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Monoclonal antibodies to monoamine oxidase B and another mitochondrial protein from human liver

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A monoclonal antibody has been generated to human liver monoamine oxidase (MAO) B by fusion of mouse myeloma cells with spleen cells from a mouse immunized with a mixture of semi-purified MAO A and MAO B. The antibody, 3F12/G10, an immunoglobulin G_1, reacts with its antigen in cryostat sections of human liver, showing an intracellular particulate distribution as demonstrated by immunoperoxidase staining. The antibody indirectly precipitates [^{3}H]pargyline-labelled human MAO B both from liver and platelet extracts but fails to precipitate MAO A from liver extracts. The antibody does not recognise rat liver MAO B, showing that the determinant is not universally expressed on MAO B. The antibody has no effect on the catalytic activity of MAO B. Other monoclonal antibodies were generated but they are directed to a protein with a subunit M_r of 54000, a contaminant of the MAO preparation. One of these antibodies, A8/C2, an IgG_{2a}, reacts with the same protein in both rat and human liver extracts.

INTRODUCTION

Monoamine oxidase [monoamine: oxygen oxidoreductase (deaminating) (flavin-containing) EC 1.4.3.4] is an integral protein of the outer mitochondrial membrane and is the major enzyme through which catecholamines and indoleamines are degraded by oxidative deamination. It is found in two different forms, with distinct catalytic properties and tissue distribution. MAO A is inhibited by the irreversible active site inhibitor clorgyline and oxidizes 5-hydroxytryptamine (Johnstone, 1968; Neff & Yang, 1974) whilst MAO B is inhibited by deprenyl and pargyline and oxidizes phenylethylamine and benzylamine (Knoll & Magyar, 1972). 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 (Callingham & Parkinson, 1979; Brown *et al.*, 1980). Studies with [^{3}H]pargyline-labelled enzyme have shown that the FAD-containing subunits of human MAO B from platelets and MAO A from placenta migrate in SDS/polyacrylamide gels with apparent M_r values of 64000 and 60000 respectively (Cawthon *et al.*, 1981) or 67000 and 64000 (Brown *et al.*, 1980). This difference in apparent M_r of the two subunits suggests that the FAD-containing peptides in the two forms of MAO are different. Indeed, it has been observed that partial proteolytic digestion of [^{3}H]pargyline-labelled MAO A and B results in distinct patterns of [^{3}H]pargyline-labelled peptides (Brown *et al.*, 1980; Cawthon *et al.*, 1981; Cawthon & Breakfield, 1979). Furthermore, a monoclonal antibody which recognizes MAO B but not MAO A has been reported (Denney *et al.*, 1982). However, attempts to distinguish MAO A and B with conventional antisera have been equivocal. Dennick & Mayer (1977) have shown that antibodies raised in sheep against purified human liver MAO, which had only MAO B activity, did not distinguish between MAO A and MAO B. On the other hand, Powell & Craig (1977) found that antibodies raised against human placental MAO (predominantly A) failed to cross-react with the platelet enzyme (predominantly B). However, they later showed that the two forms exhibit considerable immunological cross reaction but that type A enzyme possesses antigenic determinants which are apparently not shared by type B enzyme (Brown *et al.*, 1982).

We have now raised a monoclonal antibody to human liver MAO B, which does not recognize MAO A and can be used in immunohistochemical and topographical studies on the distribution of MAO B in the outer mitochondrial membrane (Russell *et al.*, 1979). In addition, we have isolated other monoclonal antibodies to epitopes on mitochondrial membrane proteins which will be useful in our work on mitochondrial protein turnover (Russell *et al.*, 1984).

MATERIALS AND METHODS

General materials were obtained from the suppliers noted previously (Billett *et al.*, 1984). Phosphate-buffered saline contained in 1 litre: 8.00 g of NaCl, 0.20 g of KCl, 1.15 g of Na_2HPO_4 and 0.20 g of KH_2PO_4, pH 7.4 (and usually 0.20 g of NaN_3).

Purification of human liver MAO

MAO was purified from mitochondrial outer membranes (MOM) isolated from normal liver at post-mortem (< 24 h after death, 100 g weight); the membranes were prepared as described previously (Billett *et al.*, 1984). MAO was purified by a modification of the method of Dennick & Mayer (1977).

