Characterization of two monoclonal antibodies obtained after immunization with human liver mitochondrial membrane preparations

E. Ellen BILLETT,* Barbara GUNN† and R. John MAYER*

*Department of Biochemistry, University Hospital and Medical School, Clifton Boulevard, Nottingham NG7 2UH, U.K., and †CRC Laboratories, University of Nottingham, University Park, Nottingham NG7 2RD, U.K.

(Received 20 February 1984/Accepted 18 April 1984)

Two monoclonal antibodies have been generated by fusion of mouse myeloma cells with spleen cells from mice immunized with human liver mitochondrial membranes. One antibody, 1H6/C12, an immunoglobulin G_{2a} (IgG_{2a}), binds to the inner membrane of rat hepatocyte mitochondria, and immunoperoxidase staining demonstrates that its epitope has an intracellular particulate distribution within rat and human hepatocytes and human brain neurons. The epitope reactive with 1H6/C12 is partially sensitive to proteinase digestion. The second antibody, 3F12/F2, an IgG₁, binds to a contaminating cell type, namely the granulocyte, but it does not bind to monocytes, lymphocytes and red cells in human blood. This antibody reacts with cells in the portal tract and sinusoids of rat and human liver, as shown by immunoperoxidase staining. The epitope for 3F12/F2 is extremely sensitive to proteinase digestion and is only exposed when granulocytes are fixed in acetone, indicating an internal localization.

During an attempt to isolate a monoclonal antibody to human liver mitochondrial membranes we have isolated an antibody, 1H6/C12, to an epitope on the MIM of hepatocytes. In addition, an antibody, 3F12/F2, which binds a protein in semi-purified human MOM preparations was isolated, but after further characterization it was clear that it was recognizing an epitope on a contaminating cell type, namely the granulocyte. This epitope is not present in monocytes, lymphocytes and red cells from human blood.

In addition to reacting with their epitopes on human liver, these monoclonal antibodies react with those on rat liver, showing useful and specific binding in tissue sections. We believe that 1H6/C12, an IgG_{2a}, is a useful marker for MIM and is the first monoclonal antibody against the MIM to be isolated. 3F12/F2 is a particularly interesting antibody, since its epitope is inside the granulocyte, unlike the epitopes of most granulocyte monoclonal antibodies. It is hoped that 3F12/F2 may prove useful for diagnostic purposes and in the study of granulocyte biology.

Abbreviations used: Ig, immunoglobulin; MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; SDH, succinate dehydrogenase; MAO, monoamine oxidase.

Materials and methods

BALB/c mice were obtained from commercial suppliers, and Wistar rats were obtained from the Joint Animal Breeding Unit, School of Agriculture, Sutton Bonnington, Leics., U.K. Tissue-culture media, bovine-foetal serum, and poly(vinyl chloride) plates (Titertek immunoassay plates) were purchased from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. The mouse myeloma line P3NS1-Ag4 was provided by Dr. C. Milstein, Department of Molecular Biology, University of Cambridge, Cambridge, U.K. Poly(ethylene glycol) 1500 was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Rabbit anti-(mouse Ig subclass) antibodies were obtained from Miles Laboratories, Slough, Berks., U.K. by Dr. M. R. Price, Cancer Research Campaign Laboratories, University of Nottingham, University Park, Nottingham NG7 2RD, U.K., who kindly carried out the subclass determination. Mouse IgG was also purchased from Miles Laboratories. Percoll and density marker beads were purchased from Pharmacia, Uppsala, Sweden.

Phosphate-buffered saline contained, in 1 litre; 8.00 g of NaCl, 0.20 g of KCl, 1.15 g of Na₂HPO₄ and 0.20 g of KH₂PO₄, pH 7.4 (and usually 0.20 g of Na₂N₃).

Purification of human MOM

MOM vesicles were prepared from 100g of a healthy post-mortem liver (<24h old) basically using the method of Martinez & McCauley (1977). Unlike Martinez & McCauley (1977), however, we mixed the MOM from the 1.2M-sucrose cushion with 2vol. of cold 1M-NaCl to remove loosely bound proteins, and centrifuged at 100000g_{av}. for 30min at 4°C. The final pellet (2.5mg of protein/ml) was sonicated in phosphate-buffered saline and stored at -70°C in small portions. The sonicated pellet contained no lactate dehydrogenase activity, but did contain succinate dehydrogenase and aryl esterase activities, indicating contamination with MIM and endoplasmic reticulum.

Immunization

BALB/c mice were injected intraperitoneally with 250μl of sterile phosphate-buffered saline containing 200μg of human MOM emulsified in an equal volume of Freund's complete adjuvant; 3 weeks later they were injected both intraperitoneally and intravenously with 100μg of MOM in sterile saline. Fusion was performed 3 days later.

Fusion of cells

Spleen cells (10⁸) were fixed with mouse myeloma P3NS1-Ag4 cells (10⁷) by using a slightly modified version of Galfré & Milstein's method (1981) (see Mayer & Billett, 1984). The mouse myeloma cell line was grown in RPMI medium supplemented with 10% (v/v) heat-inactivated foetal-calf serum and 8-azaguanine (15μg/ml). Fusion was achieved with poly(ethylene glycol) 1500, adjusted to approx. pH7.6 with NaOH, in RPMI medium containing dimethyl sulphoxide (5%, v/v, final concn.). The final cell pellet was re-suspended directly in HAT medium (15% (v/v) foetal-calf serum/0.4μM-aminopterin, 0.1mM-hypoxanthine and 16μM-thymidine; Littlefield, 1974) and distributed into four 96-well microtitre plates. All media contained streptomycin (60μg/ml) and penicillin G (100μg/ml).

