Trabajo 2

Metallothionein (MT)-III: generation of polyclonal antibodies, comparison with MT-I+II in the freeze lesioned rat brain and in a bioassay with astrocytes, and analysis of Alzheimer's disease brains

Journal of Neurotrauma ***16****: 1115-29, 1999*

JOURNAL OF NEUROTRAUMA
Volume 16, Number 11, 1999
Mary Ann Liebert, Inc.

Metallothionein (MT)-III: Generation of Polyclonal Antibodies, Comparison With MT-I+II in the Freeze Lesioned Rat Brain and in a Bioassay With Astrocytes, and Analysis of Alzheimer's Disease Brains

JAVIER CARRASCO,[1] MERCEDES GIRALT,[1] AMALIA MOLINERO,[1] MILENA PENKOWA,[2] TORBEN MOOS,[2] and JUAN HIDALGO[1]

ABSTRACT

Metallothionein-III is a low molecular weight, heavy-metal binding protein expressed mainly in the central nervous system. First identified as a growth inhibitory factor (GIF) of rat cortical neurons *in vitro*, it has subsequently been shown to be a member of the metallothionein (MT) gene family and renamed as MT-III. In this study we have raised polyclonal antibodies in rabbits against recombinant rat MT-III (rMT-III). The sera obtained reacted specifically against recombinant zinc- and cadmium-saturated rMT-III, and did not cross-react with native rat MT-I and MT-II purified from the liver of zinc injected rats. The specificity of the antibody was also demonstrated in immunocytochemical studies by the elimination of the immunostaining by preincubation of the antibody with brain (but not liver) extracts, and by the results obtained in MT-III null mice. The antibody was used to characterize the putative differences between the rat brain MT isoforms, namely MT-I+II and MT-III, in the freeze lesion model of brain damage, and for developing an ELISA for MT-III suitable for brain samples. In the normal rat brain, MT-III was mostly present primarily in astrocytes. However, lectin staining indicated that MT-III immunoreactivity was also present in microglia, monocytes and/or macrophages in the leptomeninges and lying adjacent to major vessels. In freeze lesioned rats, both MT-I+II and MT-III immunoreactivities increased in the ipsilateral cortex. The pattern of MT-III immunoreactivity significantly differed from that of MT-I+II, since the latter was evident in both the vicinity of the lesioned tissue and deeper cortical layers, whereas that of the former was located only in the deeper cortical layers. This suggests different roles for these MT isoforms, and indeed in a new bioassay measuring astrocyte migration *in vitro*, rMT-III promoted migration to a higher extent than MT-I+II. Thus, MT-III could not only affect neuronal sprouting as previously suggested, but also astrocyte function. Finally, MT-III protein levels of patients with Alzheimer's disease (AD) were, if anything, increased when compared with similarly aged control brains, which was in agreement with the significantly increased MT-III mRNA levels of AD brains.

Key words: astrocytes; GFAP; metallothionein-I+II; metallothionein-III

[1]Departmento de Biología Celular y Fisiología, Unidad de Fisiología Animal, Facultad de Ciencias, Universidad Autónoma de Barcelona, Bellaterra, Barcelona, Spain.

[2]Institute of Medical Anatomy, Section C, Panum Institute, University of Copenhagen, Copenhagen, Denmark.

INTRODUCTION

METALLOTHIONEINS (MTs) are a low molecular weight protein family, widespread in the phylogenetic scale, characterized by their ability to bind physiological (Zn, Cu) and nonphysiological (Cd, Hg) heavy-metals (Kägi and Kojima, 1987), In rodents, this family is comprised of four isoforms (MT I-IV). MT-I and II are expressed virtually in all tissues, whereas MT-III and MT-IV are localized mainly in the central nervous system (Palmiter et al., 1992; Uchida et al., 1991) and stratified squamous epithelia (Quaife et al., 1994), respectively.

