Mallory bodies—immunohistochemical detection by antisera to unique non-prekeratin components

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SUMMARY Mallory bodies (MBs) were prepared in 95% pure form from a case of human chronic alcoholic liver disease. A protein referred to as Mallory body protein (MBP), was isolated from MB by reduction and alkylation which gave one band on SDS-polyacrylamide electrophoresis. Antisera were raised to both purified MBs and MBP in rabbits and a goat. Both antisera, after absorption with spleen cells, specifically reacted immunohistochemically with MBs in frozen sections from patients with alcoholic liver disease. They also reacted with small granular structures in hepatocytes which are interpreted as a precursor or degradation product of MBs. The anti-MB serum also stained MBs in trypsinised paraffin sections in the immunoperoxidase procedure. Neither antisera reacted with normal liver or skin, and the reactivity of anti-MB and MBP sera for MBs was not abolished by absorption with prekeratin; these results indicate that MBs contain unique antigenic determinants not present in prekeratin. It is concluded that MBs are not simply composed of intermediate filament proteins.

Mallory bodies (MBs) are eosinophilic intracytoplasmic inclusions found in hepatocytes in patients with alcoholic liver disease and in other hepatic disorders such as primary biliary cirrhosis. They have a characteristic ultrastructure being composed of short filaments ranging in thickness from 14–20 nm from which thinner filaments extend.^{1,2} These filaments resemble intermediate filaments which form the cytoskeleton of many cells.² MBs also react with antibody to prekeratin, a component of intermediate filaments of epithelial cells.³ On the basis of this evidence it has been proposed that MBs may result from an unspecified defect of intermediate filament metabolism in hepatocytes damaged by alcohol or other agents.³

In this paper, it is shown that antiserum to isolated MBs react with MBs in paraffin and frozen sections of biopsies from patients with alcoholic liver disease; that an antiserum to a protein of apparent M_r 50 000 derived from MBs also reacts with MBs in frozen section only; that both antisera do not react with normal liver, skin, or prekeratin indicating that MBs contain unique antigenic determinants unrelated to prekeratin.

Methods

Bacterial collagenase (type III), n-ethylmaleimide, sucrose, sodium dodecyl sulphate (SDS), and iodoacetamide were obtained from Sigma Chemicals (England) and all other chemicals from BDH (England). Rabbit antisera against human IgG, IgA, kappa light chain, fibrinogen, α -1-antitrypsin, α 2 macroglobulin, microglobulin, and C4 were purchased from Hoechst (Germany) and all other immunohistochemical reagents were obtained from Dakopatts (Denmark). Rabbit antisera to purified actin was a gift from Dr J Holborow, Kennedy Institute, London. Rabbit antisera to whole human liver, liver specific protein,⁴ and pure Cl_q ⁵ were prepared in this laboratory. Bovine prekeratin was prepared from bovine muzzle.⁶

PURIFICATION OF MALLORY BODIES (MBS) AND MALLORY BODY PROTEIN (MBP)

MBs were isolated from livers of patients with alcoholic cirrhosis. The livers were removed at necropsy, divided into 1 cm² slices, wrapped in aluminium foil, rapidly frozen on blocks of solid CO₂, and stored at -70°C. MBs were isolated by modification of published methods.^{7,8} Briefly, the liver tissue was cut into small cubes, homogenised in 2.5 volumes of 0.25 M sucrose containing 5 mM Tris HCl and 5 mM ethylenediamine tetraacetic

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acid (EDTA) pH 8.5 with a Polytron blender at half power for one minute. The homogenate was centrifuged at $2000\times g$ for 15 minutes and the pellet was washed and resuspended in 0.25 M sucrose. Fibrous tissue was removed from this suspension by filtration through surgical gauze and nylon stocking (20 denier). The resuspended material was placed on a discontinuous sucrose density gradient composed of 40, 50, 60, and 70% sucrose and centrifuged at $99\,000\times g$ for 60 minutes; the material layering between 60 and 70% sucrose, which was enriched in MBs, was recovered and washed in 0.15 M NaCl. This material was digested with purified⁹ bacterial collagenase for 18 hours at 37°C in the presence of 0.25 mM N-ethylmaleimide. The insoluble MBs were recovered by centrifugation at $2000\times g$ for 15 minutes, washed in 0.15 M NaCl and extracted twice with deoxycholate (50 mg/ml) for 18 hours at 22°C and the suspension stored at 4°C. This material, referred to as isolated MB, was denatured and reduced in 1% SDS containing 20 mM dithiothreitol at 60°C for 20 minutes and treated with 40 mM iodacetamide for an additional 20 minutes at 60°C. The solution was extensively dialysed against phosphate buffered saline (PBS) pH 7.4 at 22°C and that material remaining in solution was stored at 4°C or in liquid nitrogen. This final material is referred to as MBP.

