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A Monoclonal Antibody Elicited to Human Platelet Monoamine Oxidase

Isolation and Specificity for Human Monoamine Oxidase B but Not A

RICHARD M. DENNEY, NUTAN T. PATEL, RICHARD R. FRITZ, AND CREED W. ABELL

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

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SUMMARY

We have isolated a mouse monoclonal antibody to human platelet monoamine oxidase (MAO) B. The antibody (MAO-1C2) was isolated from a fusion of mouse myeloma P3/X63 Ag8 to spleen cells from a BALB/c mouse immunized with a partially purified platelet preparation in which an estimated 21–31% of the protein was [^{3}H]pargyline-labeled MAO B. The antibody indirectly immunoprecipitates both [^{3}H]pargyline-labeled, catalytically inactive human MAO B, and unlabeled, catalytically active human MAO B. Binding of the antibody to MAO B has no detectable effect on catalytic activity. MAO-1C2 is specific for human MAO B, and fails to immunoprecipitate MAO A indirectly from human placenta or liver. Its ability to immunoprecipitate human MAO B but not MAO A from extracts of human liver provides a convenient technique for separating the two forms of the enzyme for comparative studies. The antibody does not recognize mouse liver MAO B, suggesting that the determinant is not universally expressed on MAO B from all species.

INTRODUCTION

MAO[1] is an integral protein of the outer mitochondrial membrane (1) and plays an important role in the degradation of dopamine, norepinephrine, epinephrine, and 5-HT (2). There are two forms of MAO, A and B, which have distinct catalytic properties, and they are expressed in various proportions in different tissues (3–6). MAO A is selectively inhibited by the irreversible active-site inhibitor clorgyline and preferentially oxidizes 5-HT (3). MAO B is selectively inhibited by deprenyl and pargyline (5), and preferentially oxidizes PEA and benzylamine. The inhibitors clorgyline, deprenyl, and pargyline contain acetylenic groups that react with the single flavin moiety which is covalently bound to a cysteinyl residue in one of the two subunits of MAO (7, 8). Despite its selectivity for MAO B, [^{3}H]pargyline will covalently label the active sites of both MAO A and MAO B under appropriate conditions (9, 10). Studies with [^{3}H]pargyline-labeled crude preparations of MAO have shown that the FAD-containing subunit of MAO A migrates differently from that of MAO B in SDS-polyacrylamide gels. The apparent molecular weights of MAO B and A from rat liver are approximately 55,000 and 60,000, respectively (9). Similarly, the apparent molecular weights of human MAO B from platelets and MAO A from placenta have been reported to differ, with molecular weights variously reported to be 64,000 and 67,000, respectively (10), or 60,000 and 64,000, respectively (11).

The molecular basis for the catalytic differences between MAO A and B is the subject of continuing debate. Differences in migration of the [^{3}H]pargyline-labeled FAD-containing subunits of MAO suggest that these subunits may differ in molecular weight. The hypothesis that the FAD-containing peptides of MAO A and B differ in primary structure is further supported by the production of distinct patterns of [^{3}H]pargyline-labeled peptides by partial proteolytic digestion of [^{3}H]pargyline-labeled MAO A and B (10–12). On the other hand, other investigators have suggested that the differences in MAO A and B can be accounted for by differences in their lipid environments (13, 14), since catalytic distinctions between MAO A and B disappear when crude preparations

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[1] The abbreviations used are: MAO, monoamine oxidase (amine:oxygen oxidoreductase, EC 1.4.3.4); 5-HT, 5-hydroxytryptamine; PEA, phenylethylamine; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline (0.2 g of KCl, 0.2 g of KH_2PO_4, 8 g of NaCl, and 1.14 g of Na_2HPO_4); IgG, immunoglobulin G; HY medium, Dulbecco-Vogt modified Eagle's minimal essential medium supplemented with (per liter) 100 ml of NCTC-135,200 units of bovine insulin, 0.49 g of pyruvic acid, 0.13 g of oxaloacetic acid, 13.6 mg of hypoxanthine, 3.87 mg of thymidine, 0.26 g of cysteine, and 20% (v/v) horse serum; NET buffer, 0.15 M NaCl, 5mM EDTA, 50 mM Tris, and 0.02 % (w/v) sodium azide (p*H* 7.4).

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of the enzymes are delipidated (14). Furthermore, MAO A and B activities appear to be stimulated by the presence of different lipids (13).

If MAO A and B differ in primary structure or covalent modifications, it should be possible to obtain antibodies which will distinguish them. Attempts to distinguish MAO A and B with conventional antisera have been equivocal. Dennick and Mayer (15) and Russell *et al.* (16) elicited antisera in rabbits against purified human and rat liver MAO which had only MAO B activity, although liver mitochondria from these species have both MAO A and B activity. Their antisera did not discriminate MAO A from MAO B in the respective species. In contast, McCauley and Racker (17) elicited an antiserum to bovine liver MAO B which specifically precipitated MAO B activity, but not MAO A activity, in extracts of bovine brain. Conversely, Powell and Craig (18) produced an antiserum against human placental MAO (predominantly A; ref. 19) which failed to cross-react with the platelet enzyme (mostly B; ref. 20).

Because of the disagreement concerning the immunological relatedness of MAO A and B, we considered it worthwhile to reinvestigate this question using hybridoma technology, as developed by Kohler and Milstein (21). The principal advantage of the hybridoma technique over that of conventional antisera is that (a) one can prepare against an antigen virtually unlimited quantities of an antibody which recognizes a single antigenic determinant, and (b) monospecific antibodies can be prepared against a component of an impure antigen preparation. We report here the isolation and characterization of a hybridoma which secretes a mouse monoclonal antibody elicited to human platelet MAO B. Here and elsewhere (22) we present evidence that the antibody does not recognize human MAO A.

MATERIALS AND METHODS

Purification of MAO from platelets. The MAO preparation used for immunization was purified from outdated human blood platelets (predominantly B-type enzyme). The details of the purification will be presented elsewhere.[2] Briefly stated, platelets were extracted with 0.5% Triton X-100, and the resulting extracts were precipitated with 50%-saturated ammonium sulfate. The sediment was suspended in and dialyzed against 0.05 M potassium phosphate buffer (pH 7.4) and applied to a DEAE-Sephacel column equilibrated with the same buffer. Active enzyme was eluted with the same buffer containing 0.5% Triton X-100. To facilitate monitoring the enzyme during subsequent purification, MAO in the combined, active fractions from the DEAE-column was labeled by treatment with 0.67 μM [^{3}H]pargyline [New England Nuclear Corporation (Boston, Mass.), specific activity 15 Ci/mmole] for 30 min at 37°. The final specific activity of the MAO was 274,500 dpm/μg of MAO protein. Estimates of quantities of [^{3}H]pargyline-labeled MAO are based on ^{3}H counts per minute, assuming the specific activity reported above, and assuming a molecular weight of 120,000 for MAO. Labeling of the enzyme with [^{3}H] pargyline under these conditions resulted in 70% inhibition of MAO activity.

