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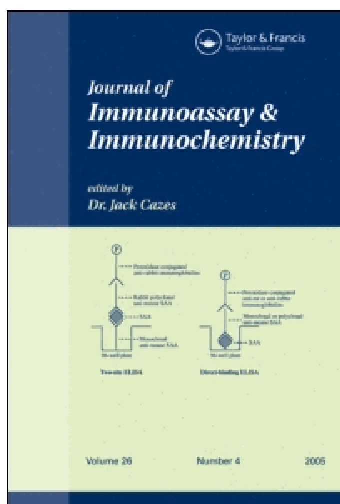

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DEVELOPMENT OF A COMPETITIVE DOUBLE ANTIBODY RADIOIMMUNOASSAY FOR RAT METALLOTHIONEIN

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ABSTRACT

A competitive double antibody radioimmunoassay (RIA) for rat metallothionein (MT) has been developed that has a detection limit of 100 pg and a range of 100 to 100000 pg. The antibody was raised in rabbits against rat MT-2 but it crossreacts equally with MT-1 and MT-2. However, when the assay is done in the presence of 2-mercaptoethanol the antibody is more specific for MT-2. Zn- and Cd- saturated MTs have similar responses in the assay. Addition of Cu(II) to Zn-MT (more than 6 mol Cu/mol MT) in non-reducing conditions modifies the reponse of the antibody, probably because of Cu(II) oxidation and later MT polymerization. Standard curves developed in the presence of cytosols from brain cortex, hypothalamus or liver did not differ from the standard curve, indicating the absence of interfering substances in the assay. Furthermore, serial dilutions of those cytosols paralleled the response of the standard curve, indicating that the response of the antibody was specific. For comparison, MT levels in some brain areas measured with the present RIA were compared with those measured with an established RIA. In addition, the expected effect of dexamethasone and stress on liver MT levels was clearly identified by this RIA. The results suggest that the present RIA can be used for quantitation of metallothionein.

(KEY WORDS: Metallothionein; radioimmunoassay; brain; liver; stress; dexamethasone)

INTRODUCTION

Metallothionein (MT) is a low molecular weight, cysteine-rich protein that lacks aromatic aminoacids and is present in a

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wide variety of species (1), principally in the liver but also in many other tissues. There are isoforms of this protein in most vertebrate species; in rodents, two isoforms have been characterized, MT-1 and MT-2. Metals ions, especially zinc, copper and cadmium are bound to MT in different proportions depending on the species, the tissue and the environment. MT can be induced by a variety of stimuli such as heavy metals, hormones or stress (1-4). However, the precise physiological role is not fully understood.

Several analytical techniques have been used to measure MT, including gel filtration chromatography, SDS-page, HPLC, metal saturation and polarographic techniques (1,5-9). However, these techniques have some lack of sensitivity and specificity, problems solved with the use of specific radioimmunoassays (RIA) that have been developed in a few laboratories (10-14). We have used two of those RIA's extensively (4,15-18), and have developed a competitive double antibody RIA following established procedures (references above) as a tool for measuring MT levels in further studies, especially in experiments where the MT levels are reduced (e.g., from cell culture experiments).

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MATERIAL AND METHODS

Isolation of rat metallothionein.

The two isoforms of MT (MT-1 and MT-2) were isolated from cytosols of rat (male Sprague-Dawley) livers that had been injected intraperitoneally with $CdCl_2$ (1 mgr Cd/kg b.w.) for 3 days. The rats were killed and the livers excised and stored at -85°C. Later, they were homogenized in a Potter-Elvehjem with ice-cold 10 mmol/L Tris-HCl, pH 8.2, containing 250 mmol/L sucrose, 10 mmol/L sodium azide, 10 mmol/L 2-mercaptoethanol and 0.1

mmol/L phenyl methylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 50,000g for 20 min at 4°C and MT was saturated with Cd by adding 25 µgCd/ml cytosol. Liver supernatant was fractionated by acetone precipitation (19); the 60-80% acetone precipitation was resuspended, clarified by centrifugation, and applied to a Sephadex G-75 column (2.6x165 cm). The proteins were eluted at 4°C with 10 mmol/L Tris-HCl, pH 8.2, at a flow rate of 0.55ml/min. The fractions from the MT peak were pooled and rechromatographied in a DEAE-Sephadex A-25 column (1x4 cm) which was preequilibrated with 10 mmol/L Tris-HCl, pH 8.2, and the proteins were eluted with a linear gradient from 10 to 150 mmol/L Tris-HCl, pH 8.2, at a flow rate of 1.44 ml/min. The MT peaks were lyophilized and then desalted in a Sephadex G-10 gel filtration column (1x60 cm).