The purity of MB preparations was assessed throughout the isolation procedure by routine electron microscopical methods. The purity of the reduced alkylated MBP was determined by electrophoresis on 7% polyacrylamide gels in the presence of SDS and 2% mercaptoethanol.

ANTISERUM TO MBS AND MBP

One milligram of MBs either in suspension or in a solution of 1% SDS was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously at multiple sites in rabbits monthly. A goat and a rabbit also received MBP (1 mg) in complete Freund's adjuvant subcutaneously. The development of antibodies to MBs and MBP was monitored by the ability of serum to stain MBs in cryostat sections of liver from a patient with central sclerosing hyaline necrosis, which contains abundant MBs (see below). All antisera were absorbed with human spleen tissue derived from cadaveric renal transplant donors before use. The spleen was cut into small pieces and homogenised in a Polytron homogeniser at half power for 30 seconds in PBS and washed in the same buffer thrice. The antisera were absorbed with an equal volume of the washed splenic tissue.

The specificity of absorbed antisera were checked by double immunodiffusion in 0.6% agarose gels

containing 2% polyethylene glycol (PEG) and by immunoelectrophoresis in 1% agarose containing 2% PEG. Specificity was also determined immunohistochemically (see below).

IMMUNOHISTOCHEMISTRY

The standard tissue for immunohistochemical detection of antibody to MBs was liver from a case of central sclerosing hyaline necrosis.¹⁰ The liver was resected 16 hours after death and numerous blocks were rapidly frozen and stored in liquid nitrogen.

Immunofluorescent detection of MBs was carried out using the sandwich technique. Cryostat sections were fixed in 1% paraformaldehyde in PBS or in acetone at -20°C for 15 minutes. The tissue was overlaid with dilutions of antiserum for one hour and after washing in PBS treated with a 1:20 dilution of fluoresceinated swine anti-rabbit or rabbit anti-goat serum for 30 minutes. The slides were then washed in PBS and mounted in glycerol. Before use the second antibodies in this technique were absorbed with a 1/10 volume of acetone dried human liver which contained MBs.

Immunoperoxidase localisation¹¹ of antibody was carried out on formalin fixed tissue processed to paraffin by routine methods. Sections (7 µm thick) dried on to glass slides at 37°C for two hours, deparaffinised, washed in PBS, and digested with 0.1% trypsin in 0.15 M NaCl adjusted to pH 7.8 with 1 M NaOH for 30 minutes at 37°C. The slides were washed in PBS and endogenous peroxidase activity abolished by treatment with 10% hydrogen peroxide in methanol for 30 minutes at 22°C; all subsequent procedures were carried out at 22°C. After another wash in PBS sections were flooded with a 1:5 dilution of normal swine serum for 10 minutes and replaced, without washing, by rabbit antiserum to MB (diluted 1:25) or goat antiserum to MBP (diluted to 1:50 or 1:300). The slides were washed in PBS and exposed to swine anti-rabbit IgG or rabbit anti-goat IgG conjugated with peroxidase for 30 minutes. In some experiments the peroxidase antiperoxidase (PAP) method was used for antibody localisation. In the PAP method the second antibody was replaced by unlabelled swine anti-rabbit or rabbit anti-sheep serum (1:20 dilution), washed in PBS and exposed to swine or rabbit peroxidase antiperoxidase conjugate (1:50 dilution) for 30 minutes; rabbit anti-sheep serum reacts with goat IgG. In both immunoperoxidase procedures, peroxidase activity was demonstrated by 0.1% diaminobenzidine—HCL in PBS containing 0.01% hydrogen peroxidase for five minutes. All antisera were diluted in normal swine serum which had first been diluted 1:5 in PBS.

Fig. 1 A. Electron micrograph of isolated MBs after collagenase digestion. The only contaminants found in these preparations are electron dense bodies (arrows). Stained with lead citrate and uranyl acetate $\times 15\,600$ (original magnification). B. Electron micrograph of isolated MBs after collagenase digestion. MBs consist of short non-branching filaments. Lead citrate and uranyl acetate, $\times 120\,000$ (original magnification).

Normal human tissues for immunohistochemical screening were obtained at necropsy; liver tissue from patients with alcoholic liver disease was obtained by percutaneous needle biopsy; bovine muzzle was obtained fresh from an abattoir. All of these tissues were fixed in 10% formalin buffered to pH 7.0 and processed to paraffin routinely. Frozen sections were prepared from liver and skin and fixed in acetone at -20°C for 15 minutes.

The MB reference tissue (see above) was stained using the PAP method with rabbit antisera to human IgG, IgA, IgM, kappa light chain, fibrinogen, α -1-antitrypsin, α 2 macroglobulin, microglobulin, C4, Cl_q , actin and with antisera to whole human liver and liver specific protein.