Feeder cells

Macrophages were obtained from 200g Wistar rats by peritoneal lavage with 20ml of RPMI medium containing 10% (v/v) heat-inactivated newborn-calf serum.

Routine screening of culture supernatants by using an indirect trace-binding solid-phase assay

This was performed in round-bottomed poly(vinyl chloride) wells. The wells were usually pre-coated with 50μl of poly-L-lysine ($M_r > 70000$;

Sigma type P1886) in phosphate-buffered saline (3mg/100ml) for 30min (Kennett, *et al.* 1981) before the addition of 1μg of human MOM in 50μl of phosphate-buffered saline. The MOM was incubated overnight at 4°C; glutaraldehyde was then added (0.25%, v/v, final concn.) and left for 15min, and the wells washed twice in phosphate-buffered saline. Non-specific adsorption was then prevented by incubation with 100mM-glycine in 0.1% (w/v) bovine serum albumin for 30min and with 1% (w/v) bovine serum albumin in phosphate-buffered saline, for 4h. The wells were then washed twice with 1% (w/v) bovine serum albumin in phosphate-buffered saline, filled with the same solution, sealed with Parafilm and stored at -20°C until needed (maximum time approx. 6 weeks). All the steps for the preparation of wells were at room temperature unless otherwise stated.

The assay was performed as follows. A 50μl portion of hybridoma supernatant (or serial dilutions of supernatant in phosphate-buffered saline) or, in controls, 50μl of P3NS1 myeloma spent medium, were added to each well and left for 2-3h at room temperature or overnight at 4°C. Wells were then washed twice in phosphate-buffered saline and twice in phosphate-buffered saline containing 1% (w/v) bovine serum albumin before addition of 50μl of ¹²⁵I-labelled rabbit anti-(mouse IgG) antibody (sp. radioactivity 5μCi/μg) in phosphate-buffered saline containing 1% (w/v) bovine serum albumin (approx. 60000c.p.m., 12ng). After 2-3h at room temperature the wells were washed as before, cut up and counted for radioactivity in a γ-radiation counter.

Other antigens were bound to the poly-L-lysine-coated wells, including rat MOM and rat MIM, but the procedure used was identical. In some experiments the wells were not pre-coated with poly-L-lysine and glutaraldehyde fixation was omitted.

Preparation of ¹²⁵I-labelled rabbit anti-(mouse IgG) antibody for screening

Rabbits were immunized four times with an IgG fraction from mouse serum at 2-weekly intervals (0.25mg/treatment), as described by Mayer & Walker (1980). The rabbit antibody was subjected to (NH₄)₂SO₄ precipitation followed by dialysis against 10mM-potassium phosphate buffer, pH8.0, to precipitate lipoproteins (Mayer & Walker, 1980). Mouse IgG was coupled to CNBr-activated Sepharose 4B (Hudson & Hay, 1980) (0.5mg/ml of Sepharose) and the rabbit anti-(mouse IgG) was affinity-purified by allowing it to bind to this column and eluting with 0.1M-glycine/HCl, pH2.5. Fractions were neutralized immediately with Tris base. For iodination, 80μg of the affinity-purified IgG was labelled with 1mCi of Na¹²⁵I (Amersham

International; code IMS 30) by using the chloramine-T method (Brown *et al.*, 1979).

Subclass determination

The IgG subclass of the monoclonal antibody was determined either by gel diffusion or by a solid-phase assay with rabbit anti-(mouse Ig subclass) antibodies.

Rat liver fractionation

The method used was based on that of de Duve *et al.* (1955). Saline-perfused livers (usually approx. 10g) from 180–200g Wistar rats were homogenized in 3 vol. of ice-cold 0.25M-sucrose in 3mM-imidazole/HCl, pH 7.4 (isolation medium) and centrifuged for 9000g-min. The pellet was rehomogenized in 1 vol. of isolation medium and the nuclear fraction was recovered by centrifuging at 9000g-min. The supernatant was centrifuged at 33000g-min to collect the mitochondria and the mitochondrial pellet was washed twice in 1 vol. of isolation medium, centrifuging at 33000g-min each time to collect the mitochondria. Lysosomes were collected from the 33000g-min supernatant by centrifuging at 250000g-min, and they were washed twice in 1 vol. of isolation medium. Finally, endoplasmic reticulum was collected by centrifuging at 3150000g-min. Each subcellular fraction was resuspended in 10ml of isolation medium and sonicated for 20s at 70W (Soniprobe; Dawe, London W.3, U.K.).

Subfractionation of rat liver mitochondria

Mitochondria were isolated from 180–200g Wistar rats and MOM detached by osmotic lysis, as described by Martinez & McCauley (1977). MOM were separated from MIM on a two-step sucrose density gradient, as described by Parsons *et al.* (1966). MOM were collected on the 1.1M-sucrose cushion (band 1) and some of the MIM (with contaminating MOM) on the 1.5M-sucrose cushion (band 2). MIM, together with intact mitochondria, formed a pellet (pellet). Bands 1 and 2 were collected separately, mixed with 2 vol. of cold water and sedimented at 100000g_{av.} for 30min. All sedimented material was sonicated in 1 ml of phosphate-buffered saline (20s at 70W; Soniprobe) before use.