MT-III was unexpectedly discovered in the human brain as a growth inhibitory factor (GIF) of neuronal survival, an action not shared by the widely expressed MT-I+II isoforms (Uchida et al., 1991). Initially MT-III was reported to be decreased in the brains of patients with Alzheimer's disease (AD; Uchida et al., 1991), in contrast to MT-I+II, which were upregulated in this important human disease (Duguid et al., 1989). However, another study (Erickson et al., 1994) could not confirm those results, since neither the MT-III protein nor the MT-III mRNA were decreased in AD brains. Since only a few brains were analyzed in each report, more studies with additional AD brains need to be carried out. MT-III regulation has been studied in a number of animal models of brain damage (Anezaki et al., 1995; Hozumi et al., 1995, 1996; Inuzuka et al., 1996; Yamada et al., 1996; Yuguchi et al., 1995a,b), which have suggested that this MT isoform is involved in reparative and/or protective processes in the brain. Unfortunately, the MT-III response has not been compared with the MT-I+II response, which is essential in order to understand the putative physiological functions of these proteins. Thus, one of the aims of this report was to produce antibodies against rat MT-III suitable for using them in immunocytochemical studies. We have examined MT-III immunoreactivity in the freeze lesioned rat brain, an animal model of brain damage where we have characterized the MT-I+II response in detail (Penkowa and Moos, 1995). The antibody was also used for developing an ELISA that allowed us to examine the MT-III content of AD brains. Finally, we have developed a different bioassay with astrocytes in culture that open new perspectives in our understanding of the putative functions of this protein.

MATERIALS AND METHODS

Recombinant Rat MT-III Purification

E. coli 1B392 Lon Δ1 transformed with the expression construct pET-29a(+)-rMT-III coding region (generously provided by Dr. P. Kille, School of Molecular and Medical Biosciences, University of Wales Cardiff) was used to induce and purify recombinant brain rat MT-III (rMT-III). Overnight cultures (20 ml) were added to 400 ml of fresh LB media containing kanamycin (50 μg/ml) and grown at 37°C. When the culture raised 0.6 absorbance units at 600 nm, the rMT-III transcription was induced with 1 mM isopropyl β-d-thiogalactopyranoside (IPTG), and 30 min later Cd or Zn was added (300 μM). Six hours later, cells were harvested, sonicated in 20 mM Tris-HCl, pH 7.6, 3 mM 2-mercaptoethanol (2-MSH), and centrifuged at 12,000*g* for 40 min. The cytosolic fraction was heated at 80°C for 15 min and the centrifugation step repeated. The supernatant was treated with thrombin (Novagen 69671-1; 3 units/mg protein) in 20 mM Tris-HCl pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$ at 37°C overnight, in order to cleavage the S·tag™ peptide fusioned at N-terminal. Digestion products were applied to a Sephadex G-75 column (2.6 × 165 cm). Proteins were eluted in 20 mM Tris-HCl, pH 7.6. The fractions from the rMT-III peak were pooled and rechromatographied in DEAE-Sephadex A-25 column (1 × 10 cm) equilibrated with 20 mM Tris-HCl, pH 7.6. Recombinant rMT-III was eluted with a 0–200 mM NaCl linear gradient in 20 mM Tris-HCl, pH 7.6. Each purification step was monitored by SDS-PAGE and atomic absorption spectrometry (AAS). The rMT-III obtained was evaluated by UV absorption spectrophotometry, SDS-PAGE, and mass spectrometry. Protein concentration of purified fractions was estimated by AAS considering 7 mol Cd (or Zn)/mol protein.

Purification of Native Rat MT-I and Rat MT-II

MT-I and MT-II were isolated from the livers of male Sprague-Dawley rats that had been injected intraperitoneally with $ZnSO_4$ (20 mg Zn/Kg b.w.) as described (Gasull et al., 1993). Briefly, livers were homogenized in a Potter-Elvehjem with ice-cold 10 mM Tris-HCl, pH 8.2, containing 250 mM sucrose, 10 mM sodium azide, 10 mM 2-MSH and 0.1 mM phenyl methyl sulfonyl fluoride. The homogenate was centrifuged at 50,000*g* for 20 min at 4°C, and the supernatant was fractionated by acetone precipitation. The 60–80% acetone precipitation was resuspended and applied to a Sephadex G-75 column (2.6 × 165 cm). Elution was done at 4°C with 10 mM Tris-HCl, pH 8.2. Fractions containing MT-I+II were pooled and applied to a DEAE-Sephadex A-25 column (1 × 4 cm), which was equilibrated with 10 mM Tris-HCl, pH 8.2. Proteins were eluted with a 10–150 mM Tris-HCl (pH 8.2) linear gradient. MT containing fractions were concentrated and desalted. Purity of samples was tested by SDS-PAGE and quantified by AAS and spectrophotometry.

