Isolation of Mallory bodies and an attempt to demonstrate cell mediated immunity to Mallory body isolate in patients with alcoholic liver disease

C GLUUD, F HARDT, J ALDERSHVILE, P CHRISTOFFERSEN, H LYON, JO NIELSEN

From the Department of Medicine, Division of Hepatology, and Department of Pathology, Hvidovre Hospital, and Second Department of Medicine, Kommunehospitalet, University of Copenhagen, Denmark

SUMMARY Mallory bodies were isolated from necropsy livers from patients with alcoholic hepatitis with and without cirrhosis with a Ficoll viscosity barrier. The purity of Mallory bodies in the isolate varied between 70 and 90%, estimated by counting Mallory bodies and non-Mallory body structures in haematoxylin-eosin stained smears. Electron microscopy confirmed the presence of Mallory bodies in the isolates. The Mallory body isolate was used as antigen in the agarose leucocyte migration inhibition test in order to test the cell-mediated immunity. No significant difference in leucocyte migration was found between controls and patients with alcoholic hepatitis, alcoholic steatosis, alcoholic cirrhosis and miscellaneous liver diseases.

Mallory bodies (MBs), or alcoholic hyalin, are an important histological hallmark of alcoholic hepatitis.¹ Since French *et al.*² described the isolation of MBs a number of investigations have dealt with the isolation and characterisation of MBs.³⁻⁶ These studies indicate that MBs are composed of branching, tubular filaments, possibly derived from intermediate filaments.^{5,6} MBs seem to be composed of five or more polypeptides⁴⁻⁷ and contain carbohydrate.^{6,7}

Levy and collaborators have reported both cell mediated⁸⁻¹² and humoral¹³⁻¹⁵ immunity to isolated MBs in patients with alcoholic hepatitis. These findings generated the hypothesis that MBs could be a neoantigen induced by ethanol. This antigen should provoke both cellular and humoral immune response, which could lead to the development of alcoholic hepatitis and progression from alcoholic hepatitis to cirrhosis.^{12,14} These findings, however, have not been reproduced by other groups.¹⁶⁻¹⁹

In the present study MBs were isolated from human liver obtained at necropsy and used as antigen in the agarose leucocyte migration inhibition test in order to evaluate the cell mediated immune response in patients with alcoholic liver disease.

Material and methods

ISOLATION OF MBS

A modification of a procedure described by HM Tinberg and SW French (personal communication, 1977) was used for the isolation of MBs. Livers were removed at necropsy (within 16 hours of death) from chronic alcoholics dying with alcoholic hepatitis with and without cirrhosis. The presence of MBs was confirmed on paraffin sections stained with haematoxylin and eosin. The livers were sliced and stored at -70°C in isopentane until use.

Frozen livers (250 g) were partially thawed, cut into smaller pieces, and ground at top speed in a Waring Blendor for $1\frac{1}{2}$ minutes in four volumes of isolation medium⁶ at 4°C . Fine grade glass wool (Pyrex Wool, Corning Glass Works) was added to the homogenate in order to absorb collagen. The homogenate was separated from the soaked glass wool by squeezing it through surgical gauze followed by filtration on a Buchner funnel lined with three layers of glass wool and one layer of surgical gauze. The glass wool was repeatedly washed with isolation medium and the rinses added to the Buchner funnel and additional isolation medium was used to wash the filter. At this point the original volume was doubled. A considerable amount of contamination with