In all immunohistochemical experiments the following controls were performed: antisera to

MBs or MBP was replaced with pre-bleed sera or antisera absorbed with its respective antigen; sections were also treated with fluorescein or peroxidase labelled antibody only.

Results

Isolated, collagenase digested, MBs were 90–95% pure as judged by electron microscopy (Fig. 1A). Isolated MBs consisted of short non-branching filaments (Fig. 1B). MBP derived from MBs, on SDS polyacrylamide electrophoresis, contained one major polypeptide band with an apparent molecular weight of 50 000 daltons and a minor band which did not enter the gel (Fig. 2). On storage the electrophoretic properties of the major band altered in that old MBs preparations contained three bands of molecular weight considerably less than 50 000 suggesting that proteolysis had occurred.

Isolated MBs are poorly immunogenic in rabbits and antibody was detected only after 12 injections over a period of one year in one of two rabbits. On the other hand, reduced alkylated MBP was a much better immunogen; antibodies were produced by a single injection of MBP after 30 days in a goat and

Fig. 2 Polyacrylamide electrophoresis of MBP in 1% SDS, 2% mercaptoethanol. There is a major protein with a molecular weight of approximately 50 000 daltons as determined from electrophoretic standards. There is additional protein which did not enter the gel.

Fig. 3 Double immunodiffusion of anti-MB against MBP. Well 1 and 4 contained pre-bleed serum and wells 2 and 3 anti-MB. Well 5 contained MBP.

after three monthly injections in a rabbit. When tested by immunodiffusion the spleen absorbed antisera produced a single line with MBP (Fig. 3) but there was no reaction with normal liver homogenate. Similarly, absorbed antisera gave a single arc on immunoelectrophoresis when tested against MBP (Fig. 4) and no reaction was observed with

Fig. 4 Immunoelectrophoresis of anti-MBP against MBP.

normal liver homogenate. MBs in physiological buffers did not produce precipitin reactions on immunodiffusion or immunoelectrophoresis with antisera because of their insolubility; SDS solubilised MBs produced nonspecific precipitation of serum proteins in agarose diffusion gels. The absorbed antisera stained only MBs in paraformaldehyde or acetone fixed frozen sections of liver from a case of central sclerosing hyaline necrosis (Fig. 5) and this reactivity was abolished by absorption with MB;

Fig. 5 Staining of MB structures in a frozen section of liver from a case of central hyaline sclerosis of the alcoholic by anti-MBP sera using the immunofluorescent technique. In addition to MBs small granules (arrows) also react with the antiserum.

Fig. 6 Staining of MB structures in a trypsinised paraffin section of liver from a case of acute alcoholic hepatitis. The antiserum stains typical MBs (A) which are frequently perinuclear and composed of individual granules—arrows—(B); in lipid laden hepatocytes the antisera stains individual granules (C) (arrows) for which no counterpart could be visualised in consecutive H and E sections. $\times 600$ (original magnification), counterstained with haematoxylin.

in the same system absorption with normal liver reduced the titre of antibodies to MBs but at the same time reduced the immunoglobulin concentrations by a similar amount. The fluorescein and peroxidase labelled reagents did not react with MBs. All of the above tests demonstrate the specificity of the antisera for MBs.

The results of staining trypsin digested paraffin sections of liver containing MBs by the immunoperoxidase procedure using rabbit anti-MB sera are shown in Fig. 6A and B; the goat antiserum did not react with MBs in paraffin sections. The rabbit antiserum gave staining reactions corresponding to the usual H and E appearance of MBs (Fig. 6A). In many biopsies, however, MBs consisted of conglomerates of small granules (Fig. 6B). The latter material was often perinuclear and sometimes surrounded the entire nucleus. Of more interest is the fact that rabbit antiserum also frequently produced a fine granular appearance in hepatocytes for which there was no readily visible counterpart in H and E

sections (Fig. 6C). In view of the latter finding MBs and MB antigen positive cells were quantified in consecutive 7μ sections stained with H and E and the immunoperoxidase procedure from two cases of acute alcoholic hepatitis. As shown in the Table the antibody demonstrated many more antigenic structures in case 1 than routine staining, whereas in case 2 both methods were equally effective. In case 1 antibody positive cells had a granular staining pattern similar to that seen in Fig. 6C. This reaction was abolished by absorption of the antisera with isolated MBs. The staining of MBs by anti-MB or MBP sera was not reduced by prior absorption of antisera with bovine muzzle prekeratin. The anti-MB serum did not react with any cell type in 10 morphologically normal liver biopsies, nor did it react with normal lung, gut, heart, kidney, intestine, lymph node, testis, or brain in paraffin or frozen sections. Of particular note, anti-MB and MBP sera did not react with human skin or bovine muzzle.