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MT saturated with Zn was obtained from rats daily injected with $ZnSO_4$ (20mg Zn/kg b.w.) for 4 consecutive days and following the above-stated protocol, except that Cd was not added to the cytosol.

Purity of MT samples was tested by gel electrophoresis and high-performance liquid chromatography (HPLC). SDS-PAGE was performed as described by Laemmli (20) and gels were stained with silver nitrate as described by Morrissey (21). Reverse-phase HPLC was performed essentially as previously described (7).

For determining MT concentration, Cd, Zn, and Cu levels of the purified MTs were measured by atomic absorption spectrometry and by absorbance of MT dissolved in HCl (10 mmol/L), using the published molar extintion coefficient (22).

Production of the primary antibody.

Rat Cd7-MT 2 isoform prepared in this way was found to be essentially pure and was used for rabbit anti-rat MT 2 antisera

production. MT (0.2 mg for each rabbit) was conjugated with bovine IgG as previously described (23) and emulsified with Freund's complete adjuvant for injecting into three female rabbits with multiple site injections (subcutaneous and intramuscular).

Primary injections were given at 0, 2 and 4 weeks, followed by boosters at 12, 24 and 48 weeks after the first injection. Serum was obtained by bleeding rabbit ears once a week after boosters and tested for the presence of antibody against MT. Blood obtained was incubated at 37ºC for two hours, centrifuged (4000 rpm), and then sera were aliquoted and stored at -85°C.

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Labelling of MT-2

MT-2 was iodinated essentially as described by the method of Bolton and Hunter (24) and later modified (10), e.g., the conjugation of the protein with N-succinimidyl-3-(4-hydroxy-phenyl) propionate (NSHPP) was performed before the radioiodination of the protein by the chloramine T reaction. NSHPP must be used because MT has no aromatic amino acids.

NSHPP (8 mg) was dissolved in 50 ml of 50% (v/v) ethyl acetate and toluene. For the reaction of NSHPP and MT the molar ratio was 10 mols NSHPP/1 mol MT. Thus, 192 µl of the dissolved NSHPP were placed into an Eppendorf tube and dried under a gentle stream of nitrogen at room temperature. Seventy four µg of MT, dissolved in 100 µl Tris HCl buffer, 50 mmol/L, pH 8, were added, and the reaction mixture was maintained in an ice bath for 30 min and vortexed for 10 sec at 5 min intervals. The reaction was stopped by the addition of 1 ml sodium phosphate buffer, 50 mmol/L, pH 7.5. The NSHPP-MT conjugate was purified in a 1x40 cm Sephadex G-25 column previously equilibrated with sodium

phosphate buffer, 50 mmol/L, pH 7.5, and eluted with the same buffer at 4°C. Twenty fractions of 20 drops were collected by gravity flow. The protein was detected by UV spectrometry at 254 nm; the fractions containing the NSHPP-MT (45-50 μg MT) were dried with a gentle stream of nitrogen to obtain a final concentration of 0.3-0.5 g MT/5-10 L.

To the 10 μl of NSHPP-MT, 10 μl of sodium phosphate buffer, 250 mmol/L, pH 7.5, were added, followed by the addition of 1 mCi of ^{125}I and 20 μl of chloramine T (2.5 g/L in sodium phosphate buffer, 250 mmol/L, pH 7.5). Thirty seconds later 100 μl of sodium metabisulphite (1.2 g/L in sodium phosphate buffer, 50 mmol/L, pH 7.5) were added to stop the reaction.