The [^{3}H]pargyline-labeled MAO was further purified by preparative isoelectric focusing (pH range 3–10) on agarose slab gels (for immunization) or by chromatofocusing (Pharmacia Inc., Piscataway, N. J.). The isoelectric-focused and chromatofocused preparations were comparable in specific radioactivity (57,600–85,100 dpm/μg of total protein). These figures indicate that MAO constituted 21–31% of the total protein. (For SDS-polyacrylamide gel profiles of human platelet MAO B purified by DEAE-chromatography or chromatofocusing, see Fig. 1).

Immunization. A BALB/c mouse was immunized by two i.p. injections of 10 μg of isoelectric-focused, [^{3}H] pargyline-labeled platelet MAO (days 1 and 7) contained in 0.1 ml of sterile PBS and emulsified in an equal volume of complete (first injection) or incomplete (second injection) Freund's adjuvant. The animal was boosted by i.p. injection of 10 μg of [^{3}H]pargyline-labeled MAO (chromatofocused material) in saline on days 54, 55, and 56, and the fusion was performed on day 57.

Fusion of cells. Spleen cells (8×10^7) were prepared on day 58 (one day following the last boost) and fused to 10^7 P3/X63 Ag8 cells (deficient in hypoxanthine phosphoribosyltransferase) with 40% polyethylene glycol 1000 (Sigma Chemical Company, St. Louis, Mo.) and plated at about 2×10^5 myeloma cells/well in 512 microculture wells in HY medium according to the procedure of Kennett *et al.* (24). Plates were incubated at 37° in 10% CO_2/air. Aminopterin (final concentration 0.018 mg/ml) was added the day after fusion to kill the myeloma cells. Cells were fed on days 7 and 14 after fusion with HY medium lacking aminopterin.

Preliminary screening of conditioned media for specific antibodies. Cell culture supernatants were screened initially for antibody capable of binding any antigen in chromatofocused MAO which would coat microtiter plates and which could be detected by a peroxidase-linked immunosorbent assay (ELISA). The ELISA protocol was similar to that of Kearney *et al.* (25). [^{3}H] Pargyline-labeled MAO (chromatofocused material) was incubated for 4 hr at 37° in Cooke polystyrene microtiter plates (150 μl/well; MAO protein, 1 μg/ml) in borate-saline buffer [per liter, 6.2 g of H_3BO_3, 9.5 g of $Na_2B_4O_7 \cdot 10H_2O$, 9.0 g of NaCl (pH 8.2)]. Conditioned media from wells containing growing clones were diluted 1:10 with PBS plus 0.05% Tween 20 (Sigma Chemical Company) and incubated in the washed wells (150 μl/well) for 4 hr at 23°. Bound mouse immunoglobulin was detected colorimetrically after a further 4-hr incubation of the washed wells with 150 μl of peroxidase-conjugated sheep anti-mouse IgG (heavy plus light chain, 1:1000 dilution from Cappel Laboratories, Cochranville, Ill.). The peroxidase reaction mixture (150 μl/well) contained, per 20 ml of citric acid buffer (pH 5), 8 mg of *o*-phenylenediamine (Sigma Chemical Company) and 4 μl of 30% hydrogen peroxide. Reactions were stopped after 2–4 min with 50 μl of 4 M sulfuric acid. Absorbance data were quantitated with an automated ELISA reader, Model MR 580 (Dynatech Laboratories, Alexandria, Va.).

[2] G. A. S. Ansari, N. T. Patel, R. R. Frits, and C. W. Abell. Purification of platelet monoamine oxidase B by HPLC. Manuscript in preparation.

Screening of conditioned media by indirect immunoprecipitation. Conditioned media from hybridomas were diluted 1:10 with the NET buffer of Kessler (26) [0.15 M NaCl, 5 mM EDTA, 50 mM Tris, 0.02% sodium azide (pH 7.4)], containing 0.1% bovine serum albumin and 0.05% NP-40 (Particle Data, Inc., Elmhurst, Ill.), and mixed with 20–40 ng of [^{3}H]pargyline-labeled MAO (isoelectric-focused preparation) contained in 50 μl of the same buffer. These samples were incubated in 96-well polystyrene microtiter plates (Cooke) at 23° for 1 hr in a rotary shaker (50 μl total volume per well). In most experiments, rabbit anti-mouse IgG (heavy plus light chain; Cappel Laboratories) was added (equivalent to 8 μg of specific antibody), and the incubation was continued for 1 hr. Heat-killed fixed *Staphylococcus aureus* Cowan I (Pansorbin A; Calbiochem, San Diego, Calif.) was then added (50 μl of a 10% suspension), and the incubation was continued for 15 min. The plate was then centrifuged for 10 min at 1700 rpm (5°) in a Cooke microplate carrier (Dynatech Laboratories) in an IEC refrigerated centrifuge (Model PR-6000).

The resulting supernatants were then assayed for [^{3}H] pargyline-labeled MAO by counting 50-μl samples dried on 2.5-cm glass-fiber filters (Reeve Angel, Clifton, N. J.) in a toluene-based scintillation fluid (containing per liter of toluene, 4 g of 2,5-diphenyloxazole and 0.05 g of dimethyl 1,4-bis[2-(5-phenyloxazolyl)]benzene) or suspended in 2 ml of PCS scintillation fluid (Amersham Corporation, Arlington Heights, Ill.). Counting efficiency for ^{3}H was 20% on dried filters or 25% in PCS when counted in a Packard Tri-Carb liquid scintillation spectrometer.