Production of Recombinant MT-III Antisera

Antisera were raised following the procedure previously described for MT-I+II (Gasull et al. 1993; Hogstrand et al., 1987). In brief, 200 μg of Cd7-rMT-III were conjugated to 400 μg of bovine IgG (molar ratio 11:1) with glutaraldehyde, diluted to 250 μL with 50 mM phosphate buffer pH 7.5 (PB) and allowed to react at 37°C for 2 h. The reaction products were brought to 2 ml in 50 mM PB and emulsified with an equal volume of Fruend's complete adjuvant and injected intramuscularly and subcutaneously in multiple sites into three female rabbits. Booster injections were given 9, 14, and 30 weeks after the first injection. Sera were obtained by bleeding animals ears 10 days after each immunization and periodically after the last immunization. Blood obtained was incubated at room temperature for 2 h, centrifuged (2,000*g*), and the serum stored at −20°C.

Detection of Antibodies and Development of an ELISA for MT-III

Sera were regularly checked by noncompetitive and competitive double-antibody ELISA assays. In the noncompetitive assay, increasing quantities (5–80 ng) of rMT-III or native rat MT-I+II were used for coating wells; this was achieved by incubating the plates with appropriate protein solutions in 100 mM carbonate buffer pH 9.6 overnight at 4°C. Plates were washed three times with 0.05% Tween 20 in phosphate-buffered saline (washing solution), and the remaining nonspecific binding sites were blocked by a 2-h incubation with 200 μL/well with PBS containing 1% BSA and 0.05% Tween 20 (blocking solution). Then the plates were rinsed twice with washing solution and incubated for 2 h at 37°C with different dilutions of antisera in blocking solution. After washing three times, 200 μL of peroxidase labeled anti–rabbit IgG (1:3,000) were added to the plates and incubated 2 h at 37°C. The plates were rinsed three times, 200 μL of substrate solution (40 mM 3-dimethyl-aminobenzoic acid, 0.8 mM 3-methyl-2-benzothiazoline hydrazone hydrochloride and 3 mM H_2O_2 dissolved in 100 mM KH_2PO_4-Na_2HPO_4, pH 7) was added, and the absorbance was read at 540 nm. All incubation steps were made in a moisture chamber.

In the competitive assay, 10 ng of rMT-III or native MT-I+II was used for coating wells. The plates were washed, blocked, washed again, and incubated for 2 h with a mixture of diluted antisera and standard curves of rMT-III or native MT-I+II in blocking solution. The rest was made as described above for noncompetitive assay.

MT-III Versus MT-I+II Response in the Freeze Lesioned Rat Brain

The polyclonal antibody generated was used for immunocytochemical studies in the rat brain in order to compare the putative differences in the response of the CNS MT isoforms to brain injury caused by a cryo-lesion model. To this end, 4 adult wistar rats (3 months old) were lesioned under tribromethanol anesthesia and were compared with 4 unlesioned rats. For the lesion, the skull over the right fronto-parietal cortex was exposed, and a focal cryo injury on the surface of the brain was produced with dry ice (−78°C) according to the protocol described previously (Lober and Torvik, 1991). Afterwards animals were recovered from the anesthesia and were able to feed and walk normally. The animals were housed in cages with free access to food and water.

After a time interval of 3 days, the lesioned and unlesioned rats were anesthetized with Brietal and fixed by vascular perfusion with 4% w/v paraformaldehyde in 0.1 M potassium phosphate-buffered saline (KPBS; pH 7.4), after perfusion with 0.3% heparin to rinse the vessels. Brains were removed, embedded in paraffin and processed for immunohistochemistry as previously described (Moos and Hoyer, 1996; Moos and Mollgard, 1993). Sections were preincubated with 10% goat serum in TBS/Nonidet (TBS: 0.05 M Tris, pH 7.4, 0.15 M NaCl) with 0.01% Nonidet P-40 (TBS/Nonidet) for 15 min at room temperature, and incubated overnight with one of the following primary antibodies: polyclonal rabbit anti–rat liver MT-III 1:1,000; polyclonal rabbit anti–rat liver MT-I+II (Gasull et al., 1993) 1:500; monoclonal mouse anti–rat ED1 (Serotec, Oxford, England, UK code MCA 341) 1:500, as a marker for microglia, monocytes and macrophages; polyclonal rabbit anti–cow GFAP (Dakopatts, DK code Z 334) 1:250, as a marker for astrocytes.