Triton X-100 extracts of MOM were loaded directly onto a Sepharose 6B column (3.2 cm internal diameter × 90 cm long; approx. 0.6 ml/min) pre-equilibrated in 20 mM-potassium phosphate buffer, pH 7.2 containing 0.1 mM-mercaptoethanol and 0.1% (w/v) Triton X-100. The fractions with the highest specific activity were pooled and the Triton X-100 removed by means of Bio-Beads SM-2 previously equilibrated in

Abbreviations used: MAO, monoamine oxidase; MOM, MIM, mitochondrial outer and inner membranes, respectively.

20 mM-potassium phosphate, pH 7.2 (Holloway, 1973). The enzyme was then precipitated with solid $(NH_4)_2SO_4$ and the precipitate was dialysed extensively against 20 mM-potassium phosphate, pH 7.2, containing 0.1 mM-mercaptoethanol. The dialysed preparation was then applied to a column (2.8 cm × 6 cm) of DEAE-cellulose previously equilibrated with 20 mM-potassium phosphate, pH 7.2, containing 0.1 mM-mercaptoethanol. Bound protein was eluted by means of a linear gradient (200 ml) of Triton X-100 (0–1%, w/v) in 20 mM-potassium phosphate containing 0.1 mM-mercaptoethanol. Fractions containing MAO activity were pooled and Triton X-100 was removed by means of Bio-Beads SM-2. The enzyme was again precipitated with $(NH_4)_2SO_4$ and the precipitate was dialysed extensively against 20 mM-potassium phosphate buffer, pH 7.2. The resultant preparation contained about 2 mg of protein. Polyacrylamide-gel electrophoresis (10%) of a sample (40 μg) in the presence of SDS showed a major band of M_r 57000 and two fainter bands of M_r 63000 and 54000. After labelling with 1.5 μM-[^{3}H]pargyline, 70% of the radiolabel was recovered in the band of protein of M_r 57000 and 30% in the band of M_r 63000.

Immunization

A BALB/c mouse was injected intraperitoneally with 250 μl of sterile phosphate-buffered saline containing 25 μg of purified human liver MAO emulsified in an equal volume of Freund's complete adjuvant; 21 days later it was injected intravenously with 20 μg of purified MAO in sterile saline; a similar injection was given on days 22 and 23. Fusion was performed on day 24.

Fusion of cells

Spleen cells (10^8) were fused with mouse myeloma P3NS1-Ag4 cells (10^7) as described previously (Billett *et al.*, 1984; Mayer & Billett, 1985) using 50% (w/v) poly(ethylene glycol) 1500 adjusted to approx. pH 7.6 with NaOH in RPMI medium containing dimethyl sulphoxide (5%, v/v, final concn.). The final cell pellet was distributed into four 96-well microtitre plates containing a macrophage feeder cell layer (Billett *et al.*, 1984).

Preliminary screening of culture supernatants

This was performed using an indirect trace binding solid-phase assay in round-bottomed poly(vinyl chloride) wells. The wells were pre-coated with 50 μl of poly(L-lysine) (M_r > 70000; Sigma type 1274) in phosphate-buffered saline (3 mg/100 ml) for 30 min (Kennett *et al.*, 1981) before addition of 2 μg of human MOM (or 0.2 μg of purified MAO) in 50 ml of phosphate-buffered saline. The antigen was incubated overnight at 4 °C and then fixed with glutaraldehyde (0.25%, v/v, final concn.) for 15 min. Non-specific adsorption was then prevented by incubation with 100 mM-glycine in 0.1% (w/v) bovine serum albumin for 30 min and with 1% (w/v) bovine serum albumin in phosphate-buffered saline for 4 h. All steps for the preparation of wells were at room temperature unless otherwise stated.

Aliquots (50 μl) of conditioned media from wells containing growing clones were incubated in the washed wells for 3 h at room temperature. Bound mouse immunoglobulin was detected after a further 3 h incubation of the washed wells with 50 μl of ^{125}I-labelled rabbit anti-(mouse IgG) antibody (specific radioactivity 5 μCi/μg; Billett *et al.*, 1984) in phosphate-buffered saline containing 1% (w/v) bovine serum albumin (approx. 60000 c.p.m., 12 ng). The wells were then washed out and the bound radioactivity counted in a γ-radiation counter.