Indirect immunoprecipitation of samples containing catalytically active enzyme (see Table 2) was done in the same way, except (a) the dilution buffer was Dulbecco's phosphate-buffered saline containing, per liter, 0.2 g of KCl, 0.2 g of KH_2PO_4, 8 g of NaCl, and 1.14 g of Na_2HPO_4, plus 0.1% bovine serum albumin and 0.05% NP-40 (PBS immunoprecipitation buffer); (b) the quantity of rabbit anti-mouse IgG added was increased to 10 μl (about 40 μg of specific antibody protein); and (c) all incubations were carried out at 0–4°. Titration curves of indirect immunoprecipitation as a function of antibody dilution were obtained as described above except that antibody samples were first serially diluted to give 4-fold decreasing concentrations of antibody in a final volume of 50 μl. Indirect immunoprecipitation of human placental and liver MAO and mouse liver MAO was done as described for immunoprecipitation screening assays (above), with the following modifications. Immunoprecipitation was done at 0° in 1.5-ml Microfuge tubes (Beckman Instrument Company, Fullerton, Calif.) by adding 200 μl of a dilution of MAO-1C2 antibody precipitated from ascites fluid with 50%-saturated ammonium sulfate and dialyzed against PBS to 10-μl samples of octylglucoside extracts of crude mitochondria from human or mouse liver. Rabbit antimouse IgG (0.56 μg of total IgG; Cappel Laboratories) was added after 1 hr, and 200 μl of a 1:10 suspension (w/v) of *S. aureus* cells after a 2nd hr. After 15 min, the tubes were centrifuged at 10,000 × *g* for 5 min in a Beckman Microfuge, and the supernatants were set aside. The pellets were suspended in 1 ml of PBS-immunoprecipitation buffer and centrifuged as before. The pellets were finally suspended in PBS immunoprecipitation buffer. Both the supernatants which had been set aside and the pellets were assayed for MAO activity.

Preparation of ascites fluids. Ascites fluids containing MAO-1C2 antibody were generated by giving pristane-primed BALB/c mice i.p. injections of 3×10^6 to 3×10^7 MAO-1C2 cells grown in HY medium (minus aminopterin) as described by Kennett *et al.* (24).

MAO activity assays. MAO was assayed for enzymatic activity by a micromethod adapted from the radiometric technique of Wurtman and Axelrod (27). Substrates were ^{14}C-labeled benzylamine (specific activity 14.6 mCi/mmole), PEA (50 mCi/mmole; New England Nuclear Corporation), or 5-HT (49.3 mCi/mmole; New England Nuclear Corporation). PEA was used at 10 μM final concentration without isotopic dilution. Benzylamine and 5-HT were used at 1 mM final concentration at a specific activity of 2 mCi/mole. Activities are expressed in nanomoles per hour. Specific activities are expressed as units per milligram of protein.

Assays of inhibition of MAO by irreversible inhibitors. Selective inhibition of MAO A or B was achieved by incubating enzyme samples with the MAO B-specific inhibitor deprenyl (5) or the MAO A specific inhibitor clorgyline (3) at concentrations of 10^{-6} M for 30 min at 37° in 0.05 M potassium phosphate buffer (pH 7.5) prior to assay. Preliminary experiments with various inhibitor concentrations showed that 10^{-6} M inhibitor concentrations gave good selectivity for MAO A and B (see Table 4). The samples were then assayed for MAO activity as described above.

Preparation of extracts of mitochondria from tissues. Human liver mitochondria were prepared from frozen liver tissue (provided by Dr. Jerome Smith, of the Autopsy Service of the University of Texas Medical branch) by homogenization in 0.05 M potassium phosphate buffer (pH 7.5) (2 volumes/g of tissue) containing 0.25 M sucrose using a Polytron tissue homogenizer [Brinkmann Instrument Company (Westbury, N. Y.), 30 sec at 4°, top speed]. The homogenate was centrifuged at 600 × *g* for 20 min at 4° in a Beckman JA-10 rotor. The supernatant was saved, and the pellet was washed once with an additional 2 volumes of buffer per gram of tissue and centrifuged; the first and second supernatants were combined. The crude mitochondria were then pelleted at 6500 × *g* for 20 min in a JA-10 rotor at 5°. The pellet was suspended in 1 volume of buffer per gram of tissue and pelleted at 6500 × *g*. This step was repeated once. The final pellet was suspended in 10 volumes of buffer per gram of washed mitochondrial pellet and frozen at −80°. Frozen pellets were stored for up to 3 months.

To extract the MAO, frozen pellets were thawed, suspended in 10 volumes of buffer, and pelleted at 12,000 × *g*. This washing step was repeated. Finally, the MAO was solubilized by the addition of 0.05 M potassium phosphate (pH 7.5) plus 0.75% (w/v) octylglucoside (Calbiochem). The suspension was stirred for 20 min at room temperature, chilled, and centrifuged at 20,000 × *g* for 15 min; aliquots of the soluble extract were frozen at −80°. Extracts of human placental mitochondria were prepared as described for liver, except that the tissue was obtained within 1 hr of delivery and was not frozen before homogenization. Placentae were obtained from the Labor and

Delivery Service of the Department of Obstetrics and Gynecology, the University of Texas Medical Branch. The spongy tissue was dissected free of fetal membranes and umbilical cord, minced, and washed free of excess blood with 0.05 M potassium phosphate buffer by gentle extrusion of excess fluid through cheesecloth. Further steps were carried out as described for liver preparations. Crude mouse liver mitochondria were prepared essentially as described for human liver. The tissue was obtained from freshly killed BALB/c females and homogenized with an all-glass Dounce homogenizer.

Protein assays. Protein was measured by the technique of Lowry *et al.* (28) or by the method of Bradford (29), with bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide slab gels (7.5% running gel, 2.5% stacking gel, 0.5-mm thick) were prepared as described by Fairbanks *et al.* (30). Samples (≤50 μl) containing 40–1000 ng of protein were denatured by boiling for 5 min in 2% SDS plus or minus 5% β-mercaptoethanol. Proteins were stained with silver as described by Merrill *et al.* (31). Radioactive profiles of ^{3}H-labeled MAO were generated by slicing lanes into 46 0.25-cm slices, digesting the polyacrylamide by overnight incubation with H_2O_2, and counting in PCS scintillation fluid (Amersham).

RESULTS

MAO was purified from outdated blood platelets as described under Materials and Methods. Since MAO loses activity during purification, we chose at an early stage to label the MAO with [^{3}H]pargyline, an MAO B-preferred inhibitor, which binds only to MAO under the conditions employed. SDS-polyacrylamide gel profiles of DEAE-purified (Lanes *3* and *4*) and isoelectric-focused (Lanes *5* and *6*) MAO are shown in Fig. 1. All of the ^{3}H in the labeled DEAE-fraction and in the isoelectric-focused or chromatofocused preparations, which were used subsequently for injection and screening, migrated as a single peak with an apparent molecular weight of 59,000 (Fig. 2).

The band of protein marked by the *arrow* in the SDS-polyacrylamide gel profile in Fig. 1 is identical with or contains the FAD-binding subunit of MAO, since (a) this band contained >90% of the ^{3}H detectable in the chromatogram and (b) further purification of the chromatofocused, [^{3}H]pargyline-labeled material to homogeneity yielded a single band of protein with the indicated mobility (22).