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

The reactivity of monoclonal antibodies with rat liver subcellular membranes, including mitochondrial subfractions, was measured as follows. The antibodies were incubated overnight at 4°C with a sonicated membrane fraction in the

presence of 1% (w/v) bovine serum albumin, 2mM-phenylmethanesulphonyl fluoride and 1mM-EDTA. The membranes were then sedimented by centrifuging at 100000g_{av.} for 1h, resuspended in 300μl of phosphate-buffered saline containing 1% (w/v) bovine serum albumin, and incubated with ¹²⁵I-labelled rabbit anti-(mouse IgG) antibody (approx. 300000c.p.m.; 60ng) for 3h at room temperature. The membranes were then collected under vacuum on glass-fibre discs (GF/C; Whatman Biochemicals, Maidstone, Kent, U.K.), washed with 20ml of phosphate-buffered saline containing 1% (w/v) bovine serum albumin, and radioactivity then measured with a γ-radiation counter.

Blood-cell fractionation

Blood cells were separated by using the method of Brandslund *et al.* (1982) with some modifications as undertaken by Dr. G. Leslie and Miss Dagny Fisker (Department of Microbiology, Queen's Medical Centre, Nottingham NG7 2UH, U.K.; personal communication), silicone-treated glassware being used throughout. In this modified method, whole blood was layered on a Percoll step gradient of 1.095g/ml (12ml) under 1.050g/ml (6ml) and centrifuged at 400g_{av.} for 5min. Lymphocytes, neutrophils and monocytes were collected at the interface between the two layers of Percoll and then subjected to a second preformed gradient of Percoll as described by Brandslund *et al.* (1982). Platelets remained above the 1.050g/ml layer and the red cells were pelleted at the bottom of the tube. Monocytes formed a band at a density of about 1.062g/ml, as indicated by density marker beads in a control gradient; lymphocytes formed a band below monocytes and neutrophils formed a band at a density of about 1.080g/ml. These cells were collected, washed and counted. A blood smear was prepared, fixed, stained for esterase activity (Horwitz *et al.*, 1977) and then stained with Methyl Green. Monocytes were identified as being esterase-positive and the other cells were identified by their morphology.

For the binding assay, 10⁵ cells were fixed in acetone at -20°C for 5min, washed twice in phosphate-buffered saline to remove excess acetone and then incubated in 0.5ml of antibody or spent myeloma medium in the presence of non-immune mouse IgG (approx. 5μg) at 4°C for 16h. They were then washed in phosphate-buffered saline containing 1% (w/v) bovine serum albumin, incubated with ¹²⁵I-labelled rabbit anti-(mouse IgG) antibody (approx. 300000c.p.m.; 60ng) for 3h at room temperature and finally washed in phosphate-buffered saline containing 1% (w/v) bovine serum albumin and counted on a γ-radiation counter.

Indirect immunoperoxidase technique

This was undertaken exactly as described by Holmes *et al.* (1982), with a mouse anti-(human osteogenic sarcoma) monoclonal antibody (791T/36/C13; Embleton *et al.*, 1981) as a control.

Proteinase and phospholipase treatment of membranes bound to poly(vinyl chloride) wells

Wells were treated as indicated in the Results section with trypsin (EC 3.4.21.4) or Pronase (*Streptomyces griseus* proteinase), both bought from Sigma. Wells were treated with a mixture of phospholipases, namely phospholipase A₂ (EC 3.1.1.4 from porcine pancreas; Sigma; 14 units/well); phospholipase D [EC 3.1.4.4; from cabbage (*Brassica oleracea*); Boehringer; 20 units/well]; phospholipase C (EC 3.1.4.3; type X from *Clostridium perfringens*; 2.5 units/well).

Biochemical assays

Monoamine oxidase [amine oxidase (flavin-containing), EC 1.4.3.4] activity was assayed in duplicate as described by Russell & Mayer (1983). Other subcellular markers were also assayed: succinate dehydrogenase (EC 1.3.99.1) (Pennington, 1961), β -N-acetyl-D-glucosaminidase (EC 3.2.1.30) (Sellinger *et al.*, 1960), lactate dehydrogenase (EC 1.1.1.27) (Kornberg, 1955) and aryl-esterase (EC 3.1.1.2) (Shephard & Hubscher, 1959). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Immunoprecipitation

Antigen preparations (human MOM, rat MIM, human liver homogenate) were labelled either by reductive methylation, with NaB³H₄ (Russell & Mayer, 1983), or with ¹²⁵I by using the chloramine-T method (Brown *et al.*, 1979), solubilized with either Triton X-100 or sodium deoxycholate and the soluble material incubated with monoclonal antibody after removal of free label and cholate by dialysis. Monoclonal antibody was added either in solution or bound to rabbit-anti-(mouse IgG)-antibody-coated *Staphylococcus aureus* cells (immunoprecipitin; Bethesda Research Laboratories) (Kennett *et al.*, 1981). In the latter method, the coated cells were then collected by centrifugation and washed four times in phosphate-buffered saline containing 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulphate and the immune complex then eluted in Laemmli (1970) sample buffer. When monoclonal antibodies were added in solution, rabbit anti-(mouse IgG) antibody was added to precipitate the immune complex, and the precipitate washed twice before addition of Laemmli sample buffer. Samples were then ana-

lysed by gel electrophoresis exactly as described by Laemmli (1970) and the radioactivity detected by fluorography (Bonner & Laskey, 1974), intensifying screens for ¹²⁵I being used.