The primary antibodies were detected by the ABC system. The secondary antibodies were mouse anti–rabbit IgG absorbed with rat serum proteins (Sigma, St. Louis, MO, code B3275) 1:400, and goat anti–mouse IgG absorbed with rat serum proteins (Sigma, code B8774) 1:200, these secondary antibodies were detected by streptavidin-biotin-peroxidase complex (StreptABComplex/HRP, Dakopatts, DK; code K377) prepared at manufacturer's recommended dilutions. These secondary and tertiary steps in the immunoreaction were performed for 30 min at room temperature. The immunoreaction was visualized using 0.015% H_2O_2 in DAB/TBS, with DAB, as a chromogen.

Unlesioned and lesioned rats were processed in parallel. Positively stained cells for MT-I+II or MT-III, defined as those cells with cytoplasmic staining, were counted from a 1-mm^2 area of unlesioned hemispheres as well as from the

ipsilateral site of the lesioned rats for statistical evaluation of the results. Cells were counted in the deeper cortical layers, where all MT isoforms were significantly upregulated.

Specificity of the antibody was first checked by preincubating the antibody with brain or liver extracts, since native MT-III is in the brain but not in the liver. Rat brains and livers were homogenized with a Politron in ice-cold 10 mM Tris-HCl, pH 8.2, and centrifuged at 15,000*g* for 10 min to obtain the cytosol. The serum was incubated overnight with the brain or liver cytosols, and then the immunostaining was carried out as described above. To further demonstrated the specificity for MT-III, we have also analyzed the immunostaining of the antibody in brains of mice carrying a null mutation in the MT-III gene (Erickson et al., 1997), which were generously provided by Dr. R. D. Palmiter, University of Washington.

Effect of rMT-III on Astrocyte Migration in Vitro

Since by immunocytochemistry MT-III is mostly found in astrocytes, suggesting that this MT isoform could have a significant effect on these cells, we sought for a putative bioassay with astrocytes. Thus, we have carried out experiments with a bioassay similar to that described previously (Faber-Elman et al., 1995). Astrocytes were cultured as previously described (Hidalgo et al., 1994). The monolayer was scratched with a sterile pipette tip, and 2 ml of fresh culture media (DMEM) were added supplemented with either 10% fetal calf serum, or BSA, MT-III and MT-I+II at the indicated concentrations and maintained 4 days in 10% CO_2 at 37°C. The migration of astrocyte/cell processes into the region, denuded by the scratch, was analyzed.

This procedure was carried out in three different cell preparations for analyzing the effect of serum, BSA and MT-III. For statistical purposes, we carried out three additional cell preparations where the migration of astrocyte/cell processes into the denuded region in the presence of serum, BSA, MT-III, or MT-I+II was quantitated as follows. Cells were stained with a Dako GFAP antibody, and semiquantitative measurements of the cell migration into the scratch were done with a Leica Q 500 MC system. The procedure was as follows: a defined frame was established where the optical density and the number of pixels were measured to obtain a comparable value for all cultures. The area analyzed was the scratch site, and therefore, the higher the cellular migration into the scratch site, the higher the absorbance and number of pixels measured. Number of pixels × optical density were calculated for each framed area and experimental condition. The values obtained in the presence of fetal calf serum and albumin were considered 100% and 0% regeneration, respectively, and those for MT-I+II and MT-III were accordingly recalculated.

MT-III Protein and MT-III mRNA Levels in AD Brains

With the ELISA above described, MT-III protein levels were measured in 8 AD brains and compared with those of 8 normal brains. To this end, cortex samples were obtained from the Neurological Tissue Bank, Hospital Clínic i Provincial de Barcelona, Universidad de Barcelona. For ELISA determinations, samples were homogenized with a Potter-Elvehjem in PBS, centrifuged at 20,000 *g*, and the cytosols used for MT-III determinations. For MT-III mRNA measurements, total RNA was extracted (Chomczynski and Sacchi, 1987), electrophoresed in a 1% agarose gel and transferred to nylon filters as previously described (Hernández et al., 1997). A northern-blot was carried out using a cDNA kindly provided by Dr. Peter Kille, School of Molecular and Medical Biosciences, University of Wales Cardiff, following a procedure previously described (Hernández et al., 1997).