A BALB/c mouse was immunized with isoelectric-focused, platelet MAO labeled with [^{3}H]pargyline. On the basis of the level of [^{3}H]pargyline per microgram of protein, MAO constituted about 21–31% of the total protein in the injcctcd preparation. The corresponding figure, based on the proportion of silver bound by this band (31), was estimated to be ~12% as judged by microdensitometric scanning of the gel profile. Spleen cells from the immunized mouse were fused with the mouse myeloma P3/X63 Ag8 as described under Materials and Methods. Seventeen days after fusion, conditioned media from the 288 wells showing cell growth (56% of the wells plated) were assayed for antibody which would bind any component of the injected antigen as detected by an ELISA assay.

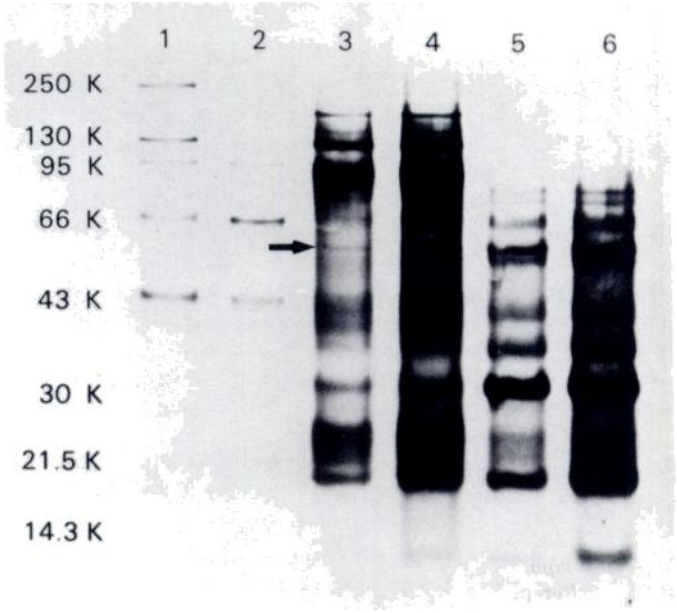

FIG. 1. *SDS-polyacrylamide gel profiles of protein samples used in the study*

Gels were run and stained for protein with silver or sliced and counted for ^{3}H as described under Materials and Methods. Lane *1*, high molecular weight standards (Bio-Rad; 0.1 μg/band): (from *top* to *bottom*) myosin, 250,000; β-galactosidase, 130,000; phosphorylase B, 95,000; bovine serum albumin, 66,000; ovalbumin, 43,000. Lane *2*, low molecular weight standards (Bio-Rad; 0.1 μg/band): (from *top* to *bottom*) phosphorylase B, 95,000; bovine serum albumin, 66,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,338. Lane *3*, 1.4 μg of protein from [^{3}H]pargyline-labeled DEAE-purified human platelet MAO. Lane *4*, same as Lane *3*, 2.8 μg of protein. Lane *5*, 1.25 μg of total protein from [^{3}H]pargyline-labeled human platelet MAO purified by chromatofocusing; Lane *6*, same as Lane *5*, 2.5 μg of protein.

Results of assays of 240 of the 288 supernatants tested are shown in Fig. 3. Conditioned media from 31 wells gave color reactions judged to be significantly above background (>0.4; Fig. 3). (Conditioned medium from P3

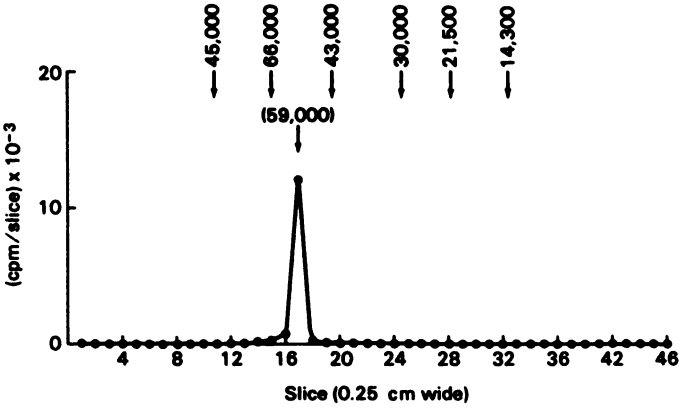

FIG. 2. *SDS-polyacrylamide gel profiles of [^{3}H]pargyline-labeled MAO used for screening*

SDS-polyacrylamide gels were run as described under Materials and Methods. Shown is the ^{3}H content of each of 46 slices of gel after electrophoresis of the [^{3}H]pargyline- labeled, chromatofocused fraction of human platelet MAO (6.25 μg of protein containing 105,600 cpm of [^{3}H]pargyline-labeled MAO applied). The sample illustrated was the same as that in Lanes *5* and *6*, Fig. 1.

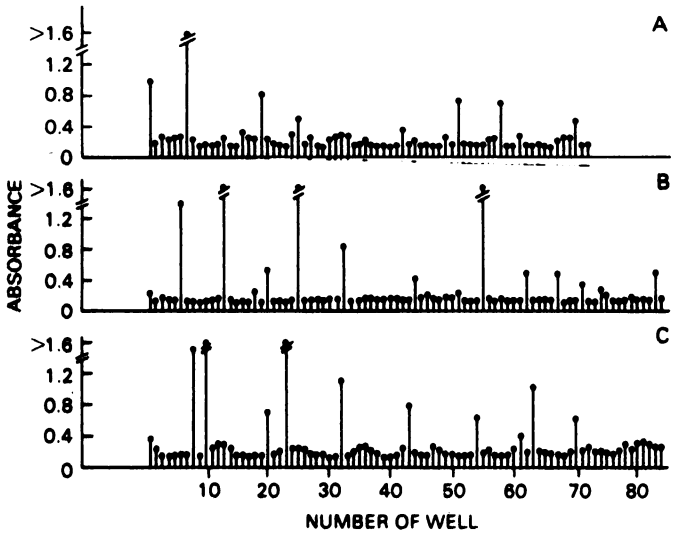

FIG. 3. *Detection of hybridomas secreting immunoglobulins which bind to components of partially purified human platelet MAO*

Microtiter plates coated with chromatofocused, [^{3}H]pargyline-labeled human platelet MAO (see gel profile in Fig. 1, Lanes *5* and *6*) were incubated with medium from 288 wells containing growing cells 17 days after fusion (data from 240 wells are shown here). Bound immunoglobulin was detected by using sheep antimouse IgG conjugated to peroxidase, and a colorimetric assay for bound peroxidase as described under Materials and Methods. Data from 3 plates containing 240 supernatants are shown. A, Plate 1 (72 supernatants tested); B, Plate 2; C, Plate 3. Cells from the 31 wells which gave absorbance values of >0.4 (28 from the plates shown plus 3 from another plate) were split to fresh wells and retested 4 days later.