Immunoblotting

This was carried out as described by Towbin *et al.* (1979). Proteins (120 µg of rat MIM, 150 µg of human MOM) were separated on 10% (w/v)-polyacrylamide denaturing gels (Laemmli, 1970), transferred to nitrocellulose membranes, incubated with monoclonal antibody (or spent medium plus non-immune mouse IgG) and finally incubated with ¹²⁵I-labelled rabbit anti-(mouse IgG) antibody.

Immuno-affinity chromatography

The monoclonal antibodies were subjected to (NH₄)₂SO₄ precipitation followed by dialysis against 10 mM-potassium phosphate buffer, pH 8.0, to precipitate lipoproteins (Mayer & Walker, 1980). The monoclonal antibodies (7 mg of each) or non-immune mouse IgG (1 mg) were then coupled to 1 ml of CNBr-activated Sepharose 4B (Hudson & Hay, 1980). Extracts (human MOM and human liver homogenate) were labelled by reductive methylation (Russell & Mayer, 1983), solubilized with Triton X-100, and incubated overnight at 4°C with the immunoabsorbent. After washing six times in phosphate-buffered saline, bound protein was eluted in turn with (1) 0.25 M-glycine/HCl, pH 10.0, +0.5 M-NaCl + 0.1% cholic acid, (2) 4 M-KSCN and (3) Laemmli sample buffer, and the eluted protein was analysed by gel electrophoresis (Laemmli, 1970).

Results

Fusion products

Over 90% of the microtitre wells contained hybridomas and were screened by using the trace-binding solid-phase assay. At least 15 of these cultures showed strong reactivity towards the human MOM preparation, but many were unstable and subsequently became negative. Four cultures survived and three of these, 4H11, 1H6 and 3F12, were cloned twice by limiting dilution on peritoneal-exudate cell-feeder layers and stable clones were established. All 3F12 clones gave similarly positive values on screening, indicating a clonal origin. Some of the 1H6 clones gave negative binding, whereas the rest gave similar positive values, suggesting that the original culture consisted of a non-secreting clone and a secreting clone. All 3F12 clones and all positive 1H6 clones showed the same specificity when tested on cryostat sections. The clones 1H6/C12 and 3F12/F2 were selected for re-cloning and further culture.

1H6/C12 and 3F12/F2 consistently gave increased binding (5–30 times) of rabbit anti-(mouse IgG) against human MOM when compared with binding of myeloma spent medium. A titration assay showed that half-maximal binding to 1 μ g of human MOM was achieved at a dilution of 1:300 for 3F12/F2 and a dilution of 1:125 for 1H6/C12 (Fig. 1). In addition, 1H6/C12 also bound rat MOM to a similar extent as human MOM; binding of 3F12/F2 to rat MOM was very small.

The subclass of 3F12/F2 was IgG₁, that of 1H6/C12 IgG_{2a}, both with κ light chains.

Fig. 1. Reactivity of culture supernatants with human MOM bound to poly(vinyl chloride) wells

Culture supernatants (●, 1H6/C12; ▲, 3F12/F2) were added undiluted or diluted in phosphate-buffered saline to wells containing 1 μ g of human MOM and binding was monitored by using the indirect trace-binding solid-phase assay as described in the Materials and methods section.

Association of 1H6/C12 and 3F12/F2 with rat liver subcellular membranes

Binding of the antibodies to several membrane preparations (1 mg of protein) was monitored by using ¹²⁵I-labelled rabbit anti-(mouse IgG) and a filter binding assay (see the Materials and methods section). The results are shown in Fig. 2, together with marker-enzyme distributions. 1H6/C12 bound to every membrane fraction (Fig. 2a), but the general profile, determined in three separate experiments, indicated that binding mimicked most closely the distribution of the mitochondrial enzymes succinate dehydrogenase and monoamine oxidase (Fig. 2c and 2d). 3F12/F2 binding, on the other hand, was much smaller than that shown by 1H6/C12, but showed a more discrete distribution (Fig. 2b), with the endoplasmic-reticulum-enriched fraction showing maximum binding. Rather unexpectedly, no binding to the mitochondrial fraction was detected.

Association of 1H6/C12 with mitochondrial subfractions

A purer fraction of MOM was obtained from rat liver by using a two-step gradient (see the Materials and methods section and Table 1), and binding of the two monoclonal antibodies to 100 μ g of each fraction was measured by using the filter binding assay, 3F12/F2 acting as a control. The very small (approx. 3%) contamination of mitochondrial pellet by the endoplasmic-reticulum marker, arylesterase, explains the lack of binding of 3F12/F2 to any mitochondrial subfraction (Table 1; binding ratio of 1.0 or less). Band 1 contains a high ratio of MAO to SDH activity (1.66 for band 1, 0.252 for band 2 and 0.275 for the pellet), indicating that it is enriched in MOM. 1H6/C12, however, did not bind to band-1 membranes (binding ratio 0.94), but to bands 2 and 3 (binding ratios of 3.22 and 4.72 respectively). This demonstrates that 1H6/C12 is recognizing an epitope on MIM.