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Labelled MT was separated from unreacted iodide by gel filtration in a 1x40 cm Sephadex G-25 column, equilibrated and eluted with Tris buffer, 50 mmol/L, pH 7.5, containing 1 g/L gelatin. Twenty drop fractions were collected. To identify the peak protein, a 10 μl sample of each fraction was counted for 1 min in a gamma counter. The radioactivity incorporated into the protein was determinated by the addition of 500 μl of Tris-gelatin buffer and 500 μl of tannic acid (10% tannic acid in 1 mol/L HCl). The tubes were mixed and incubated for 10 min at 4°C, and then were centrifuged at 10000g for 15 min. Those 2-3 tubes of the peak that showed more than 95% protein bound radioactivity were pooled and diluted using Tris-gelatin buffer containing 2 g/L sodium azide and frozen until use. The specific radioactivity of the ^{125}I-labelled protein was generally 120-130 Ci/g MT.

Competitive binding radioimmunoassay.

The assay was performed as follows: 300 μl of TrisHCl-gelatine buffer, 50 mmol/L, pH 8.0, with 1 g/L gelatine and 1 g/L

sodium azide, 100 µl of unknown or standard, and 50 µl of first antibody solution (final dilution: 1 in 6400), which contains EDTA 50 mmol/L and normal rabbit serum 25 ml/L, were incubated for 48 h at 4°C. The tracer was then added (50 µl of labelled MT-2, about 200 pg of MT or 15000 cpm) and further incubated at 4°C for 8 h. The second antibody solution (100µl of 1:9 diluted goat-anti-rabbit antibody in Tris-gelatine buffer) was then added and the tubes were incubated overnight at 4°C. The assay tubes were centrifuged at 4000 rpm for 60 min after the addition of 2 ml of Tris-HCl buffer, 50 mmol/L, pH 8.0, the supernatants aspirated and the pellets counted in a gamma counter.

Standard curves were usually plotted using Logit-log data transformation.

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Tissues preparation.

Basal rats were killed by decapitation and livers and brains were immediately removed and frozen at -85°C. Livers were homogenized in a Potter-Elvehjem with Tris-HCl buffer, 10 mmol/L, pH 8.2, containing 2-mercaptoethanol 2 mmol/L, sucrose 250 mmol/L, sodium azide 10 mmol/L and PMSF 0.1 mmol/L. Brain areas were dissected out, homogeneized by sonication with 1 ml of the same buffer used for liver and frozen again. The homogenates of both liver and brain areas were centrifuged at 50,000 g for 1 hour at 4°C. The supernatats were stored frozen. Cytosols from liver, cerebellum and brain cortex were used for RIA validation. In addition, levels of MT measured by the present RIA (shown as pgMT/g tissue) in different brain areas were compared with those obtained for MT-I with the RIA developed by Bremner's laboratory (12).

Two experiments were made to check the reliability of the present RIA. In one, some rats were injected with dexamethasone

(2 mg/kg) and were killed 8 hours later; control rats received saline. In the other experiment, rats were subjected to 14 hours of immobilization stress (4) and then killed along with control rats.

Statistical analysis.

Results were evaluated with the Student "t" test, one-way or two-way analysis of variance (ANOVA). When multiple comparisons were made a posteriori, the SNK (Student-Newman-Keuls) procedure was used. Data were log transformed when needed to achieve homogeneity of variances.

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RESULTS AND DISCUSSION

Sephadex G-75 profile from the 60-80% acetone fractionation of rat liver, as expected (25), showed a single major protein peak corresponding to MT, characterized by a high absorbance at 250nm (Cd-thiolate bonds) and a minimum at 280 nm due to an absence of aromatic amino acids (not shown).

The MT peak was applied to a DEAE-Sephadex column and MT-1 and MT-2 were eluted as described in Methods. As expected (1,2), 2 peaks corresponding to the 2 MT isoforms, MT-1 and MT-2, were obtained (not shown).

The purity of the isolated MTs was checked by SDS-PAGE (not shown) and by reverse phase HPLC (Fig. 1a and 1b). MT-2 was found to be considerably purer than MT-1 by both methods, and therefore was the isoform selected for raising the polyclonal antibodies. The HPLC profiles obtained are comparable to published results (7).