glass wool was noticed. Because of this, the homogenate was filtered through two layers of nylon mesh (pore size 40 and 10 μm) on a Buchner funnel. The filtrate was centrifuged at 365 g for 10 min and the supernatant was sucked off leaving a yellow-green-white pellet. The latter was resuspended in isolation medium by vigorous shaking and centrifugation was repeated 2-3 times until the supernatant was clear. The pellet was then digested⁵ at 37°C overnight in 0.05 mol/l Tris buffer pH 7.5 containing 0.1% DNAase, 0.1% RNAase, 0.1% collagenase (Worthington Biochemical Corp), 0.36 mmol/l CaCl_2 , 0.1 mmol/l MgSO_4 , and 0.1% Thiomersal. Then the suspension was washed three times in ice-cold deionised water. The pellet was incubated in 5% deoxycholate at 37°C overnight, washed three times in ice-cold deionised water and resuspended in 0.25 mol/l sucrose using an MSE homogenizer (top speed, 2 min) to a total volume of 30 ml. Five ml were layered on 30 ml Ficoll 400 (40% wt/wt) and centrifuged for 65 min at 20 000 rpm (48 200 g) at 4°C (Sorvall Super Speed Refrigerated Centrifuge). The pellet was washed in 20 volumes of deionised water and stored at -20°C in 0.01% acetic acid until use.

Light microscopy

During the isolation procedure smears of fractions and final pellets were stained with haematoxylin and eosin and examined for presence and purity of MBs.

Electron microscopy

Isolates of MBs were fixed in 3% glutaraldehyde in 0.1 mol/l sodium phosphate buffer (pH 7.4) for two hours. After fixation the isolate was washed in phosphate buffer and embedded in Epon. Thick sections (1 μm) were stained by toluidine blue. Thin sections (500 Å) were stained in uranyl acetate and lead citrate and examined in a Jeol 100 electron microscope.

Solvents

In order to solubilise MBs for application in the leucocyte migration inhibition test the following solvents were tested for their ability to dissolve MBs and their effect on spontaneous leucocyte migration index: (a) Guanidine buffer (guanidine HCl 2 mol/l, mercaptoethanol 50 mmol/l, disodiumphosphate 10.5 mmol/l, pH 7.5);¹² (b) guanidine HCl 6 mol/l; (c) NaOH 1 mol/l and 40 mmol/l; (d) sodium dodecyl sulphate (SDS) 1%.

Preparation of MB isolate for the leucocyte migration inhibition test

MB isolate was dissolved in SDS 1% and dialysed

overnight against 1200 ml NaOH 40 mmol/l. The protein concentration was 1.8 mg/ml. This material was used as antigen in the leucocyte migration inhibition test after dilution with phosphate buffer to a concentration giving rise to no inhibition of migration by normal controls (see Results).

Leucocyte migration inhibition test

The agarose leucocyte migration inhibition test was used as described in detail elsewhere.²⁰ In summary, leucocytes were prepared from controls and patients to a final concentration of 2.2×10^8 leucocytes per ml. 2×10^8 leucocytes were incubated with 10 μl MB isolate prepared as described above. As control, 2×10^8 leucocytes were incubated in phosphate buffer without MB isolate. Seven μl of the leucocyte-antigen mixture were placed in three wells, and the control mixture was placed in two other wells in the agarose plate. The plates were incubated for 24 h at 37°C in a 2% CO_2 in air saturated with water vapour. The migration area was read under a projection microscope and migration index was calculated

$$\text{MI} = \frac{\text{Ra}^2 - \text{r}^2}{\text{Rc}^2 - \text{r}^2}$$

where Ra is the radius of migration with antigen, Rc migration in the control, and r radius of the wells.

PATIENTS

Twenty-nine patients and 16 healthy staff members were included. The median age of patients was 52 years (range 17-77 years) and seven were women. A liver biopsy was performed in 27 of the patients, and based on histological, clinical and biochemical findings, the patients were classified into the following groups:

Alcoholic hepatitis ($n = 11$). All had abused alcohol and the diagnosis was confirmed by biopsy ($n = 9$) and/or by the classical symptoms of fever, jaundice, leucocytosis, and upper quadrant pain.¹ Two patients had no biopsy due to low coagulation factors. Seven of the nine biopsies also showed cirrhosis.

Alcoholic cirrhosis ($n = 6$). All had abused alcohol and had a biopsy showing cirrhosis compatible with an alcoholic aetiology.