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

Band	Total protein recovered (mg/10g of liver)	Marker enzymes (arbitrary units/mg of protein)		Monoclonal-antibody binding* (c.p.m./100 μ g of protein)			Binding ratio†	
		SDH	MAO	1H6	3F12	P3NS1 spent medium	1H6	3F12
1	1.3	0.9	1.5	9990 $\pm$ 1514	9690 $\pm$ 752	10317 $\pm$ 291	0.96	0.94
2	8.8	11.9	3.0	20235 $\pm$ 3188	5025 $\pm$ 1073	6921 $\pm$ 716	3.22	0.80
Pellet	9.8	13.8	3.8	29415 $\pm$ 5751	3528 $\pm$ 683	6225 $\pm$ 207	4.72	0.60

* Binding of antibodies to 100 μ g of protein monitored by using ¹²⁵I-labelled rabbit anti-(mouse IgG). Results are means $\pm$ S.D. (n = 3).

† Mean c.p.m. with monoclonal antibody divided by mean c.p.m. with P3NS1 spent medium.

Fig. 2. Reactivity of culture supernatants with rat liver membrane fractions. Binding of 1 ml of 1H6/C12 (a), and 3F12/F2 (b), to 1 mg of nuclear (N), mitochondrial (M), lysosomal (L) and endoplasmic-reticulum (E) fractions determined by the filter-binding assay. Distribution of enzyme markers are given in (c)–(g) (P = particle-free supernatant). Binding was expressed as c.p.m./mg of protein; enzyme distributions are presented in arbitrary activity units/mg of protein. Assays were performed in duplicate, and did not differ by more than 10%.

Fig. 3. Immunoperoxidase staining of human liver sections

(a) Negative control stained with 791T/36/C12 (magnification $\times 70$). (b) Stained with 1H6/C12, showing unstained portal-tract area (magnification $\times 70$). (c) Stained with 1H6/C12, showing unstained sinusoidal cells (arrow), but particulate staining in parenchyma cells (magnification $\times 70$). (d) Stained with 3F12/F2, showing staining in sinusoids (magnification $\times 70$). (e) Same as (d), but showing staining of blood in portal tract (arrow) (magnification $\times 35$).

Immunoperoxidase staining of liver

The cellular distribution of the epitopes was further examined by the immunoperoxidase staining of cryostat human and rat liver sections. The pattern of staining reproducibly obtained for both antibodies is shown in Fig. 3 together with negative controls; the pattern was identical in human and rat liver (result not shown). 1H6/C12 stained the liver parenchyma and showed a particulate distribution throughout all positive cells (Figs. 3*b* and 3*c*), with human cells staining stronger than rat cells; 1H6/C12 did not stain cells of the portal tract and non-hepatocytic parenchymal sinusoidal cells. 3F12/F2, on the other hand, stained the non-hepatocytic parenchymal sinusoidal area (Figs. 3*d* and 3*e*) and blood in the portal vessels (Figs. 3*e* and 3*h*); it is assumed that 3F12/F2 stains blood elements in the sinusoids. Both antibodies were also used to stain cell bodies of neurons, and 1H6/C12 again showed a particulate distribution in the cytoplasm (Fig. 4*a*), whereas 3F12/F2 stained residual blood elements on capillary walls (Fig. 4*b*).

Reactivity of 3F12/F2 towards human blood cells

3F12/F2 clearly showed strong binding to granulocytes (mainly neutrophils), but not to any other cell type when cells were fixed in cold acetone (Table 2). Binding was negligible, however, when the cells were not fixed in acetone, indicating that the epitope is not on the outer surface of the granulocytes.

Table 2. Reactivity of 3F12/F2 with human blood cells
For the definition of binding ratio, see Table 1.
Results are means of duplicate measurements.

Cell type	Monoclonal-antibody binding (c.p.m./10 ⁵ cells)		Binding ratio
	Spent medium	3F12/F2	
Monocytes	2742	5624	2.05
Lymphocytes	789	600	0.76
Granulocytes	1866	22104	11.84
Red cells	1395	1026	0.73

Fig. 4. Immunoperoxidase staining of human brain neurons
An enriched neuronal fraction (Allsop *et al.*, 1983) stained with (a) 1H6/C12 and (b) 3F12/F2. In (b) capillary walls (arrow) are stained. Magnification $\times 250$.

Chemical nature of antigens

(i) *Proteinase treatment.* In one experiment to test the proteinase-sensitivity of the 1H6/C12 epitope, rat liver MIM (1.6 mg of protein) was incubated with 100 μ g of trypsin at 37°C for 5 h; trypsin inhibitor (200 μ g) was then added, and the membranes centrifuged to form a pellet before incubation with the antibody. Binding of 1H6/C12 to untreated MIM, as assessed by the filter binding assay, was 102135 ± 8687 c.p.m. (s.d., three determinations), but binding to trypsin-treated MIM was only 63261 ± 5331 c.p.m. (s.d., three determinations), i.e. approximately half the binding of non-proteinase-treated membranes, suggesting that the epitope is at least partially sensitive to trypsin.