Three rabbits were immunized with conjugated MT-2 as described in Methods. One of the bleedings was selected for the

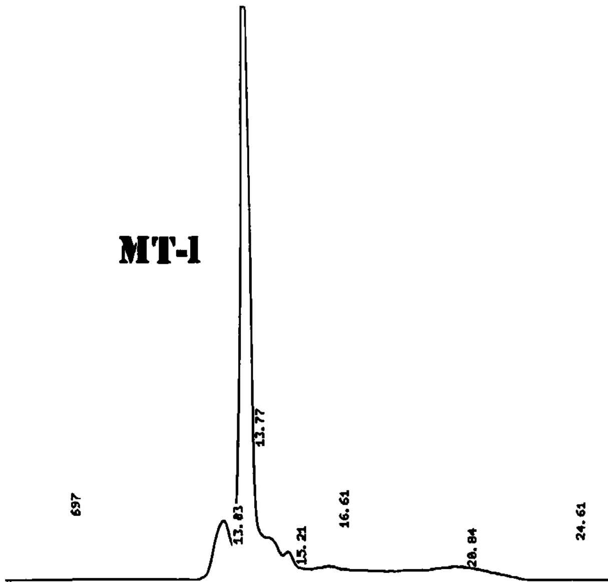

Figure 1.a. Reversed-phase high-performance liquid chromatography of the MT-1 peak from the DEAE-Sephadex A-25 chromatography in a C-18 column (1Ø μ particle size). The proteins were eluted with a two-step linear gradient of Ø-1Ø% B for Ø-5 min followed by 1Ø-2Ø% B from 1Ø-3Ø min at room temperature and at a flow rate of 1 ml/min. Buffer A was 1Ø mmol/L sodium phosphate, pH 7.Ø, and buffer B was 6Ø% acetonitrile in buffer A. The effluent of the column was continuously monitored at 25Ø nm.

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present study of the development of a RIA for rat MT. Serial dilutions of the serum indicated that an antiserum dilution of 1/64ØØ bound near 3Ø% of labelled antigen in the first two weeks after the preparation of the tracer. This percentage decreased progressively with time because of the deterioration of the tracer, but the results were still acceptable 6 weeks after its preparation. The titre of our antibody is therefore less than reported for other antibodies (1Ø,12). Our nonspecific binding was around 3%, a value comparable to the established RIAs.

Figure 2 shows a typical standard curve for MT-1 and MT-2 using the RIA protocol described in Methods; this double-

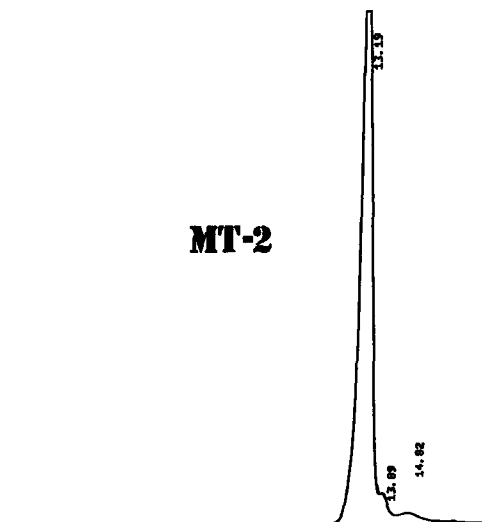

Figure 1.b. RP-HPLC of the MT-2 peak under the same conditions. As expected, MT-2 elutes before than MT-1, and, in agreement with the SDS-PAGE analysis, was more pure than MT-1.

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antibody, competitive RIA has a detection limit of 1,000,000-2,000,000 pg/L as determined by serial dilutions of MT, and a range of 1 µg/L to 1,000 µg/L of MT, with an interassay coefficient of variation of 10-15% for most MT amounts within the range of the curve, and an intraassay coeficient of variation of about 5%. The inhibition curves suggest that the antiserum has equal affinity for the two rat isoforms, a result previously reported for other antibodies (10,11,14), and therefore a 50/50% mixture of MT-1 and MT-2 is being used in the standard curves in normal conditions.

Figure 3 indicates that standard curves developed in the presence of liver, as those developed in the presence of brain tissue (not shown), are not different from those without tissue addition, indicating that there are no tissue matrix effects.