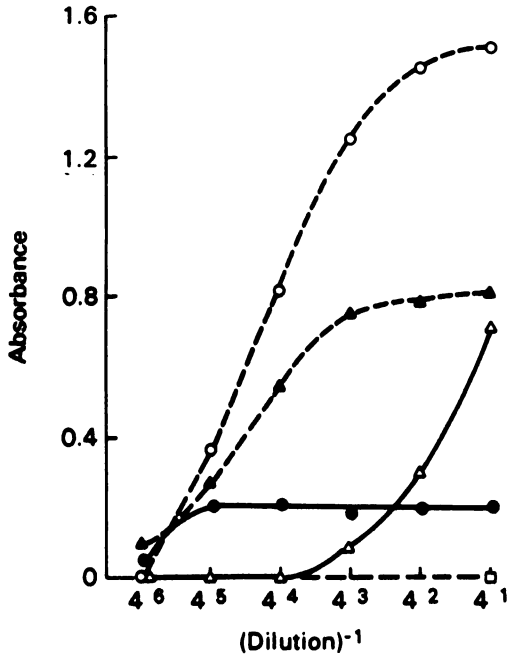

FIG. 4. *Titration of detectable antibody in ELISA-positive primary cultures*

Conditioned media from the 14 clones which were still ELISA-positive at the time of freezing were titered by ELISA assay on plates coated with chromatofocused, [^{3}H]pargyline-labeled human platelet MAO. Bound immunoglobulin was detected colorimetrically by assaying peroxidase activity after incubation of wells with peroxidase-conjugated sheep antimouse IgG, as described under Materials and Methods. The four titration curves illustrated were chosen to represent the diversity of the ELISA data among the 14 conditioned media assayed. ○---○, Well 3G2; ●——●, Well 6G5; △——△, Well 1C5; ▲---▲, Well 1C2; □, P3 (control) supernatant.

cells gave a reading of 0.15 in this assay.) After expansion of the cell populations to about 5×10^6 cells for freezing in liquid nitrogen, 14 primary wells were strongly positive by ELISA assay. The titration curves, four of which are presented in Fig. 4, differed markedly in shape from clone to clone, suggesting that the conditioned media contained diverse antibodies which apparently recognized a variety of antigenic determinants. Antibody from well 1C2, which we show below to be specific for MAO B, saturated sites on the plate at dilutions of <1:16 to 1:64 and bound an intermediate level of peroxidase at saturation.

In order to determine directly whether any of the ELISA-positive antibodies could immunoprecipitate MAO, we mixed samples of conditioned medium from each of 12 wells with a small amount of [^{3}H]pargyline-labeled MAO and assayed their ability to immunoprecipitate the labeled protein in the presence or absence of the secondary reagents, heat-killed, fixed *S. aureus* cells (24), or a combination of both anti-mouse IgG and *S. aureus* cells. These assays revealed that only 1 of the 12 conditioned media tested, henceforth called MAO-1C2 medium, efficiently immunoprecipitated [^{3}H]pargyline-labeled MAO (Table 1). MAO-1C2 medium immunoprecipitated the enzyme only when rabbit antimouse IgG was included in the indirect assay. Direct determination of the counts per minute bound to the washed pellets demonstrated that MAO-1C2 medium immunoprecipitated 10 times more MAO than any other conditioned medium tested (Table 1).

Hybridoma MAO-1C2 was grown continuously for 9 months (about 90 generations) without apparent loss of specific antibody secretion. In the meantime, 11 subclones were isolated, and supernatants from 6 of 6 subclones tested were found to secrete MAO-binding antibody as judged by the ability of their secreted antibodies to immunoprecipitate [^{3}H]pargyline-labeled MAO in indirect immunoprecipitation tests. One of these subclones, MAO-1C2 no. 8, was injected into pristane-primed mice in order to generate ascites fluid. Indirect immunoprecipitation titration curves of antibody concentrated 5-fold from conditioned medium by ammonium sulfate precipitation and of an active ascites fluid are compared in Fig. 5. Indirect immunoprecipitation of [^{3}H]pargyline-labeled MAO by the ammonium sulfate-precipitated antibody from cell culture medium showed the expected sigmoid curve, with 50% of the label precipitated at dilutions between 4^{-4} and 4^{-5}. However, the ascites fluid failed to precipitate significant amounts of ^{3}H-labeled MAO indirectly at dilutions of $\leq 4^{-8}$, presumably because MAO-1C2 antibody was present in excess of the immunoglobulin-binding capacity of the rabbit anti-mouse IgG present in the mixture. At dilutions of 4^{-4} to 4^{-6}, virtually all of the [^{3}H]pargyline-labeled MAO was precipitated. At dilutions of $\geq 4^{-6}$, the expected sigmoid curve was observed (Fig. 5). Taking $4^{-7.5}$ as the dilution of ascites fluid which gave half-maximal binding of [^{3}H]pargyline-labeled MAO present in the assay (44 ng), we estimated that 1 ml of ascites fluid could bind the equivalent of 14.1 mg of MAO under appropriate conditions. Independent titrations of ascites fluids from eight different animals over a period of 6 months have given 50% immunoprecip-

TABLE 1

Indirect immunoprecipitation of [^{3}H]pargyline-labeled platelet MAO by conditioned media

Samples of conditioned media were used to immunoprecipitate 88 ng of [^{3}H]pargyline-labeled MAO contained in a chromatofocused preparation of the enzyme (see Materials and Methods). Both anti-mouse IgG and *Staphylococcus aureus* cells were used as secondary reagents. Following assay of the unprecipitated [^{3}H]pargyline-labeled MAO, the bacterial pellets were washed twice with 1 ml of NET buffer by centrifugation and suspended in 100 μl of NET buffer; 50 μl were assayed directly for bound ^{3}H.

Source of antibody	% cpm left in solution	cpm bound to bacteria
P3/X63 Ag8	93.5	105
1C2	15.6	2836
1C5	87.4	106
1E5	81.1	120
3E2	94.2	70
3G2	90.1	76
4C6	83.5	142
5F7	93.5	98
5G6	88.5	110
5F10	92.3	138
6B10	72.9	102
6D6	68.1	90
6E10	76.5	128
6G5	69.6	232

itation titers of 4^{-7} to 4^{-8}. MAO-1C2 no. 8 was recloned after an additional 3 months in culture, and seven of nine secondary subclones secreted anti-MAO antibody. Therefore, specific antibody secretion by this hybridoma appears to be reasonably stable. All subsequent experiments were performed with MAO-1C2 no. 8 ascites fluid.