Cloning

Two cultures, 3F12 and 2A8, were cloned by limiting dilution and were found to be clonal in origin. One sub-clone of each, 3F12/G10 and 2A8/C2, were injected into pristane-primed mice in order to generate ascites fluid. The IgG subclass of 3F12/G10 was found to be type 1 and that of 2A8/C2 was type 2a by using a solid-phase assay with rabbit anti-(mouse Ig subclass) antibodies.

Indirect immunoperoxidase technique

This was undertaken exactly as described by Holmes *et al.* (1982) and was kindly carried out by Dr. D. Allsop, Department of Biochemistry, University Hospital and Medical School, Nottingham. A mouse anti-(human osteogenic sarcoma) monoclonal antibody (791T/36/C13; Embleton *et al.*, 1981) was used as a control.

Immunoprecipitation

Human and rat MOM (Billett *et al.*, 1984; 2 mg of each) were labelled either by reductive methylation with NaB^3H_4 (7.5 mCi; Russell & Mayer, 1983) or with 1.5 μM-[^{3}H]pargyline (New England Nuclear, Boston, MA, U.S.A.; specific radioactivity 20.3 Ci/mmol) for 60 min at 30 °C. The membranes were then collected by centrifugation at 100000 *g* for 30 min, solubilized with Triton X-100 (Triton:protein 2:1, w/w) in the presence of 10^{-3} M-phenylmethanesulphonyl fluoride for 1 h at room temperature and the soluble material was collected by centrifugation at 100000 *g* for 30 min. Aliquots of the solubilized labelled protein (approx. 20 μg) contained in 25 μl were incubated with hybridoma culture supernatants in the presence of 0.1% bovine serum albumin and 0.02% NaN_3 for 16 h at 4 °C. Rabbit anti-(mouse IgG) antibody was added to precipitate the immune complex and the precipitate was washed four times in phosphate-buffered saline containing 0.1% (v/v) Triton X-100 and 0.1% (w/v) SDS before addition of Laemmli sample buffer for gel electrophoresis.

Immunoaffinity chromatography

Ascitic fluid derived from the hybridoma 3F12/G10 was subjected to $(NH_4)_2SO_4$ precipitation followed by dialysis against 10 mM-potassium phosphate buffer, pH 8.0, to precipitate lipoproteins (Mayer & Walker, 1980). This antibody or another, control monoclonal antibody, (4 mg of each) was then coupled to 1 ml of CNBr-activated Sepharose 4B (Hudson & Hay, 1980). Solubilized extracts containing radiolabelled MAO were incubated overnight at 4 °C with the immunoadsorbent. After washing six times in phosphate-buffered saline, bound protein was eluted with 4 M-KSCN. The KSCN was immediately removed from the protein by filtration on a Sephadex G-25 column pre-equilibrated with phosphate-buffered saline.

Binding of monoclonal antibodies to subcellular fractions (filter binding assay)

The reactivity of monoclonal antibodies with mitochondrial subfractions was measured as described (Billett *et al.*, 1984), binding being monitored by using

^{125}I-labelled rabbit anti-(mouse IgG) antibody. Mitochondrial subfractions were prepared from mitochondria subjected to osmotic lysis, using a method based on that of Martinez & McCauley (1977), modified as described by Billett *et al.* (1984).

Preparation of blood platelets

Platelet-rich plasma was prepared exactly as described by Billett *et al.* (1983).

SDS/polyacrylamide-gel electrophoresis

This was undertaken exactly as described by Laemmli (1970) and the radioactivity was detected by fluorography (Bonner & Laskey, 1974).

Biochemical assays

Protein was determined by the method of Lowry *et al.* (1951) or by a biuret method, with bovine serum albumin as standard. MAO activity was assayed in duplicate as described by Russell & Mayer (1983) and succinate dehydrogenase (EC 1.3.99.1) as described by Pennington (1961).

RESULTS

Fusion products

At 14 days after fusion, conditioned media from the 176 wells showing hybridoma growth (44% of the wells plated) were assayed for antibody which would bind to human MOM as detected by the indirect trace-binding solid phase assay. Conditioned media from 11 wells gave positive reactions to human MOM. These media also gave positive reactions to purified human MAO and all populations were expanded and frozen in liquid N_2.

