve of rabbit anti-rat hepatic MT 2 serum and rat hepatic ^{109}Cd-MT 2. 0.1 ml of various concentrations (9.5 to 54.7 μg /ml) of ^{109}Cd-MT 2 was mixed with 0.1 ml of 10-fold diluted antiserum or preimmune serum. After subtracting the background radioactivity obtained by using preimmune serum, the net percent binding was plotted.

MTs, since low molecular weight proteins or peptides produce non-precipitating antibodies (17,18). The ammonium sulfate precipitation method clearly demonstrated the formation of the specific antibodies to rat hepatic MT 2. The titer, however, was not high enough to measure less than 0.1 μg of the protein.

The data presented here is the first report demonstrating the immunogenic similarities among MTs of different mammalian origins. This phenomenon may be attributable to similar conformations of these MTs. According to recent reports, the amino acid sequences of equine hepatic and renal MTs, human hepatic MT 2 and mouse hepatic MTs (19-23) are strikingly similar. The immunogenic reactivities of various preparations of MTs as described in the present report were not identical. This might have been due to the differences in their affinities for the antibody and also to varying degrees of purity.

Surprisingly, even rabbit hepatic MT peak 1 and 2 cross-reacted with the antiserum. In our laboratory MT was detected in plasma only after excessive exposure to Cd, and its appearance in the circulation was followed by nephrotoxicity (8). Injected Cd-MT has been shown to be nephrotoxic (24,25). The possible role of MT in the development of an autoimmune response and its significance in the nephrotoxicity remains to be investigated.

Our present results also showed that human MT reacted with the antiserum to rat MT. This suggests that rabbit anti-rat hepatic MT serum could be used

Table 1

Cross-reactivity of MTs with rabbit anti-rat hepatic MT 2 serum

Metallothionein	Radioactivity in Precipitate (cpm)				Net % total cpm precipitated
	Antiserum (A)	Preimmune serum (P,mean)		Net (A)-(P)	
Rat hepatic MT 1[a]	2005	423	(428)	1577	18.8
	2200	434		1772	21.1
Rat hepatic MT 2[a]	540	63	(61)	479	15.7
	543	58		482	15.9
Rat renal MT 1[a]	1008	151	(163)	845	34.4
	977	175		814	33.1
Rat renal MT 2[a]	1193	138	(136)	1057	30.3
	1178	135		1042	29.9
Rabbit hepatic Peak 1[b]	696	76	(80)	616	9.2
	731	83		651	9.7
Rabbit hepatic Peak 2[b]	521	54	(69)	452	9.8
	505	83		436	9.5
Human renal MT[c]	1051	138	(130)	921	14.6
	1091	122		961	15.2

(a) Rat hepatic MT 1 (83900 cpm/ml) and 2 (30420 cpm/ml), renal MT 1 (24570 cpm/ml) and 2(34870 cpm/ml) from the disc electrophoresis step.
(b) Rabbit hepatic peak 1 (66840 cpm/ml) and 2 (46090 cpm/ml) from the DEAE Sephadex step.
(c) Human kidney MT (63020 cpm/ml) from the Sephadex G-75 step.

The protein concentration in each preparation was 10μg/ml.

to measure human MT. Establishment of a radioimmunoassay would be of great use for the evaluation of health status of people exposed to cadmium compounds in their living and work environments (8).

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