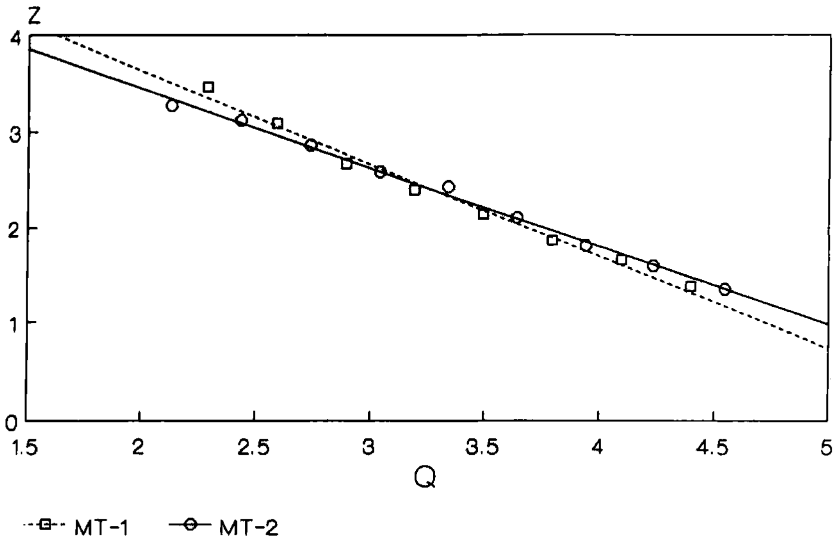

Figure 2. Logit-log plots for radioimmunoassay ussing MT-1 (▪) and MT-2 (o) inhibitory curves with standards ranging from 100 to 50000 pg/tube. The two isoforms have identical behavior.

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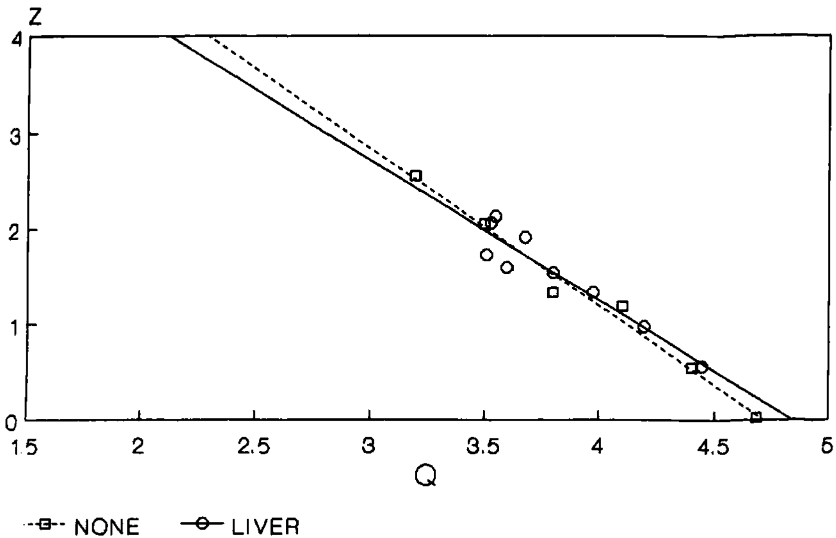

Figure 3. Logit-log plots for MT without (▪) or with addition (o) of liver cytosol. The curve with liver cytosol addition was plotted considering the actual amount of competitor in the X axis that results after including the endogenous MT content.

This is in agreement with other established RIAs. By contrast, for the quantitation of sera, standard curves must be set up in the presence of serum (not shown). In addition, serial dilutions of cerebellum, cortex and liver cytosols have parallel slopes with that of the control standard curve (not shown), indicating identity of a substance in the tissues with purified MT.