Immunoperoxidase staining of liver

The cellular distribution of the epitopes was examined by the immunoperoxidase staining of cryostat human liver sections. Three of the conditioned media showed weak positive staining of the parenchyma and six showed strong positive staining of the parenchyma, all with a particulate distribution in the cytoplasm (see Figs. 1*a* and 1*b* for representative staining of two of the antibodies, 3F12 and 2A8). These antibodies did not appear to stain the non-hepatocytic parenchymal sinusoidal cells. This intracellular particulate distribution would be consistent with a mitochondrial localization. Another culture medium, on the other hand, did not stain the cytoplasm of these cells but stained areas around the portal tract and around the sinusoidal cells, suggesting an epitope localized in the extracellular matrix (3A12, Fig. 1*c*).

Immunoprecipitation from labelled MOM preparations

To determine whether any of the antibodies were specific to MAO we tested their ability to precipitate the enzyme from MOM labelled with either NaB^3H_4 or [^{3}H]pargyline; the immune precipitates were analysed on 10% polyacrylamide gels. When radioactively methylated human MOM was used as an antigen preparation one culture medium, 3F12, precipitated a protein with an M_r of 57000 (Fig. 2), corresponding to MAO B (see later); the media from five cultures, 4F12, 2A8, 4B10, 4F4 and 2H4, precipitated an M_r 54000 protein (see Fig. 2 for precipitation by 2A8) and one culture medium, 3A12, precipitated an M_r 100000 protein. All these culture media also precipitated an M_r 66000 protein, corresponding to serum albumin, from the methylated MOM preparation (see Fig. 2); this precipitation appears to be non-specific and cannot be removed by washing in buffers containing 0.1% Triton X-100 and 0.1% SDS. This albumin is derived from the endoplasmic reticulum which is known (Billett *et al.*, 1984) to contaminate the MOM preparation. When reductively methylated rat MOM was used instead of human MOM as the antigen preparation, no proteins were precipitated by the 3F12 antibody and only the 2A8 medium precipitated the M_r 54000 protein (results not shown); none of the cultures precipitated serum albumin.

Incubation of human MOM with [^{3}H]pargyline resulted in the labelling of the two forms of MAO (Fig. 3, lane *b*) with the B form (apparent M_r 57000) being predominantly labelled compared with the A form (apparent M_r 63000), as expected from the relative concentrations of the enzymes in the liver. The 3F12 culture medium precipitates only the M_r 57000 [^{3}H]-pargyline-labelled protein, i.e. MAO B (Fig. 3, lane *c*). The other culture media did not precipitate [^{3}H]pargyline-labelled MAO (e.g. 2A8, lane *d*). No labelled proteins were precipitated by the culture media from [^{3}H]pargyline-labelled rat MOM.

When [^{3}H]pargyline-labelled human blood platelets, which predominantly contain MAO B (top trace, Fig. 4) were incubated with these media, only the 3F12 culture medium precipitated the labelled protein (Fig. 4, middle trace). It is therefore apparent that although the antibody was raised against active MAO it is able to react with pargyline-labelled, i.e. inactive, MAO.

Association of 3F12/G10 and 2A8/C2 with human liver mitochondrial subfractions

Binding of the antibodies to mitochondrial sub-fractions (100 μg of protein) was monitored with ^{125}I-labelled rabbit anti-(mouse IgG) and a filter binding assay (see the Materials and methods section). The results are shown in Table 1 together with marker enzyme distributions. Band 1 contains a higher ratio of MAO to succinate dehydrogenase activity than Band 2 (5.6 for Band 1, 1.75 for Band 2), indicating that it is enriched in MOM. One important feature, however, is that the binding of 3F12/G10 is directly correlated with MAO activity. Indeed, binding of 3F12/G10 per unit of MAO activity (c.p.m./unit of MAO) is relatively constant (Band 1, 243; Band 2, 207; pellet, 264), indicating that the membrane-bound enzyme is accessible to the antibody and is being recognized by the antibody. This is very important because solubilized preparations of MAO were used as an antigen. The binding of 2A8/C2, an antibody used as a control, did not mimic MAO activity and was more closely correlated with succinate dehydrogenase activity, suggesting an inner mitochondrial membrane localization.