Since MAO-1C2 was elicited to pargyline-inhibited platelet MAO B and detected by its ability to bind the same material, we tested its ability to bind the catalytically active human enzyme. We used in these experiments extracts of frozen-thawed, washed human liver mitochondria which were rich in MAO B. Oxidation of benzylamine was used to assay MAO B activity. Control experiments indicated that >90% of the benzylamine-oxidizing activity was sensitive to 10^{-6} M deprenyl. Therefore, in this experiment we measured MAO B, and not benzylamine oxidase. The addition of MAO-1C2 to a sample of the liver preparation did not inhibit or precipitate MAO B activity (Table 2). The rabbit anti-mouse IgG, MAO-1C2, and *S. aureus* cells added had negligible MAO B activity (<0.5 unit) as compared with the liver extract added (5.2 units) (Table 2). However, the addition of both MAO-1C2 and rabbit anti-mouse IgG to the liver enzyme resulted in precipitation of 76% of the activity (3.9 units in the pellet, 1.2 units in the supernatant). Since the total activity recovered in the pellet and supernatant (5.1 units) was close to the activity added to the assay (5.2 units), the precipitated enzyme appeared to be fully active (Table 2). Further addition of *S. aureus* cells did not result in additional precipitation of enzyme activity under these conditions (Table 2). These data demonstrate that the majority of liver MAO B activity can be precipitated by the antibody, and that indirect immunoprecipitation of MAO B activity in extracts of liver mitochondria requires only MAO-1C2 and rabbit anti-mouse IgG. Excess *S. aureus* cells are routinely added, even though they are not always essential, to ensure quantitative precipitation at low ratios of monoclonal antibody to rabbit anti-mouse IgG.

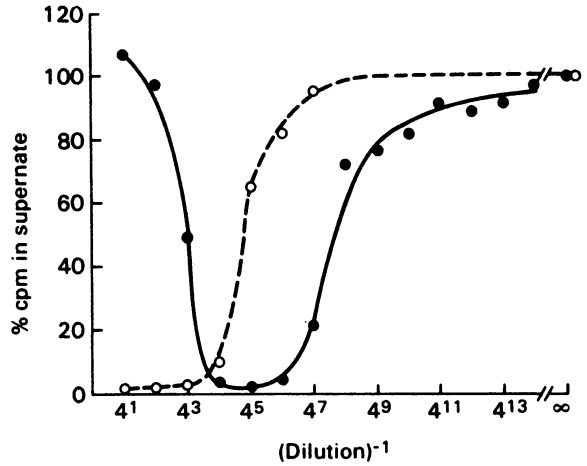

FIG. 5. *Indirect immunoprecipitation of [^{3}H]pargyline-labeled human platelet MAO by MAO-1C2 from conditioned medium and ascites fluid*

Samples of [^{3}H]pargyline-labeled human platelet MAO from a DEAE-fraction (containing 4224 cpm, 44 ng of labeled MAO, and 7.2 μg of total protein) were incubated in microtiter plates containing 50 μl of serial dilutions of MAO-1C2 precipitated from conditioned medium (O---O) or from untreated ascites fluid from a BALB/c mouse bearing a tumor of MAO-1C2 cells (●——●). Rabbit antimouse IgG (40 μg of antibody protein) and *Staphylococcus aureus* cells (50 μl of 10% suspension) were added and the ^{3}H remaining in the medium was assayed by scintillation counting, as described under Materials and Methods.

TABLE 2

Effect of MAO-1C2, anti-mouse IgG, and Staphylococcus aureus cells on the activity and solubility of MAO

Samples of an extract of human liver mitochondria (50 μl; 0.835 mg of protein) were mixed with the indicated combinations of (a) 50 μl of a dilution (1:256) of MAO-1C2 ascites fluid, (b) 10 μl of rabbit anti-mouse IgG, and (c) 50 μl of *S. aureus* cells as described under Materials and Methods, and incubated in 96-well microtiter plate. All samples were made up to the same final volume (115 μl) with PBS immunoprecipitation buffer. After appropriate incubations, the plate was centrifuged, and 20-μl samples were assayed in duplicate for MAO activity, using benzylamine as substrate.

Additions	Units of MAO activity[a]	
	Pellet	Supernatant
	nmoles/hr/sample	
MAO-1C2 only	<0.5	<0.5
S. aureus only	<0.5	<0.5
Rabbit anti-mouse IgG	<0.5	<0.5[b]
MAO only	<0.5	5.2 ± 0.06
MAO + MAO-1C2	<0.5	4.9 ± 0.03
MAO + MAO-1C2 + rabbit anti-mouse IgG	3.9 ± 0.30	1.1 ± 0.01
MAO + MAO-1C2 + rabbit anti-mouse IgG + *S. aureus*	3.8 ± 0.04	1.1 ± 0.12
MAO + rabbit anti-mouse IgG	<0.5	4.4 ± 0.17
MAO + *S. aureus*	0.5 ± 0.81	4.3 ± 0.90

[a] Activities of <0.5 are considered negligible, and represent less than 40 cpm over a background of 172 cpm in the assay. Values are expressed as mean of duplicate assays ± the standard deviation.

[b] Single assay only.

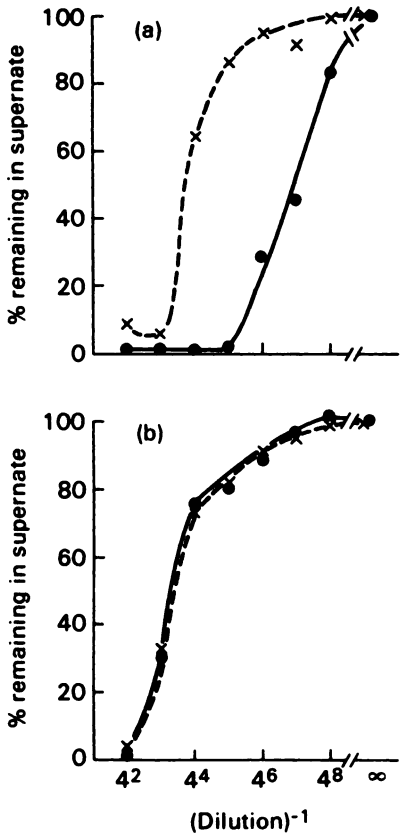

FIG. 6. *Titration of [^{3}H]pargyline-labeled and catalytically active human platelet MAO*