The antisera to immunoglobulin, actin, C4, Cl_q ,

Table Quantification of Mallory bodies in tissue sections*

Case no.	Field no.	H and E	Immunoperoxidase
1	1	0	15
	2	1	15
	3	0	10
	4	0	32
	5	1	8
2	1	23	29
	2	23	28
	3	14	19
	4	3	5
	5	4	3

*Mallory body structures were counted in five standard areas (using an eyepiece graticule) of consecutive 7 μ paraffin sections from cases of acute alcoholic hepatitis stained with H and E or the immunoperoxidase technique for MBs.

whole human liver, and liver specific protein did not stain MBs in the reference liver tissue.

Discussion

This study demonstrates that antisera to isolated MBs and MBP are specific reagents for the identification of MBs in sections of liver biopsies from patients with alcoholic liver disease. Rabbit anti-MB serum stains MBs in trypsin digested liver sections but goat anti-MBP is not active on the same material. It is possible that the goat anti-MBP serum reacts with a determinant which is destroyed by paraffin embedding. It has been claimed that the PAP reagent (peroxidase-antiperoxidase conjugates) bind to MBs in unfixed frozen sections¹² and that horseradish peroxidase label MBs in formaldehyde fixed frozen sections¹³ and that both of the reagents are specific for MBs. These observations have not been confirmed in this laboratory. It is clear, however, that neither PAP nor horseradish peroxidase bind to MBs in paraffin sections as shown in this investigation and others.¹³ Unfixed MBs also bind non-immune rabbit sera¹³ but they do not have this property in paraffin embedded material. Furthermore, they did not bind a variety of specific antisera against numerous immunoglobulins, complement components, liver specific protein and actin, indicating that these components are not present in MBs or, alternatively, that their antigenic determinants are lost in processing. These negative results, and the finding that the reactivity of anti-MB and anti-MBP sera for MBs is abolished by absorption with isolated MBs, exclude the possibility that anti-MB and anti-MBP sera bind to MBs in tissue sections non-specifically. The greater sensitivity of immunohistochemistry over conventional staining for MB related structures, is demonstrated in the Table.

Anti-MB and MBP sera react with MB antigen which exists in granular form in hepatocyte cytoplasm; this antigenic material is not readily visible by conventional staining. These granules of MB material may represent a precursor and or a degradation product of fully formed MBs but the present results do not distinguish between these possibilities. Recently, similar antigenic structures have been recognised in frozen sections of liver from griseofulvin fed mice and Denk *et al.*³ concluded that they may represent both precursors and degraded forms of whole MBs. It is interesting to recall that Mallory,¹⁴ using conventional staining, speculated that MBs resulted from the coalescence of smaller cytoplasmic granules. The methodology described here, being applicable to routinely processed biopsies, may be useful in retrospective and prospective studies of the genesis of MBs in alcoholic liver disease and in analysing the antigenic structure of MBs in other liver disorders. Preliminary investigations have shown that MBs in conditions such as primary biliary cirrhosis may be antigenically similar to those found in alcoholic liver disease.¹⁵

Epithelial¹⁶ mesenchymal¹⁷ muscle,¹⁸ and neural cells¹⁹ contain, in addition to microfilaments and microtubules, a third system of cytoskeletal filaments which are generically termed intermediate filaments. Intermediate filaments have an average diameter of approximately 10 nm in most cells but the proteins of which they are composed differ from one cell type to another—for example, epithelial intermediate filaments contain prekeratin while mesenchymal intermediate filaments contain vimentin but lack prekeratin.¹⁷ As MBs are filamentous and also react with antisera to prekeratin, it has been postulated that MBs are related to the epithelial intermediate filament class.³ The present observations show that MBs contain antigens distinct from prekeratin, as anti-MB and anti-MBP sera do not react with prekeratin containing cells (human skin and bovine muzzle) and the staining of MBs by these reagents was not diminished by absorption with purified prekeratin. These antigens may be unique to MBs because anti-MB sera did not react with any other tissue tested. The fact that anti-MB serum reacts with MBs in paraffin and frozen sections and anti-MBP sera stains MBs in frozen sections only argues that MBs contain at least two distinctive antigens. This evidence, together with the fact that MB filaments have a larger average diameter, 15 nm,^{1,2} than intermediate filaments, indicates that MB filaments contain components other than prekeratin.

Isolated MBs consist of five or six polypeptides ranging in molecular weight from 32 000–66 000 daltons.^{2,20} MBP isolated from MBs by denaturation,

reduction and alkylation may correspond to one of these polypeptides but this has not actively been proven. As antisera to MBP react only with MBs it can be concluded that this protein is specific to MBs and is one of the unique antigens referred to above. The hypothesis that MBs contain unique antigenic determinants has been strengthened by the recent observation that some monoclonal antibodies produced by the hybridoma technique also react only with MBs.²¹

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