When rat mitochondria, rat MOM and rat MIM were analysed on a 10% polyacrylamide gel and stained with Coomassie Blue, the M_r 54000 band, i.e. the band precipitated by 2A8/C2, was clearly present (results not shown). This band was also present in fractions enriched in lysosomes and nuclei, both of which are contaminated with mitochondria, but it was absent from the endoplasmic reticulum fraction. Our MOM preparations contain slight contamination with endoplasmic reticulum (see Billett *et al.*, 1984) but, since the M_r 54000 band is

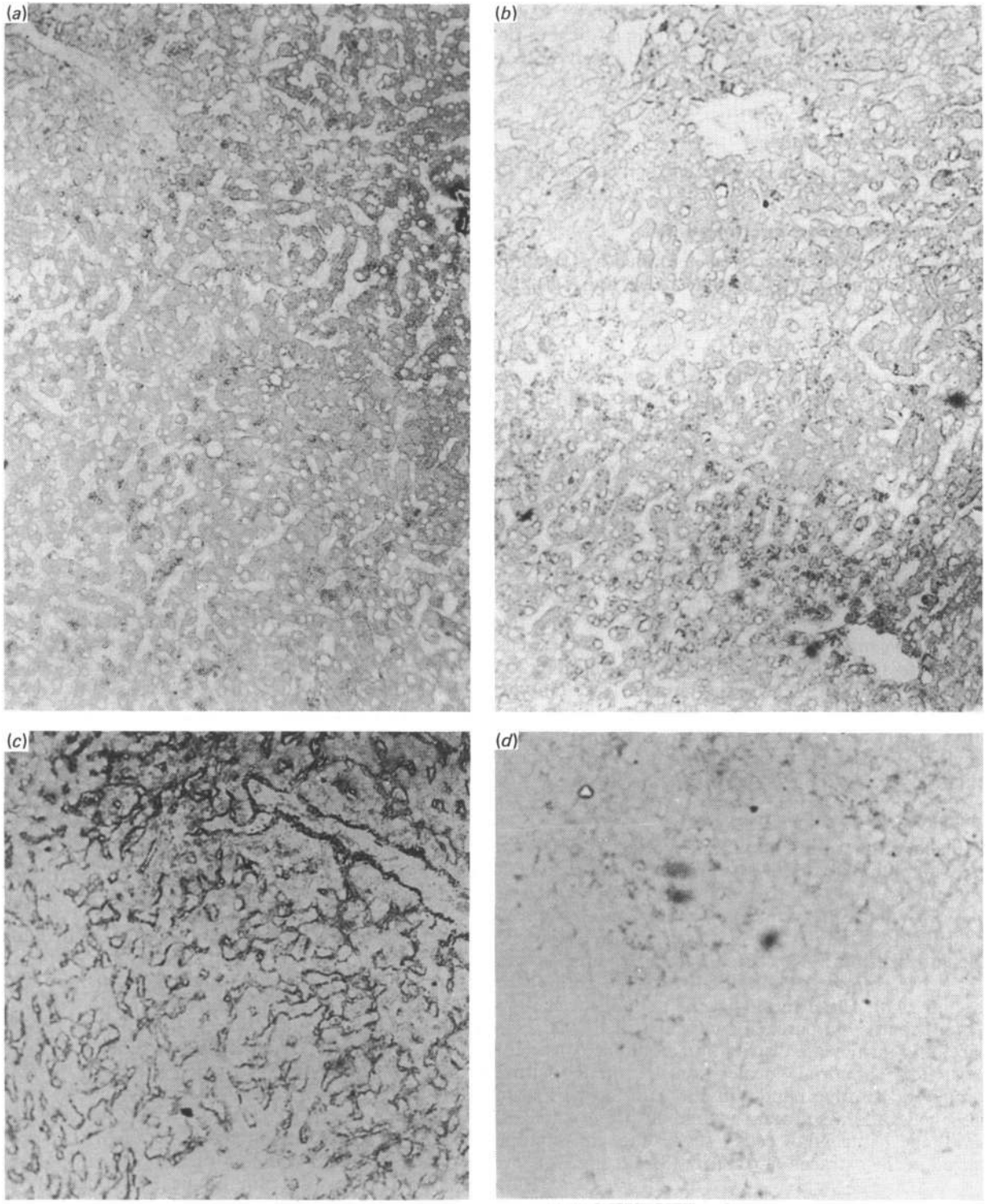

Fig. 1. Immunoperoxidase staining of human liver sections

(*a*) Stained with 3F12/G10, showing unstained sinusoidal cells, particulate staining of parenchyma cells; (*b*) stained with 2A8/C2, showing same distribution of stain as in (*a*); (*c*) stained with 3A12, showing staining of membranes around the sinusoidal cell and no stain in the parenchyma cells; (*d*) negative control stained with 791Y/36/C12. Magnification in (*a*)–(*d*), ×70.

not present in this fraction, it suggests that the 2A8/C10 antibody is indeed recognizing a mitochondrial antigen.