Alcoholic steatosis ($n = 4$). All had abused alcohol and biopsies showed steatosis of the liver (and fibrosis in one).

Miscellaneous liver diseases ($n = 8$). According to clinical, serological and histological criteria this group comprised one patient with Gilbert's syndrome, three with acute viral hepatitis, two with cryptogenic

Fig. 1 *Light microscopy of Mallory body isolate from human liver stained with haematoxylin and eosin. The preparation consists mainly of Mallory bodies. The small particles are glass wool $\times 250$.*

Fig. 2 *Electron microscopy of Mallory body isolate. The characteristic filamentous structure is well preserved. Uranyl acetate and lead citrate $\times 10\,000$.*

cirrhosis, one with chronic active hepatitis and cirrhosis, and one with cirrhosis and α_1 -antitrypsin deficiency.

Results

ISOLATION OF MBS

Counting MB and non-MB structures in haematoxylin and eosin stained smears (Fig. 1) the purity of MBs in the isolates varied between 70 and 90%. Most of the contaminating material consisted of nuclei and unidentifiable debris. Introducing a nylon mesh in the isolation procedure reduced the contamination of glass wool, but not totally. Yields of protein in the MB isolate averaged 2-5 mg protein per 100 g of liver wet weight.

Electron microscopy of the isolates confirmed the presence of MBs (Figs 2 and 3). The characteristic filamentous ultrastructure and tubular appearance was seen and did not differ from that of MBs in freshly obtained liver biopsy specimens. According to the classification of Yokoo *et al.*,²¹ both type II and

type III MBs were identified, but no type I MBs were observed.

SOLUBILISATION OF MBS AND TOXICITY OF SOLVENTS

Varying and usually small amounts of the MB isolate could be solubilised using the guanidine buffer, guanidine HCl 6 mol/l, and NaOH. Using SDS 1% about 90% of the MB isolate could be solubilised.

Evaluating the spontaneous effect of these solvents on leucocyte migration index of normal persons showed that guanidine buffer, guanidine HCl 6 mol/l, SDS 1% and NaOH 1 mol/l all were toxic and gave small migration indices (< 0.30) compared to phosphate buffer. NaOH (40 mmol/l) did not affect the migration index and diluting this concentration further did not increase the migration index. Diluting the guanidine buffer and guanidine HCl 6 mol/l 10-20 times and SDS 1% 200 times abolished the inhibition of migration. MB isolate of high purity was hereafter dissolved in SDS 1% (5 ml) and dialysed against 1200 ml NaOH 40 mmol/l as described in Material and methods. This material was used for further studies.

Fig. 3 Electron microscopy of Mallory body isolate. The filamentous nature is evident. Uranyl acetate and lead citrate $\times 99\,000$.

LEUCOCYTE MIGRATION INHIBITION IN CONTROLS AND PATIENTS

In Fig. 4 the effect of the dissolved MB isolate on the migration of leucocytes from control persons is shown. The antigen had to be diluted 40-60 times before no significant inhibition of migration occurred. A 40 times dilution (corresponding to a protein concentration of 0.045 mg/ml) was used hereafter.

Fig. 4 Effect of Mallory body isolate on migration index of leucocytes from normal controls. Mallory body isolate was prepared as described in Material and methods. Broken horizontal line represents mean migration index.

In Fig. 5 the migration indices of controls and patients with liver diseases are shown. In 13 healthy controls the mean migration index was 0.85; a significant inhibition of leucocyte migration was defined as a migration index less than 0.69, corresponding to the mean minus two standard deviations. As shown no significant difference between normal controls and patients with alcoholic hepatitis, alcoholic cirrhosis and alcoholic steatosis was observed. Two patients with alcoholic hepatitis and one with steatosis showed inhibition of leucocyte migration according to the criteria outlined above. Patients with various acute and chronic liver diseases did not differ significantly from the previous mentioned groups.