When rat MIM bound to uncounted poly(vinyl chloride) wells (i.e. not treated with polylysine, not fixed with glutaraldehyde) was treated with Pronase or trypsin, approximately half of the 1H6/C12-binding epitope was removed in 3 h (Fig. 5e). Treatment of human MOM bound to uncoated wells with proteinases for 90 min completely abolished both 1H6/C12 and 3F12/F2 binding (Figs. 5a and 5b). However, if the membranes were fixed with glutaraldehyde to polylysine-coated wells, no effect of proteinases on 1H6/C12 and 3F12/F2 binding was observed (Figs. 5c, 5d and 5f). It appears that glutaraldehyde fixation of the membranes renders the epitopes resistant to proteinase digestion. This is not a general phenomenon, however, since the binding of another monoclonal antibody, 2D1, to human MOM fixed to the

Fig. 5. Effect of proteinase digestion of membranes attached to poly(vinyl chloride) wells on subsequent binding of monoclonal antibodies

Human MOM (H-MOM) was bound to uncoated (a and b) or poly-L-lysine-coated [c and d, plus glutaraldehyde (glut.) fixation] wells, and rat MIM (R-MIM) was similarly bound to uncoated (e) or coated (f, plus glutaraldehyde fixation) wells, before incubation with 50 μ l of trypsin (■) or Pronase (□) (100 μ g/ml) for 3 h at room temperature. Control wells (●) were incubated with 50 μ l of a mixture of trypsin and trypsin inhibitor (100 μ g and 200 μ g/ml respectively). After proteinase treatment, 50 μ l of trypsin inhibitor was added (200 μ g/ml) to each well and the wells incubated at room temperature for 15 min. All wells were then washed six times with phosphate-buffered saline containing 1% (w/v) bovine serum albumin and the binding of 1H6/C12 and 3F12/F2 to the wells then determined by using the routine trace-binding assay. Assays were performed in triplicate, and did not differ by more than 10%.

Table 3. *Effect of trypsin pretreatment on the binding of 1H6/C12 and 3F12/F2 to mitochondrial membrane preparations*
Mitochondrial membranes were treated with trypsin, attached to poly(vinyl chloride) wells and binding of monoclonal antibodies to the wells then determined by using the trace-binding assay. Values are expressed as c.p.m. $\pm$ s.d. ($n = 3$) and corrected for binding of spent medium. Values in parentheses represent percentages of the control (i.e. no treatment) value.

Antigen	Treatment	Radioactivity bound (c.p.m.)	
		1H6/C12	3F12/F2
Human MOM	None	8751 $\pm$ 353 (100)	8748 $\pm$ 401 (100)
	Trypsin		
	30 min	6768 $\pm$ 763 (77)	791 $\pm$ 329 (9)
	180 min	6869 $\pm$ 216 (78)	717 $\pm$ 73 (8)
	Heat (100°C, 15 min)	9108 $\pm$ 586 (100)	288 $\pm$ 232 (3)
Rat MIM	None	9096 $\pm$ 533 (100)	—
	Trypsin		
	30 min	5724 $\pm$ 168 (63)	—
	180 min	3909 $\pm$ 930 (42)	—
	Heat (100°C, 15 min)	7089 $\pm$ 416 (78)	—

wells was decreased, after Pronase and trypsin digestion, by 80 and 60% respectively (results not shown).

It is possible that the epitopes of 1H6/C12 and 3F12/F2 are not themselves in proteins but that they are attached to proteinase-sensitive proteins, so that, after proteinase treatment, binding is abolished. This possibility was tested by incubating the membrane preparations with trypsin (or subjecting them to heat treatment) before attaching them to the poly(vinyl chloride) wells; in this case the complete incubation mixture was used to coat the wells. The 3F12/F2 epitope on human MOM was extremely sensitive to both proteinase digestion and heat treatment (Table 3). After a 3 h incubation with trypsin, the binding of 1H6/C12 to rat-MIM was decreased by 60% and to human MOM by 20%, indicating only limited proteinase-sensitivity. Boiling had little effect on the 1H6/C12 epitope.

3F12/F2 binds an epitope on a protein (Fig. 5 and Table 3), whereas 1H6/C12 binds an epitope that is associated with a protein but is not completely sensitive to proteinases and is extremely resistant to heat (Fig. 5 and Table 3); experiments were therefore undertaken to establish whether or not the epitope is a lipid.

(ii) *Treatment with phospholipases and total lipid extraction.* 1H6/C12 and 3F12/F2 did not bind to a lipid extract (chloroform/methanol, 2:1, v/v) of a rat liver MIM preparation and did not bind to a pure preparation of the phospholipid cardiolipin, a predominant MIM constituent. The effects of phospholipases on the binding of 1H6/C12 to rat MIM bound to uncoated poly(vinyl chloride) wells are shown in Table 4. Phospholipases had little

Table 4. *Effect of phospholipase treatment on the binding of 1H6/C12 and 3F12/F2 to rat liver MIM*

Details of assay are given in the Materials and methods section. Values are expressed as c.p.m. $\pm$ s.d. ($n = 3$) and corrected for binding of spent medium. Values in parentheses represent percentages of the control (i.e. no treatment) value.