Immunoaffinity chromatography with 3F12/G10

Mitochondrial membranes (2.65 mg) were labelled with [^{3}H]pargyline and the labelled protein was extracted from the membranes with Triton X-100. When this extract was incubated overnight with a 1 ml column of Sepharose–3F12/G10, between 12 and 28% of the radioactivity was bound to the column and over half of this radioactivity was eluted with 4 M-KSCN. When the eluate was analysed by electrophoresis on 10% polyacryl-

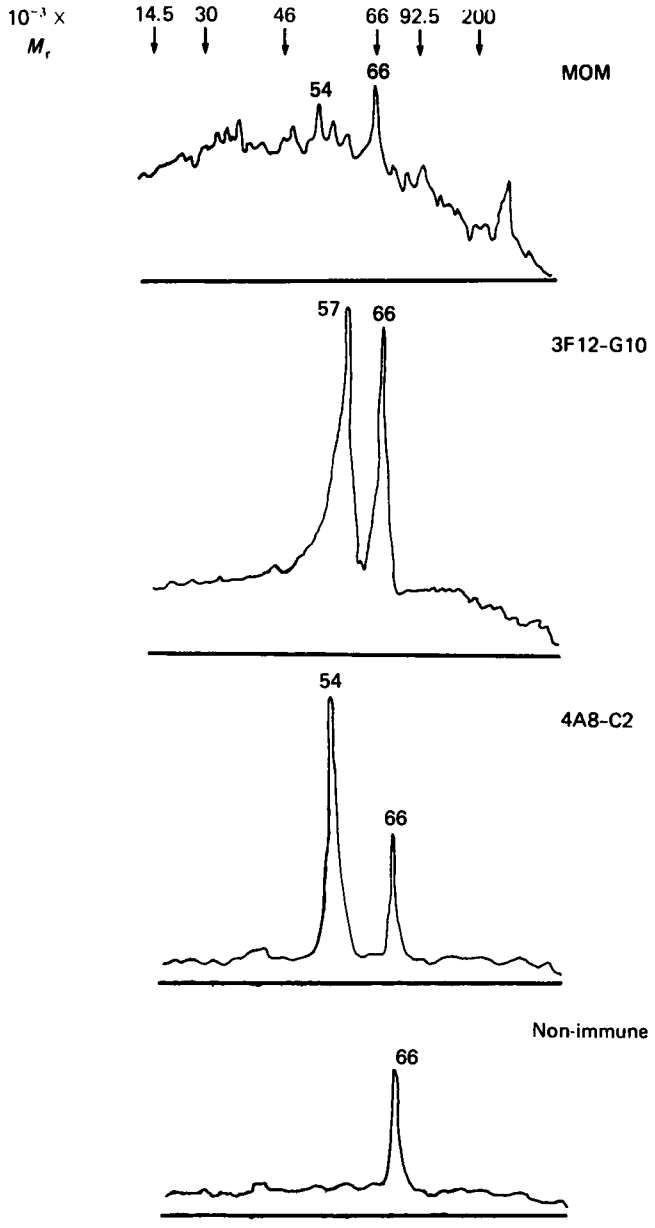

Fig. 2. Indirect immunoprecipitation of ^{3}H-labelled human MOM proteins by 3F12/G10 and 2A8/C2

Human MOM was labelled with NaB^3H_4 by reductive methylation, solubilized with Triton X-100 and subjected to immunoprecipitation as described in the Materials and methods section. The immunoprecipitates were analysed by electrophoresis on a 10% polyacrylamide gel and the radioactivity was detected by fluorography and measured by densitometry. The top trace represents Triton-solubilized proteins from MOM. ^{14}C-labelled *M*r standards were obtained from Amersham Research Products and consisted of a mixture of myosin (M_r 200000), phosphorylase *b* (92500), bovine serum albumin (66000), ovalbumin (46000), carbonic anhydrase (30000) and lysozyme (14500). These are indicated by arrows.