Statistical Analysis

Results were evaluated by the Student *t* test when only two groups were compared (cell countings in the cryolesion experiment; AD versus control brains) and by two-way analysis of variance (ANOVA) when more than two groups were compared (astrocyte bioassay). In this case, type of MT isoform and concentration were the main factors.

RESULTS

Purification of Recombinant Rat Brain MT-III and Generation of Polyclonal Antibodies

Recombinant rat brain MT-III was produced as described above and purified as follows. The fusion proteins (S·tag™ +rMT-III) were visualized in SDS-PAGE as a single band with an apparent molecular weight (MW) of approximately 25 kD, which was dramatically induced in IPTG-treated *Escherichia coli* cultures. To purify rMT-III we took advantage of some well-known biochemical properties of MTs, such as their heat stability, spectroscopic properties, heavy metal binding capacity and low molecular weight (Kägi and Kojima, 1987). Thus, after heating the cytosols of the *E. coli* cultures most proteins denaturalized and were lost upon centrifugation, but fusion proteins remained in soluble form and become the predominant protein species as judged by SDS-PAGE (data not shown). The soluble fraction was treated with thrombin to cleave the fusion protein generating rMT-III and a 25-aminoacid fragment (S·tag™). Different thrombin concentrations and incubation times were examined,

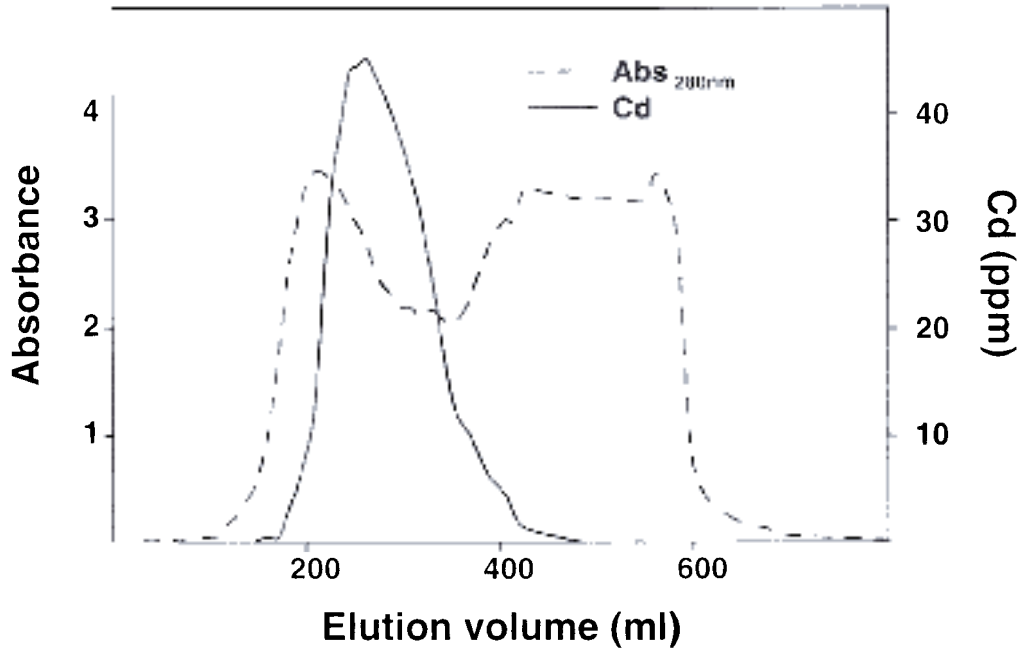

FIG. 1. Sephadex G-75 chromatography. Digestion products of thrombin treatment (30 ml) were applied to a G-75 column (2.6 × 115 cm). Recombinant rat MT-III was eluted in 20 mM Tris-HCl, pH 7.6. The flow rate was 15 ml/h. Fractions were analyzed by measuring the optic density at 230, 250, and 280 nm and by Cd-atomic absorption spectrometry. Cd content was used as indicator of Cd7-rMT-III. The rMT-III peak was not perfectly symmetrical suggesting heterogeneity, probably due to a partial loss of metals, aggregation or oxidative processes. Similar results were obtained with the ZnrMT-III.