Period of digestion with phospholipase (min)	Monoclonal-antibody binding (c.p.m.)	
	1H6/C12	3F12/F2
0	4215 $\pm$ 253 (100)	6204 $\pm$ 392 (100)
90	3687 $\pm$ 572 (87)	7044 $\pm$ 386 (113)
180	3723 $\pm$ 341 (88)	5769 $\pm$ 211 (93)

effect on the binding of this antibody, and it is therefore unlikely that the epitope is a lipid.

Attempts to identify the molecules containing the epitopes

Several antigen preparations, for example, human MOM, human liver extract and rat MIM, were labelled with ^{125}I or NaB^3H_4 , solubilized and incubated with a sample of 1H6/C12 or 3F12/F2 before precipitation (see the Materials and methods section). The immune precipitates in incubations using the two monoclonal antibodies contained more radiolabel than did the controls using spent medium and non-immune mouse IgG, but no distinct labelled component was detected after gel electrophoresis.

Binding of the antibodies to polypeptides blotted on to nitrocellulose was also tested, but no specific binding was observed. In addition,

immunoaffinity chromatography was attempted but no specific protein was eluted from the monoclonal-antibody columns.

Discussion

In the present study we have isolated a monoclonal antibody, 1H6/C12, obtained from a fusion of splenocytes from a mouse immunized with human mitochondrial membranes, and murine myeloma cells, which binds to MIM. We believe that this is the first monoclonal antibody reported with this specificity. Immunoperoxidase staining demonstrated that the epitope reactive with 1H6/C12 has an intracellular particulate distribution within at least human and rat liver parenchyma cells and the cell bodies of brain neurons, consistent with a mitochondrial localization.

Our results suggest that the epitope to which 1H6/C12 binds is more likely to be a protein than a lipid, and the epitope is associated with a protein that is proteinase-sensitive (Figs. 5a and 5e; Table 3). However, it is much less sensitive to proteinase digestion than the epitope for 3F12/F2 (Table 3). 1H6/C12 failed to immunoprecipitate a specific protein from several labelled membrane preparations, despite using incubation conditions that were successful with another antibody in our laboratory [an anti-(monoamine oxidase) monoclonal antibody; E. E. Billett, & R. J. Mayer, unpublished work]. In addition, immunoblotting and immunoaffinity chromatography have also failed to identify the molecule containing the epitope, a common problem with monoclonal antibodies (Noonan *et al.*, 1981). Nevertheless, the antibody may be a useful marker in the study of mitochondrial diseases, for example, primary biliary cirrhosis (Berg *et al.*, 1982) and mitochondrial encephalomyopathies (Morgan-Hughes *et al.*, 1982), diseases that often have their cause in defects in the mitochondrial respiratory chain (Busch *et al.*, 1981). In addition it could be used in our work on the study of mitochondrial protein degradation in isolated rat hepatocytes (Evans & Mayer, 1983, 1984). 1H6/C12 could be used with the protein A-gold complex (which will bind 1H6/C12, an IgG_{2a}) to identify precisely the MIM location of the epitope by electron microscopy (Roth *et al.*, 1978).

In addition to the anti-mitochondrial antibody, another monoclonal antibody, 3F12/F2, was also isolated that reacted with an epitope on granulocytes. Immunoperoxidase staining of both human and rat liver cryostat sections demonstrated that the epitope is present in blood vessels and in blood in the sinusoids. However, granulocytes isolated from human blood only reacted with 3F12/F2 if

permeabilized by fixing in acetone, suggesting that 3F12/F2 reacts with an internal epitope. We consider that, since the epitope is extremely proteinase- and heat-sensitive, 3F12/F2 recognizes an intracellular protein constituent. Mast-cell granules are known to contaminate mitochondrial fractions in rat liver (Haas *et al.*, 1979), and, since the granules of granulocytes are morphologically very similar to mast-cell granules, and similar in size to mitochondria (Barrett *et al.*, 1981), it is very likely that specific and azurophil granules would contaminate the human liver mitochondrial preparation.

3F12/F2 did not react with normal peripheral blood lymphocytes, monocytes or red cells. Since there were very few basophils and eosinophils in the granulocyte preparation, it is highly likely that the antibody is binding to neutrophils. It would be interesting to check, by using bone-marrow cells (Mannoni *et al.*, 1982), whether only specific stages in the neutrophil maturation sequence are recognized. It is unlikely that this antibody bound non-specifically to cells by Fc receptors, since it was non-reactive with lymphocytes and monocytes, which have large numbers of these Fc receptors.

Other monoclonal antibodies to neutrophils are known (e.g. Schienle *et al.*, 1982; Zola *et al.*, 1981; Cotter *et al.*, 1981; Civin *et al.*, 1981), but these usually bind to the cell surface, or to both the surface and a cytoplasmic constituent, and the chemical nature of the epitopes are not known. Although we failed to immunoisolate the 3F12/F2 antigen, we have shown that it is a protein. In addition, in one experiment there was evidence of 3F12/F2 binding to the endoplasmic-reticulum fraction (Fig. 2), the site of synthesis of the enzymes located in the leucocyte granules (Wheater *et al.*, 1979), which is consistent with this conclusion. We consider that, as with other neutrophil monoclonal antibodies, 3F12/F2 could be used for diagnostic purposes, for example, using the immunogold method for optical microscopy as described by De Waele *et al.* (1983). In addition, 3F12/F2 may be useful in the study of granulocyte function and differentiation.