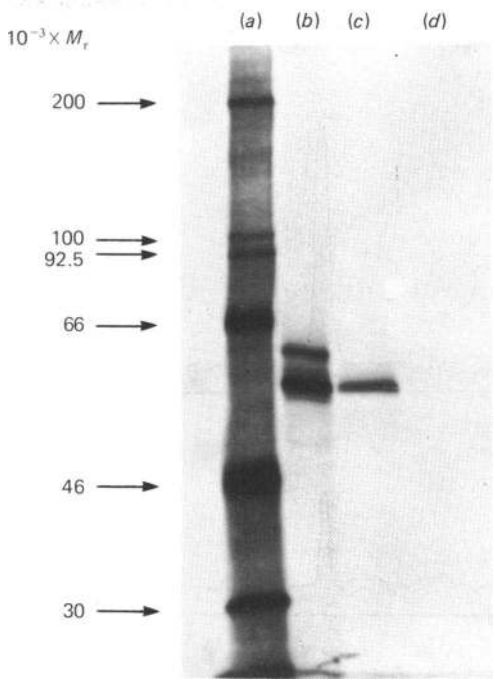

Fig. 3. Indirect immunoprecipitation of [^{3}H]pargyline-labelled human MOM proteins by 3F12/G10 and 2A8/C2

Human MOM was labelled with [^{3}H]pargyline, solubilized and subjected to immunoprecipitation as described in the Materials and methods section. The immunoprecipitates were analysed by electrophoresis in 10% polyacrylamide gels and the radioactivity detected by fluorography. Lane (*a*), ^{14}C-labelled standards; (*b*), [^{3}H]pargyline-labelled MOM; (*c*), MOM precipitated with 3F12/G10; (*d*), MOM precipitated with 2A8/C2. Radiolabelled standards are as in Fig. 2.

amide gels followed by fluorography, a major band of MAO B (M_r 57000) was detected. Prior to fractionation on the column the MAO B/MAO A ratio for the mitochondrial membranes was 2.40; protein which did not bind to the immunoaffinity column had a MAO B/MAO A ratio of 0.70, indicating considerable enrichment in MAO A. This shows that the immunoadsorbent has the potential to select MAO B from a crude extract, but optimal conditions for this selection have yet to be determined.

Effect of 3F12/G10 on catalytically active MAO

Culture filtrates and ascites fluid derived from 3F12/G10 had no effect on the activity of MAO extracted from human liver mitochondrial pellets with octylglucoside. Activities measured using [^{14}C]tyramine (1 mCi/mmol) as a substrate were as follows, expressed as d.p.m. $\pm$ S.D. ($n = 4$): no addition, 2316 ± 242; plus 3F12/G10 culture supernatant, 2521 ± 363.

DISCUSSION

In the present study we have used a mixture of catalytically active MAO A and MAO B from human liver to raise a monoclonal antibody, 3F12/G10, which recognizes human MAO B whether it is embedded in the outer mitochondrial membrane (Fig. 1, Table 1) or rendered soluble by detergent extraction (Figs. 2–4). As far as we know we are the first to use an anti-MAO monoclonal antibody to stain MAO in cryostat sections. In view of its reactivity with the native protein in the membrane, it is hoped that 3F12/G10 can be used to

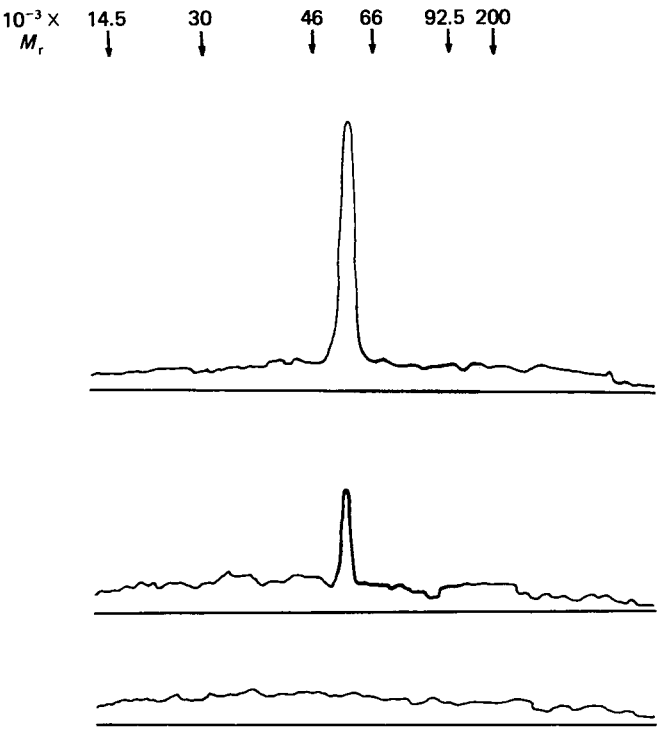

Fig. 4. Indirect immunoprecipitation of MAO B from [^{3}H]pargyline-labelled platelets by 3F12/G10