Samples of a [^{3}H]pargyline-labeled DEAE-fraction of human platelet MAO, (containing 13.2 ng of labeled enzyme), unlabeled platelet DEAE-fraction (containing an estimated 0.83 μg of unlabeled MAO protein), or a mixture of the same quantities of the two were incubated with serial dilutions of MAO-1C2 (ammonium sulfate-precipitated from conditioned medium). Rabbit antimouse IgG (40 μg of antibody protein) and *Staphylococcus aureus* cells were added as indicated under Materials and Methods. Samples were incubated at 4°.

a. Separate titration of labeled and unlabeled MAO: ×---×, fraction of enzyme activity not precipitated as determined by oxidation of benzylamine (100% = 11.6 units in each well); ●——●, fraction of [^{3}H] pargyline-labeled MAO not bound to bacteria as determined by scintillation counting (100% = 1320 cpm, counting efficiency 20%).

b. Titration of a mixture of [^{3}H]pargyline-labeled and unlabeled human platelet MAO. ×---×, enzyme activity not bound to bacteria; ●——●, [^{3}H]pargyline-labeled MAO not bound to bacteria. The quantities of labeled and unlabeled MAO added per well were the same as in a.

We next asked whether MAO-1C2 could distinguish between [^{3}H] pargyline-labeled, catalytically inactive human platelet MAO and unlabeled, catalytically active MAO. Samples of DEAE-purified, unlabeled, catalytically active human platelet MAO or [^{3}H]pargyline-labeled enzyme (830 and 13.2 ng of enzyme, respectively) were mixed with serial dilutions of MAO-1C2, and the enzyme-antibody complexes precipitated as usual (Fig. 6a). The immunoprecipitation of [^{3}H]pargyline-labeled MAO was very efficient [virtually no ^{3}H was left in the supernatant in wells containing dilutions of $<4^{-5}$ (Fig. 6)]. Immunoprecipitation of catalytically active MAO required about 64 times more antibody than did precipitation of the labeled enzyme (Fig. 6B), which was consistent with the presence of about 60 times more MAO protein in the unlabeled MAO titration (830 ng compared with 13.2 ng). When labeled MAO and unlabeled MAO were mixed, their immunoprecipitation curves were superimposable, despite the fact that the two enzymes were present at very different concentrations (Fig. 6b). Since MAO-1C2 did not preferentially precipitate either [^{3}H] pargyline-labeled or unlabeled MAO in the mixture, we conclude that MAO-1C2 does not distinguish between catalytically active and [^{3}H]pargyline-labeled MAO.

Considering the disagreement in the literature concerning the immunological similarity of MAO A and B, it was important to determine whether MAO-1C2 recognized MAO A. Since human placental mitochondria are a rich source of MAO A (32), we prepared crude placental mitochondria, extracted them with octylglucoside, mixed samples containing active MAO A with serial dilutions of MAO-1C2, and assayed the ability of the antibody to immunoprecipitate MAO A activity. We assayed unprecipitated MAO in the supernatant and precipitated MAO in the bacterial pellet with the substrates 5-HT and PEA. In addition, we performed the assays with and without prior treatment of the samples with the inhibitors clorgyline and deprenyl at concentrations which we had found previously to inhibit selectively MAO A (clorgyline) and MAO B (deprenyl). The results of the titration of placental material (Table 3) show that very little of the recovered 5-HT-oxidizing activity was found in the pellet (unbound, 29 units; bound, <1 unit). The 5-HT-oxidizing activity was due to MAO A, since it was sensitive to 10^{-6} M clorgyline and insensitive to 10^{-6} M deprenyl (Table 3). From these data we conclude that MAO-1C2 immunoprecipitated <3.5% of placental MAO A under the conditions employed.

TABLE 3

Immunoprecipitation of MAO A and B activity in extracts of mitochondria from human placenta and liver

Duplicate samples of octylglucoside extracts of human liver mitochondria (40 μl) were immunoprecipitated as described under Materials and Methods. MAO activities in the supernatant ("unbound") and in the suspended bacterial immunoprecipitate ("bound") were assayed using 1 mM 5-HT and 10 μM PEA as substrates with and without prior treatment of the samples with 10^{-6} M clorgyline or deprenyl. Values underlined are the best indicators of MAO A activity (clorgyline-sensitive 5-HT oxidation) or MAO B activity (deprenyl-sensitive PEA oxidation). Assays of duplicate samples differed by <10%. The minimal detectable levels of PEA oxidation and 5-HT oxidation (<0.02 and <1.0 nmole/hr/sample, respectively) represented 2-fold over background, and differ because of the different specific activities of the substrates used (see Materials and Methods).

Substrate	MAO activity			
	Placenta		Liver	
	Unbound	Bound	Unbound	Bound
	nmoles/hr/sample			
PEA				
No inhibitor	0.18	0.32	0.50	5.2
Clorgyline-sensitive	0.16	<0.02	0.14	<0.02
Deprenyl-sensitive	<0.02	0.31	0.38	5.1
5-HT				
No inhibitor	29	<1.0	18	<1.0
Clorgyline-sensitive	29	<1.0	18	<1.0
Deprenyl-sensitive	<1.0	<1.0	<1.0	<1.0

Compared with the 5-HT-oxidizing activity, the PEA-oxidizing activity of the placental mitochondrial extract was very low. The results indicate that some of this PEA-oxidizing activity was immunoprecipitated by MAO-1C2 (0.32 unit bound, 0.18 unit unbound; Table 3). The PEA-oxidizing activity which was immunoprecipitated by MAO-1C2 (0.32 unit) was sensitive to 10^{-6} M deprenyl, but insensitive to 10^{-6} M clorgyline, like MAO B. On the other hand, the PEA-oxidizing activity which was not immunoprecipitated by MAO-1C2 (0.18 unit) was sensitive to clorgyline and insensitive to deprenyl, like MAO A. The simplest interpretation of these results is that, although some of the low PEA-oxidizing activity in placenta is due to MAO A, there is a component of this activity which has the catalytic and immunological characteristics of MAO B.

Corresponding data for immunoprecipitation of MAO A and B from extracts of human liver mitochondria are also shown in Table 3. Again, very little 5-HT-oxidizing activity (<1.0 unit) was precipitated, whereas most of the activity (18 units) remained in the supernatant. In contrast, most of the PEA-activity (5.2 units) was precipitated, whereas <10% (0.5 unit) remained in the supernatant (Table 3). The clorgyline and deprenyl sensitivities of the activities support the interpretation that, under these conditions, 5-HT oxidation was due primarily to MAO A, and PEA oxidation to MAO B.