Discussion

Using the method of Tinberg and French (personal communication, 1977), with modifications as described, MBs were isolated with a purity of about 80%. This is in accordance with previously published results.^{3 4 7} MBs have been isolated in semipure and pure fractions by other investigators using the method of Okamura *et al.* employing partitioning in a two phase polymer system.^{4 7 14} In our hands,

Fig. 5 Migration index of leucocytes from normal controls, patients with alcoholic liver disease and miscellaneous liver diseases after addition of Mallory body isolate. Broken horizontal line represents mean migration index and solid horizontal line represents 2 SD.

however, only small and contaminated preparations of MBs could be obtained by this method. Similar difficulties have recently been reported by others.⁶

The MBs isolated in the present study appear very much as in situ with a characteristic rope-like appearance when seen by light microscopy. Electron microscopy confirmed the presence of MBs, and both type II and III MBs were identified according to the classification of Yokoo *et al.*²¹ No type I MBs were identified, which is in accordance with previous observations.⁶

MBs are composed of branching filaments, and are thought to be derived from a normal protein constituent of the liver cell cytosol. Reports that MBs were related to actin²² have not been confirmed in more recent studies.^{5 23} The constituent that resembles MB filaments best is the intermediate filament.²³ Like MBs they do not bind heavy meromyosin, and intermediate filaments can absorb MB-specific antibody.⁵ Other investigators have stressed that MBs are related to—but not identical with—intermediate filaments.²⁴ Antibodies towards neurofilaments, known to bind to intermediate filaments, did not bind to MBs,⁵ and antibody against human glial fibrillary acidic protein did not react with isolated MBs (C Gluud and E Bock, unpublished observations). The glial filament is an intermediate filament.²⁵

In contrast with the observations of Zetterman *et al.*^{10 12} but in accordance with MacSween,¹⁸ patients with alcoholic hepatitis did not show any significant inhibition of migration index when incubated with MB isolate. The reason for the lack of cell-mediated immunity to MBs in our patients could be due to methodological differences. First, different isolation procedures were employed which

may have led to differences in the antigenic composition. Secondly, MBs were solubilised in SDS followed by NaOH dialysis in the present study. Zetterman *et al.*¹² used the guanidine buffer. MBs are difficult to solubilise^{3 5 23} and varying and rather small fractions of the MB isolate could be solubilised using this buffer. Further, the guanidine buffer is toxic to leucocytes when undiluted, an observation also made by MacSween.¹⁸ The guanidine buffer contains mercaptoethanol, which can convert non-adherent (macrophage-free) cells into a population with the functional immune properties of macrophages. Therefore, mercaptoethanol may affect leucocytes from patients with alcoholic hepatitis in an unpredictable way producing migration inhibition.²⁶ Thirdly, Zetterman *et al.*¹² used the capillary migration test, while in the present study the agarose leucocyte migration inhibition test was employed. This method is easier to reproduce and uses smaller amounts of antigen. MacSween noted that the leucocyte migration of patients with alcoholic hepatitis was reduced more after preincubation with MBs rather than by adding leucocytes directly to the culture chamber, although the results did not achieve statistical significance.¹⁸ The protein concentration used in our leucocyte migration test is comparable with that used in earlier studies.²⁰

The pathogenetic role of MBs in the development of alcoholic liver diseases still seems to be controversial, and further studies to clarify the role of MBs and autoimmune mechanisms in the progression of alcoholic liver disease are needed.

Addendum

Since the preparation of this manuscript, Triggs *et al.*²⁷ have published results which showed migration inhibition to MBs in some patients with alcoholic hepatitis. Using the capillary migration test, they found only migration inhibition when using preincubation. As in the present paper Triggs *et al.*²⁷ also reported a toxic effect for leucocytes of a number of solvents. They therefore used sonicated suspensions of MBs in saline as antigen material.

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Requests for reprints to: Christian Gluud, Department of Medicine, Division of Hepatology, Hvidovre Hospital, DK-2650 Copenhagen, Denmark.