Human platelets were labelled with [^{3}H]pargyline, solubilized and subjected to immunoprecipitation, etc., as described in Fig. 3. The radioactivity was measured by densitometry. The top trace represents Triton-solubilized proteins from platelets; middle, platelets precipitated with 3F12/G10; bottom, precipitation with 2A8/C2. Radiolabelled standards are as in Fig. 2 and are indicated by arrows.

study the topography of this form of MAO in the membrane, for example, by electron microscopy using the immuno-gold method of detection (Roth *et al.*, 1978). This will allow definitive assessment of the proposed orientation of MAO B in the outer surface of the outer mitochondrial membrane (Russell *et al.*, 1979). In addition, it will be a useful tool for monitoring the distribution of the enzyme in various tissues.

The 3F12/G10 antibody is also reactive with [^{3}H]-pargyline-labelled MAO, i.e. it can recognize MAO which has been rendered catalytically inactive by this suicide inhibitor. Therefore, binding of the antibody appears not to be dependent on any structural features of the FAD-binding site of the enzyme which will be modified when pargyline binds covalently to the enzyme, and it is also clear that pargyline binding does not have a large effect on the conformation of the enzyme. In addition, the antibody does not interfere with binding of a substrate, for example, tyramine, to MAO, suggesting that the antibody also does not bind the catalytically active site of the enzyme.

When the 3F12/G10 antibody is incubated with a mixture of MAO A and MAO B it can specifically bind to MAO B and, provided a secondary anti-immunoglobulin is added, it can immunoprecipitate this form of the enzyme. The antibody can therefore be used to separate the two enzyme forms for comparative studies. Since the monoclonal antibody appears to recognize an epitope which is present only on the B form of the enzyme it confirms the conclusion of Brown *et al.* (1982) and Denney *et al.* (1982) that there are structural differences between the A and B forms of MAO. It also suggests that the immunodominant area on MAO B is situated some distance away from the catalytic site. In addition, since it can react with and precipitate MAO B in human platelets, 3F12/G10 may possibly be used in an immunoassay for MAO B protein in extracts of human platelets. It may therefore have clinical potential in understanding psychiatric disorders (Needham *et al.*, 1981).

Although 3F12/G10 appears to react with MAO B in both human liver and platelet extracts it does not bind to MAO B in rat liver extracts. We have not undertaken an extensive survey of the reactivity of the antibody, but it is clear that the determinant is not universally expressed on MAO B from all species.

Five other antibodies were isolated which could precipitate a protein of M_r 54000 from a crude human liver extract and also showed an intracellular particulate distribution of the antigen in liver parenchyma cells. These antibodies are clearly directed to a protein contaminant in the MAO preparation and the particulate distribution and gel profiles are consistent with a mitochondrial localization. One of the antibodies, A8/C2, precipitates the protein from rat liver extracts, demonstrating the presence of the same determinant on

Table 1. Association of monoclonal antibodies with human liver mitochondrial subfractions

Band	Total protein recovered/10 g of liver (mg)	Marker enzyme activities (arbitrary units/mg of protein)		Monoclonal binding* (c.p.m./100 μg of protein)	
		Succinate dehydrogenase	MAO	3F12/G10	2A8/C2
1	1.9	6.6	37.0	9014	4050
2	6.4	31.6	55.5	11500	11576
Pellet	11.8	5.1	28.3	7474	6428

* Corrected for non-specific binding, monitored by using spent medium.

the protein in two species. These antibodies will be very useful tools, especially since they precipitate a specific protein, in our work on mitochondrial protein degradation in tissue culture cells. The A8/C2 antibody will be particularly useful for this purpose since we are currently involved in monitoring the turnover of mitochondrial membrane proteins in rat hepatoma cells (Russell *et al.*, 1984).

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