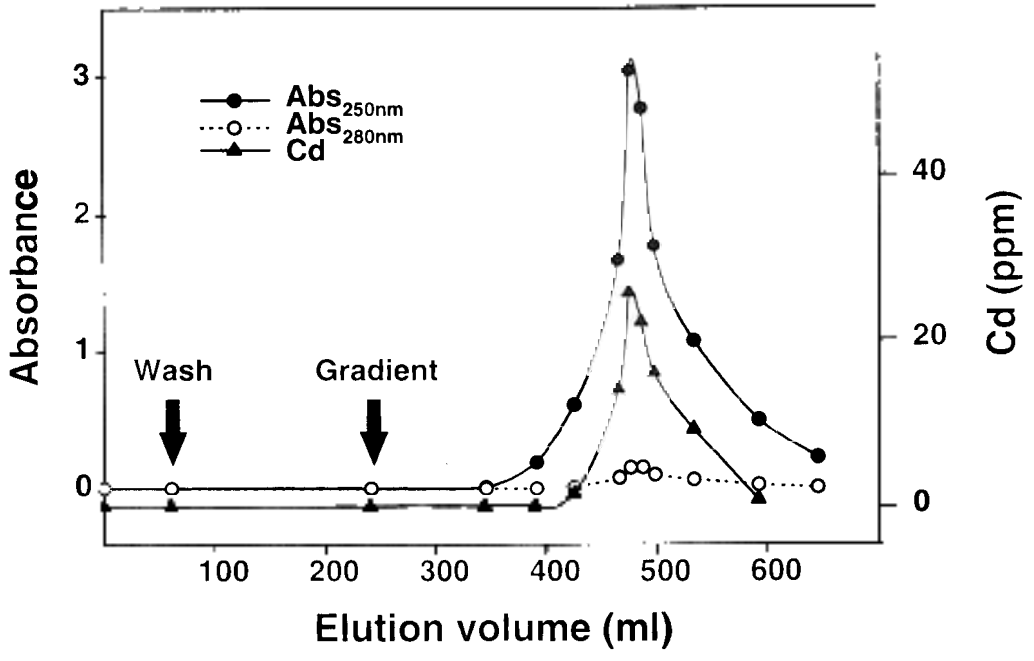

FIG. 2. Sephadex DEAE A-25 chromatography. Central fractions of the rMT-III peak of the Sephadex G-75 chromatography were rechromatographied in a DEAE A-25 column as described in material and methods. Fractions were assessed by UV spectrophotometry at 230, 250, and 280 nm; in addition, Cd content was determined by atomic absorption spectrophotometry. Optical density and Cd concentration curves were parallel suggesting the presence of a unique main protein. Recombinant rat MT-III peak eluted at 120 mM NaCl. Peak central fractions were pooled, concentrated and used for immunizing the animals.

and typically we now use overnight incubations with 3 units/mg protein of thrombin. As expected, in SDS-PAGE MT-III migrated faster than hepatic MT-I+II isoforms as predicted by its more negative net charge (Faller and Vasák, 1997; Pountney et al., 1994), and carboxymethylation of the proteins was needed to obtain the expected MW (data not shown).

After the treatment with thrombin, the cytosol was applied to a Sephadex G-75 column. The low MW peak containing the rMT-III was identified spectrophotometrically and by measuring the Cd content in the fractions (Fig. 1). DEAE-Sephadex A-25 ion-exchange chromatography resolved rMT-III as a major peak at approximately 120 mM NaCl (Fig. 2). Peak central fractions were pooled and precipitated with acetone. The 60–80% acetone precipitate was resuspended in buffer or stored at −80°C. Typical yields using this procedure were 15 mg protein/l of culture. The isolated proteins were checked by SDS-PAGE, and no protein contamination was noted by gel staining with coomassie blue (not shown). This was supported by the absorption spectra obtained (Fig. 3), since high and low absorbance at 250 nm and 280 nm was observed, indicative of Cd-thiolate bonds and lack of aromatic amino acids, respectively (Pountney et al., 1994). Also as expected, decreasing the pH caused the loss of Cd from MT-III and the subsequent shift of the MT-III absorbance at 250 nm (Fig. 3). Finally, the purified rMT-III was also evaluated by TOF mass spectrometry using the native MT-I+II as controls, and the expected MW was obtained (data not shown).

The purified rMT-III was injected into three rabbits and the MT-III immunoreactivity of the sera was followed by noncompetitive double-antibody ELISA using rMT-III or native MT-I+II for coating the plates. Ever since we first obtained sera it showed a positive reaction against rMT-III but not against purified rat liver MT-I+II. Sera immunoreactivity increased in successive bleedings, raising a maximum 8 months after the first immunization. A typical result is shown in Fig. 4. The specificity of the antibody was further demonstrated by other approaches as detailed below.