Fig 4 shows the effect of the metal (Zn, Cd, Cu) bound to MT in the RIA. Zn-MT and Cd-MT showed the same response in the assay, as reported by others (10,12). The results for Cu are not definitive, since more experimental work has to be done. We incubated Zn-MT with different amounts of Cu(II). This metal is known to have higher affinity for MT than Zn (1,2); however it is also known that Cu(I) is bound by the protein in physiological conditions with a stoichimetry of up to 12 Cu per molecule of MT (1,2). Cu (II) is bound by the protein (7 Cu(II) per molecule), but it tends to be oxidized to Cu(I) causing the polymerization of MT if reducing conditions are not maintained. In our assay we did not maintain reducing conditions, and therefore it is expected that such an event was taking place, which could explain why MT incubated with increasing Cu (II) amounts showed increasing responses in the RIA, an effect already described by others (26). Since in most physiological conditions MT is not saturated with Cu but is mixed with Zn (1,2), it seems that the present antiserum can be used in most conditions for MT quantification.

In the RIA developed by Mehra and Bremner (12) 2-mercaptoethanol is used in the assay, in spite of the fact that the binding capacity of the antiserum decreases considerably, an effect also seen by Nolan and Shaikh (26). We found that 2-MSH depressed the binding capacity of the antiserum, and a

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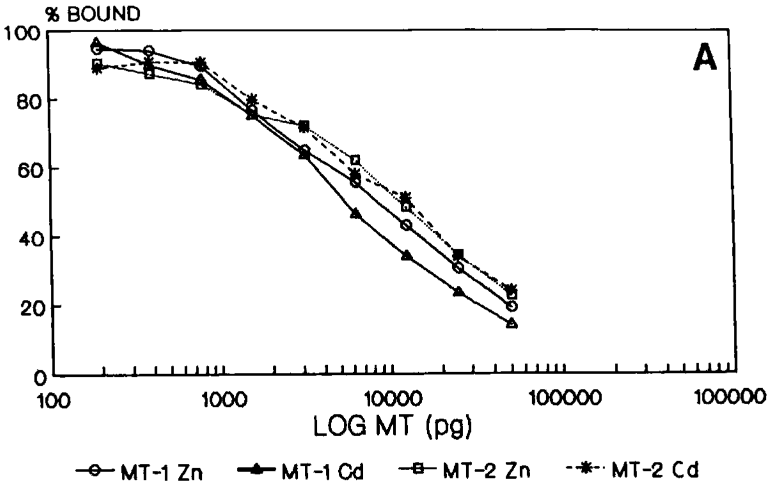

Figure 4.a. Percent bound as a function of log MT is shown for standard curves using Zn and Cd saturated metalloforms of both MT isoforms as competitors. MT-1 Zn (o), MT-1 Cd (Δ), MT-2 Zn (▪) and MT-2 Zn (*).

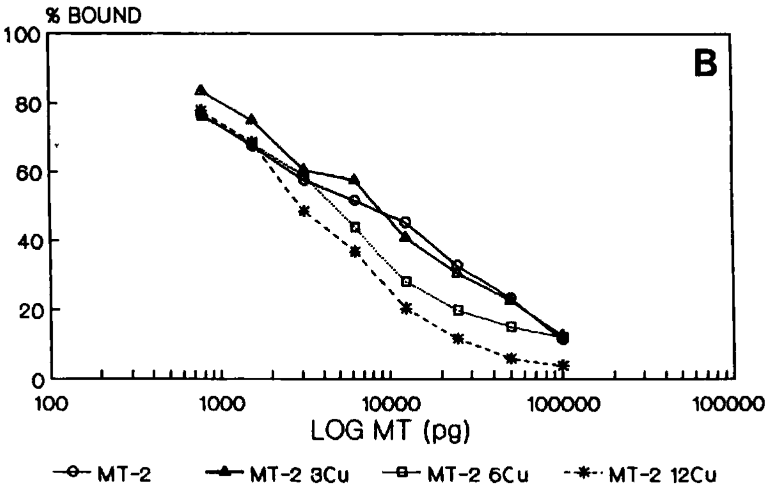

Figure 4.b. Percent bound as a function of log MT is shown for standard curves after addition of copper (II) to Zn-7 MT-2 at different molar ratios: Ø (o), 3 (Δ), 6 (▪), and 12 (*) mol Cu(II) per mol of Zn7-MT-2. The results for MT-1 were comparable.