We thank the Wellcome Trust for financial support for this work and Mrs. Alison Fisk and Dr. Chris Holmes for technical assistance with the immunoperoxidase technique.

References

- Allsop, D., Landon, M. & Kidd, M. (1983) *Brain Res.* **259**, 348-352
- Barrett, S. G., Hansen, K. S. & Bainton, D. F. (1981) *Br. J. Haematol.* **48**, 491-500
- Berg, P. A., Klein, R., Lindenborn-Fotinos, J. & Kloppel, W. (1982) *Lancet* **ii**, 1423-1426

- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Brandslund, I., Rasmussen, J. M., Fisker, D. & Svebag, S.-E. (1982) *J. Immunol. Methods* **48**, 199–211
- Brown, J. P., Tamerius, J. D. & Hellstrom, I. (1979) *J. Immunol. Methods* **31**, 201–209
- Busch, H. F. M., Jennekens, F. G. I. & Scholte, H. R. (eds.) (1981) *Mitochondria and Muscular Disease*, B.V. Mefar, Amsterdam
- Civin, C. I., Mirro, J. & Banquerigo, M. L. (1981) *Blood* **57**, 842–845
- Cotter, T. G., Spears, P. & Henson, P. M. (1981) *J. Immunol.* **127**, 1355–1361
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- De Waele, M., De Mey, J., Moeremans, M., De Brabander, M. & Van Camp, B. (1983) *J. Histochem. Cytochem.* **31**, 376–381
- Embleton, M. J., Gunn, B., Byers, V. S. & Baldwin, R. W. (1981) *Br. J. Cancer* **43**, 582–587
- Evans, P. & Mayer, R. J. (1983) *Biochem. J.* **216**, 163–175
- Evans, P. & Mayer, R. J. (1984) *Biochem. J.* **219**, 61–72
- Galfré, G. & Milstein, C. (1981) *Methods Enzymol.* **73B**, 1–45
- Haas, R., Heinrich, P. C. & Sasse, D. (1979) *FEBS Lett.* **103**, 168–171
- Horwitz, D. A., Allison, A. C., Ward, P. & Knight, N. (1977) *Clin. Exp. Immunol.* **30**, 289–298
- Holmes, C. H., Gunn, B., Austin, E. B., Embleton, M. J. & Baldwin, R. W. (1982) *Int. J. Cancer* **29**, 559–565
- Hudson, L. & Hay, F. C. (1980) *Practical Immunology*, 2nd edn., Blackwell Scientific Publications Ltd., Oxford
- Kennett, R. H., McKearn, T. J. & Bechtol, K. B. (eds.) (1981) *Monoclonal Antibodies: Hybridomas: a New Dimension in Biological Analyses*, Plenum Press, New York and London
- Kornberg, A. (1955) *Methods Enzymol.* **1**, 441
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Littlefield, J. W. (1974) *Science* **145**, 709–710
- Lowry, O. M., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mannoni, P., Janowska-Wieczorek, A., Turner, A. R., McGann, L. & Turc, J.-M. (1982) *Hum. Immunol.* **5**, 309–323
- Martinez, P. & McCauley, R. (1977) *Biochim. Biophys. Acta* **497**, 437–446
- Mayer, R. J. & Walker, J. H. (1980) *Immunochemical Methods in the Biological Sciences: Enzymes and Proteins*, Academic Press, London and New York
- Mayer, R. J. & Billett, E. E. (1984) *Techniques in Life Sciences: Techniques in Protein Chemistry*, Elsevier Amsterdam, in the press
- Morgan-Hughes, J. A., Hayes, D. J., Clark, J. B., London, D. N., Swash, M., Stark, R. J. & Rudge, P. (1982) *Brain* **105**, 553–582
- Noonan, K. D., Starling, J. J. & Seitz, T. L. (1981) in *Monoclonal Antibodies in Endocrine Research* (Fellows, R. & Eisenbarth, G., eds.), p. 41, Raven Press, New York
- Parsons, D. F., Williams, G. R. & Chance, B. (1966) *Ann. N.Y. Acad. Sci.* **137**, 643–666
- Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654
- Roth, J., Bendayan, M. & Orci, L. (1978) *J. Histochem. Cytochem.* **26**, 1074–1081
- Russell, S. M. & Mayer, R. J. (1983) *Biochem. J.* **216**, 163–175
- Schienle, H. W., Stein, H. & Muller-Ruchholtz, W. (1982) *J. Clin. Pathol.* **35**, 959–966
- Sellinger, O. Z., Benfray, H., Jacques, P., Doyen, A. & de Duve, C. (1960) *Biochem. J.* **74**, 450–456
- Shephard, E. H. & Hubscher, G. (1959) *Biochem. J.* **113**, 429–440
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Wheater, P. R., Burkitt, H. G. & Daniels, V. G. (1979) *Functional Histology*, Churchill-Livingstone, Edinburgh, London and New York
- Zola, H., McNamara, P., Thomas, M., Smart, I. J. & Bradley, J. (1981) *Br. J. Haematol.* **48**, 481–490