MT-III Versus MT-I+II Response in the Freeze Lesioned Rat Brain

The specificity of the anti–MT-III antibody was evidenced by the fact that the immunoreactivity observed in rat brain sections was eliminated by preincubating the antiserum with rat brain but not liver extract (Fig. 5). Furthermore, the antibody recognized the mouse MT-III protein, and, consequently, the immunoreactivity completely disappeared in MT-III null mice (Fig. 5).

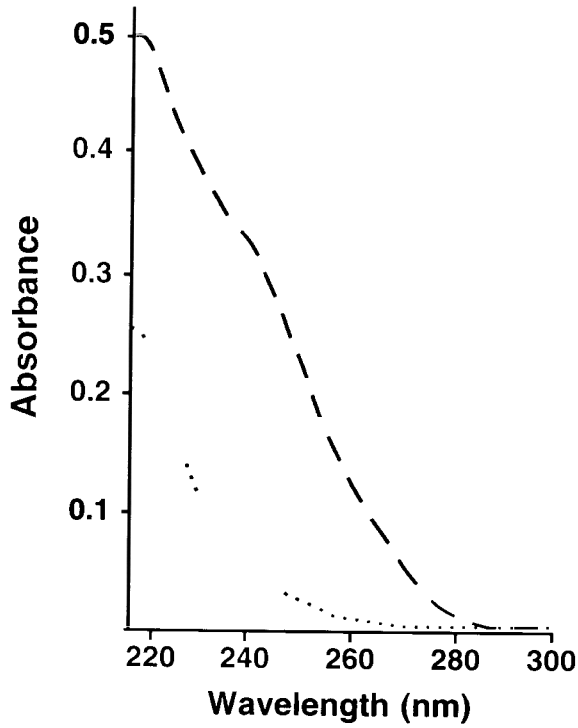

FIG. 3. Absorption spectra of purified Cd-rMT-III. Solid line, Cd-rMT-III in 20 mM Tris-HCl, pH 7.6. Dashed line, rMT-III spectra after adding 5 μl/ml buffer of HCl 37%. As expected the absorbance at 250 nm in acid medium was dramatically reduced due to the loss of Cd-thiolate bonds. Note the low absorbance at 280 nm, suggesting that the protein is free of significant contaminants.

Regarding MT-I+II and MT-III immunoreactivities, in normal unlesioned rats MT-III was observed in both superficial and deeper cortical cell layers and in the glia limitans, whereas MT-I+II was present primarily in the superficial cortex (Fig. 6). The MT-III–positive cells in the cortex were grey matter protoplasmic astrocytes with a typical stellate appearance, and ramified microglia. A few MT-III–positive neurons were detected in the basal nuclei. MT-III–positive cells in the leptomeninges and lying next to major vessels had a round amoeboid appearance and were lectin positive. All animals examined, inclusive lesioned rats, showed positive MT-I+II staining in ependymal cells and in the choroid plexus epithelial cells of the third, fourth and lateral ventricles together with immunoreactivity in arachnoid and pia mater. In the cortex, MT-I+II expression in unlesioned rats was somewhat decreased compared to that of MT-III (3 versus 10 positive cells/mm^2 cortex). However, the scarce MT-I+II was observed in both astrocytes and a few ramified microglia.

Rats subjected to the cryo lesion were clinically unaffected, determined by their ability to feed and walk nat-