Since the monoclonal antibody was elicited by injection of human MAO B into a mouse, it was of interest to determine whether the antibody would immunoprecipitate mouse MAO B. We therefore attempted to immunoprecipitate MAO from an octylglucoside extract of crude mouse liver mitochondria, which was rich in MAO B (4). The unprecipitated material was assayed for MAO activity with PEA and 5-HT as substrates (Table 4). A control assay, in which MAO-1C2 was omitted, was done in parallel. The results showed that no significant PEA- or 5-HT-oxidizing activity was removed from the supernatant. We conclude, therefore, that MAO-1C2 did not immunoprecipitate significant amounts of mouse MAO A or B.

DISCUSSION

To our knowledge, we have isolated the first hybridoma-derived antibody to MAO. Since the original hybrid cell population has been twice subcloned with no change in the specificity of the secreted antibody, the antibody appears to be monoclonal. Although hybridoma MAO-1C2 was isolated from among the fusion products of spleen cells from a mouse immunized with [^{3}H]pargyline-labeled, inactive MAO, immunoprecipitation of mixtures of labeled and unlabeled enzyme failed to demonstrate any ability of the antibody to distinguish catalytically active from [^{3}H]pargyline-labeled platelet MAO (Fig. 6). Therefore, binding of the antibody appears not to be dependent on any structural features of the enzyme which are covered up or altered when pargyline binds covalently to the enzyme. MAO-1C2 also binds MAO B activity in human liver mitochondrial extracts and a small amount of MAO B-like activity in the mitochondrial extracts from human placenta. Our observation that MAO-1C2 does not inhibit the catalytic activity of human MAO B (Table 2) further suggests that the antibody does not bind the active site of the enzyme.

Complexes of MAO and MAO-1C2 do not pellet when centrifuged at low speed, which suggests that, under the conditions employed, the antibody does not precipitate the enzyme in the absence of secondary immunoglobulin reagents. However, MAO-1C2 will immunoprecipitate human MAO B when secondary anti-immunoglobulin is added. The ability of the antibody to immunoprecipitate >90% of both [^{3}H]pargyline-labeled, inactive platelet MAO and catalytically active MAO indicates that the antigenic determinant is probably expressed on most or all of the MAO B molecules. We have shown elsewhere (20) that MAO-1C2 binds a single discrete polypeptide component in crude extracts of human liver mitochondria, with the subunit molecular weight expected for MAO B. This high degree of specificity indicates that the determinant is not commonly expressed on mitochondrial polypeptides, and may in fact be unique to MAO B. We could find no evidence that MAO-1C2 binds human MAO A or mouse MAO A or B. These data demonstrate the high degree of specificity of MAO-1C2 for human MAO B. We expect that the antibody will cross-react with MAO B from at least some mammals more closely related to humans. Using MAO-1C2, we are currently developing a specific, competitive radioimmunoassay for MAO B protein levels in crude extracts of human platelets.

Although MAO-1C2 was elicited to MAO B from human platelets, it appears to bind equally well to the MAO B activity detected in human liver and placenta. Our identification of MAO A and B in these tissues was based on the ability of clorgyline to inhibit MAO A selectively (3) and the ability of deprenyl to inhibit MAO B (5). Under our conditions, oxidation of 10^{-3} M 5-HT by extracts of liver and placental mitochondria was virtually completely sensitive to clorgyline and resistant to deprenyl, and thus appeared to be quite selective for MAO A (Table 3). Therefore, our monoclonal antibody appears to recognize a structural feature which is present only on those molecules with the catalytic specificity of MAO B. Since the antibody does not appear to bind the active site of the enzyme, MAO A and B may differ in at least two parts of the molecule; the active site, which presumably determines the substrate specificity, and the site located elsewhere which is recognized by MAO-1C2. Al-

TABLE 4

Indirect immunoprecipitation of mouse MAO A and B activity by MAO-1C2

A sample (40 μl) of an octylglucoside extract of crude mouse liver mitochondria was immunoprecipitated with MAO-1C2 as described under Materials and Methods. The MAO activity remaining in the supernatant was assayed using PEA and 5-HT as substrates. Rabbit anti-mouse IgG and *Staphylococcus aureus* cells were used as secondary reagents. The control immunoprecipitation assay was performed exactly like the other except that MAO-1C2 was omitted.

	Activity remaining in supernatant	
	PEA oxidation units	5-HT oxidation units
	nmoles/hr/sample	
Plus MAO-1C2	15.2 ± 0.02	4.8 ± 0.28
Control (no MAO-1C2)	15.7 ± 0.06	4.1 ± 0.06

though these data are most simply interpreted as indicating that MAO A and B are structurally different polypeptides, we cannot at this time rule out the possibility that the covalent binding of a small molecule, such as a carbohydrate or lipid, might so alter the conformation of a single enzyme species that it both modifies the substrate specificity of the enzyme and changes the antigenic determinant recognized by MAO-1C2. The final resolution of the controversy concerning the molecular basis of the difference between MAO A and B will require detailed structural studies of the purified proteins. Such a study of human MAO B will be facilitated by our ability to purify the enzyme using MAO-1C2 immunoaffinity columns (22).

The ability of MAO-1C2, coupled with secondary immunoglobulin reagents, to immunoprecipitate MAO B without inhibiting the enzyme or altering its substrate or inhibitor specificity makes possible a highly sensitive test for the presence of low levels of human MAO B in extracts such as those from placenta, which contain a high level of MAO A. This test is done by exposing the extract to be tested to MAO-1C2, anti-mouse IgG, and *S. aureus* cells; washing the pellet; and assaying the MAO B activity bound in the pellet. High specificity for MAO B in the assay can be achieved by using the usual approach of inactivating residual, nonspecifically bound MAO A in the pellet with clorgyline and assaying the MAO with a B-preferred substrate, such as PEA at low (10 μM) concentrations. This scheme has two advantages in the detection of low levels of MAO B. First, the immunoprecipitation effectively concentrates the MAO B from the test extract, thereby making the assay more sensitive; second, it physically separates MAO B from the bulk of the MAO A, thus facilitating the discrimination of B from A. With this approach, we detected what appears to be a low but significant level of MAO B activity in extracts of human placental mitochondria. We cannot be certain whether the MAO B is present in the placental tissue itself or is contributed by blood cells (especially platelets) which are difficult to separate completely from the placental tissue. It will be of interest to use this combined immunoprecipitation and inhibitor assay to quantitate the levels of MAO B in other cells or tissues which have been reported to have various levels of MAO B activity.

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Send reprint requests to: Dr. Richard M. Denney, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Tex. 77550.