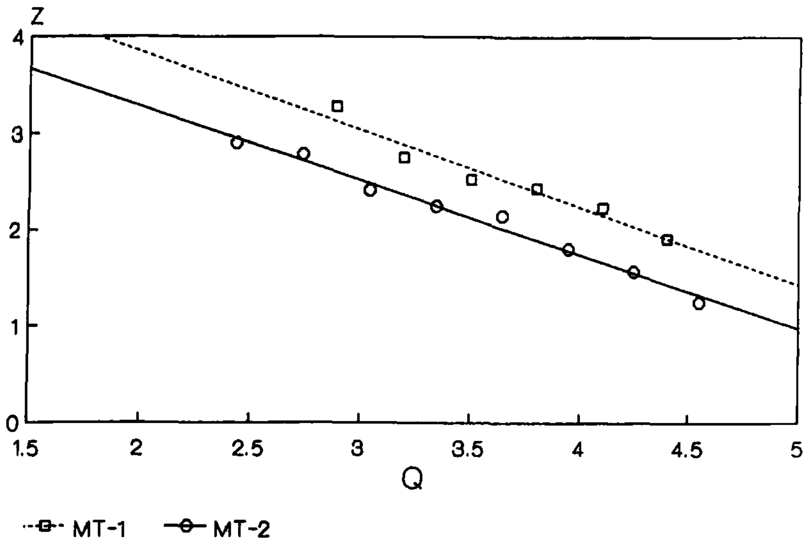

Figure 5. Logit-log plots for MT-1 (▪) and MT-2 (o) in the presence of 10mM 2-mercaptoethanol (final concentration) added along with the tracer. In these conditions, the antibody is more specific for MT-2.

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significant shift was seen in the behavior of the antibody against the two MT isoforms (Fig 5). Thus, in the presence of 2-MSH 600 pg of MT-2 caused the same inhibition as that of 3000 pg of MT-1, indicating that in the quantification of unknowns MT-2 would be the isoform mostly measured.

For comparison (Table 1), MT levels in brain areas were measured with our RIA and were compared with those measured with an established RIA for MT-I (12). The MT-II/MT-I ratio appears to differ between different brain areas (MT-I+MT-II/MT-I= 1.64, 3, 2.36 and 4.4 for pons+medulla, frontal cortex, hippocampus and hypothalamus, respectively), suggesting functional differences between the MT isoforms that deserves further attention.

Table 2 shows the effect of dexamethasone and immobilization stress on liver MT levels. As expected (1,4),

TABLE 1

Comparison of MT-1 and MT-1+MT-2 levels (ng MT/g tissue) measured by RIA*

Rat	Pons+medulla		Frontal cortex		Hippocampus		Hypothalamus	
	MT-1	MT-1 + MT-2	MT-1	MT-1 + MT-2	MT-1	MT-1 + MT-2	MT-1	MT-1 + MT-2
1	647	1282	850	3929	2711	6488	2340	8564
2	529	1529	883	3359	2637	6121	1000	7611
3	681	812	998	3061	2527	4926	2340	8999
4	1049	1489	1509	3818	1489	4929	2380	11334
5	1038	1352	903	2944	2486	5391	2180	8442
6	958	1573	1366	3059	2212	5324	1900	9301

* MT-I levels were measured in the laboratory of Dr. Ian Bremner.

MT-I+MT-II levels were measured with the present RIA.

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TABLE 2

Effect of dexamethasone and stress on liver MT levels

	Liver MT (μg/g)
Exp. 1	
Saline	12.1±1.72
Dexamethasone	24.1±2.80 *
Exp. 2	
Control	9.4±1.23
Stress	186.3±19.5 *

Results are mean±SE (n=8-10). * $p<0.01$

dexamethasone had a slight effect (two-fold induction), in contrast to immobilization stress (twenty-fold induction). The present RIA, therefore, appears to be reliable in that it identifies the expected increases of liver MT induced by known factors such as dexamethasone and stress.

In sum, the present results suggest that the polyclonal antibody raised is suitable for MT quantification in biological fluids by means of a double antibody RIA. In addition to brain and liver tissue, we have used it succesfully for kidney and for cultured hepatocytes and astroglia (not shown). Preliminary data indicate that this antibody is also suitable for developing an ELISA for rat MT and for immunocytochemistry.

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