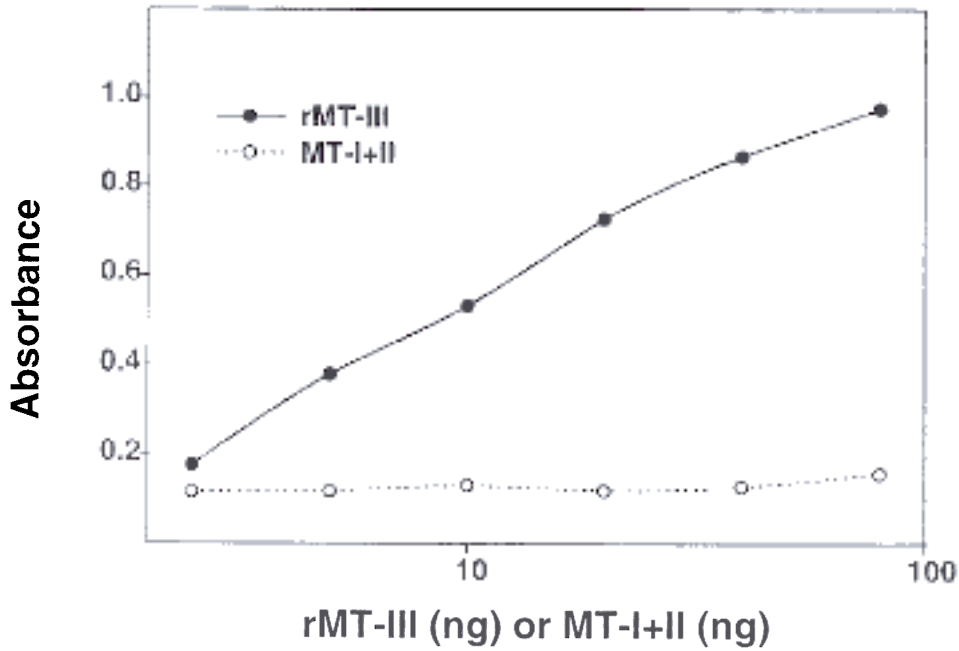

FIG. 4. Noncompetitive assay. Immunoreactivity was assessed in plates coated with 2.5, 5, 10, 20, 40, and 80 ng of rMT-III or native MT-I+II as described in materials and methods. Results are expressed in absorbance units measured at 540 nm. Absorbance increased linearly with the amount of protein used for coating (in log scale) when rMT-III was used. In contrast, no response was observed with native MT-I+II as coating.

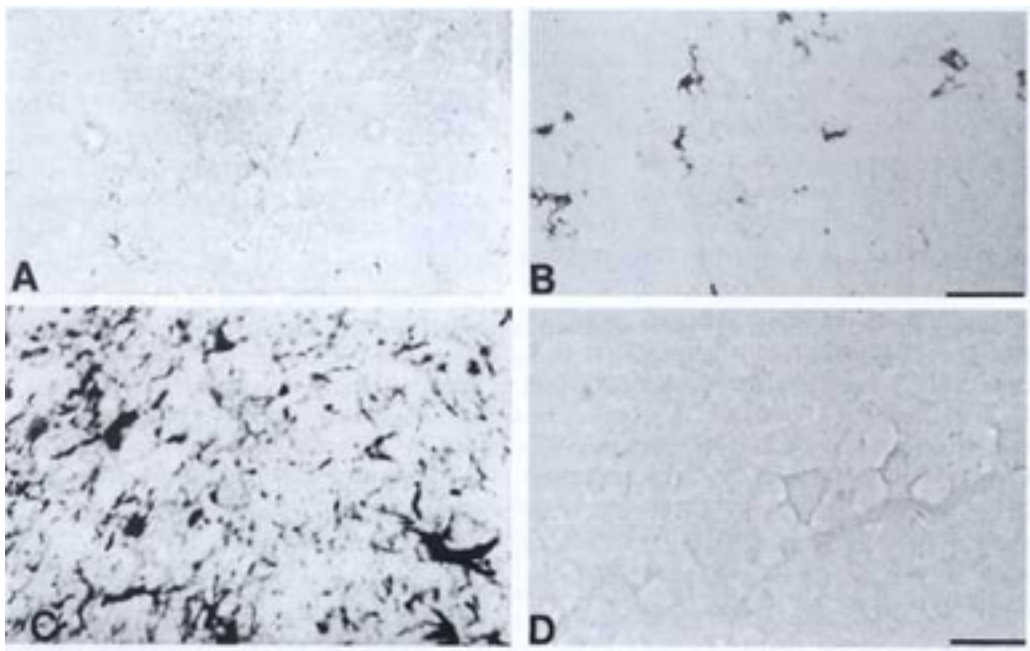

FIG. 5. Specificity of the antibody. (A) MT-III immunoreactivity in rat brain following preincubation of the MT-III antibody with rat brain extract. (B) MT-III immunoreactivity in rat brain following preincubation of the MT-III antibody with liver extract, showing that the generated MT-III antibody reacts against MT-III in mice. (C) MT-III immunoreactivity in mouse brain without preincubation of the antibody. (D) MT-III immunoreactivity in brain of MT-III null mice, showing no immunoreactivity. Bar = 57 μm (A, B), 20 μm (C,D).