Comparison between liver and serum concentrations of mannan binding protein

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Abstract

Aims: To investigate staining patterns for mannan binding protein (MBP) by immunocytochemistry in liver biopsy specimens from patients with various hepatic disorders; to measure the serum MBP concentration in the patients at the time of biopsy; and to compare these to define further the role of MBP in disease.

Methods: Fifty seven consecutive patients with a variety of types of liver disease were studied. Fresh liver biopsy specimens were immunostained with anti-MBP and graded for intensity of staining. Serum MBP concentrations were measured on samples obtained on the day of biopsy, as were a full range of liver blood tests.

Results: MBP was only detectable in liver biopsy specimens from patients with morphological evidence of liver disease. MBP was most prominent in the livers of patients with severe alcoholic liver disease; livers harbouring metastases or showing biliary disease had moderate concentrations. Patients with liver disease were more likely to have raised serum MBP concentrations, but there was no correlation between these values and those found in the biopsy specimens. There was also no significant correlation between either of these concentrations and liver blood test abnormalities.

Conclusions: Patients with liver disease tend to have raised MBP concentrations in both the liver and serum, but the exact relation between the two is as yet undefined.

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Mannan binding protein (MBP) was first

described in 1978 in rabbit liver,¹ since when it has been isolated from the liver and serum of humans and a wide range of animals.²⁻⁵

It is a Ca²⁺ dependent lectin which structurally resembles the complement component C1q. It can bind to the cell walls of micro-organisms including Gram negative bacteria, mycobacteria, and yeasts. It is thought to perform several functions, including acting as a focus for complement activation on the surface of micro-organisms,⁶ acting as an opsonin in defence against some micro-organisms,⁷ preventing infection of H9 lymphoblasts by HIV in vitro,⁸ and it may also behave as an acute phase reactant.⁹ MBP deficiency has been reported to result in recurrent severe infections in some children.⁶

A previous study investigating immunohistochemical staining for MBP in human tissues,¹⁰ in which a wide range of tissues from three post mortem cases and 71 liver biopsy specimens were studied, showed that the liver was the only tissue to contain clinically important amounts of MBP. An interesting finding was that morphologically normal liver contained virtually no stainable MBP (seven biopsy and three post mortem cases); liver tissue from patients with a range of hepatic diseases showed a variable degree of positive staining. The most noticeable staining was seen in patients with severe alcoholic liver disease (cirrhosis) and those with metastatic tumours in the liver, with moderate staining seen in patients with biliary disease.

There is a wide range for the normal serum concentration of MBP, with 90% of values lying between 33 and 2656 µg/l (these values having now been revised by a factor of 16.6 from those originally quoted.)¹¹ As there are also wide variations in the concentrations of MBP in the liver, as detected by immunohistochemistry,¹⁰ we designed a study to investigate serum MBP concentrations in a group of patients with liver disease, and compared these concentrations with the amount of MBP detected in the liver biopsy specimens taken at the same time as the serum samples.

Methods

The patient group tested consisted of 57 patients undergoing liver biopsy in Oxford over an eight month period from whom fresh frozen tissue was available. The average age of the patients was 46 (range 13-78 years), the sex ratio was 1:1.4 (women:men). The racial origin of most of the patients was Caucasian.

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Table 1 MBP in patients with normal liver biopsy specimens

Case No	Liver biopsy specimen appearance	Serum MBP (µg/l)	Tissue MBP	AST	AP	GGT	Albumin	Bilirubin
1	Normal	1207	0	47	373	114	46	9
2	Normal	4532	0/1	25	220	9	50	5
3	Normal	292	0	34	198	75	55	10

Tissue MBP expressed as strength of staining of liver biopsy specimens with anti-MBP on a scale 0-3.

AST = aspartate aminotransferase, normal range 10-35 IU/l

AP = alkaline phosphatase, normal range 100-300 IU/l

GGT = gamma glutamyl transpeptidase, normal range 15-35 IU/l

Albumin: normal range 35-50 mmol/l

Bilirubin: normal range 3-17 µmol/l

Table 2 MBP in patients with alcoholic liver disease

Case No	Liver biopsy specimen appearance	Serum MBP ($\mu\text{g/l}$)	Tissue MBP	AST	AP	GGT	Albumin	Bilirubin
4	Fatty change	2832	0/1	44	266	257	49	11
5	Fatty change	1888	1	53	266	134	44	13
6	Fatty change	1771	1	46	376	413	44	8
7	Fatty change	2266	1	20	91	11	44	8
8	Fatty change	1814	2	69	288	679	45	7
9	Hepatitis	2546	1	95	181	37	50	7
10	Hepatitis	6122	1/2	67	336	113	29	17
11	Hepatitis	3407	1/2	55	182		49	4
12	Fatty + fibrosis	98	2	72	378	146	42	15
13	Fibrosis	125	2	25	499	79	31	10
14	Hepatitis + fibrosis	17306	1/2	29	176	79	44	9
15	Hepatitis + fibrosis	13197	2	113	486	778	44	35
16	Hepatitis + fibrosis	1954	2	113	420	273	33	9
17	Hepatitis + fibrosis	4532	2	262	826	2861	28	184
18	Hepatitis + fibrosis	3021	2/3	72	252	240	29	18
19	Hepatitis + fibrosis	926	3	82	206	293	47	13
20	Severe fibrosis	2201	2/3	25	225	295	27	6
21	Cirrhosis	604	2	118	388	73	29	478
22	Cirrhosis	7558	2	121	753		30	79
23	Cirrhosis	354	3	60	541	243	37	6
24	Cirrhosis + hepatitis	544	3	96	399	218	43	20
25	Cirrhosis + hepatitis	493	3	68	235	559	42	15
26	Cirrhosis + hepatitis	769	3	134	336	124	23	52

Tissue MBP is strength of staining of liver biopsy specimens with anti-MBP on a scale 0–3. See table 1 for normal ranges of liver blood tests.

About 0.5–1.0 cm of fresh tissue from each biopsy specimen was snap frozen and stored in liquid nitrogen within 30 minutes of the procedure, as part of our routine protocol with liver biopsy specimens. The rest of the tissue was processed routinely (paraffin wax sections) for diagnostic purposes. Frozen sections (5 μm) were cut on to gelatine-chrome alum coated multispot slides, and air dried for 2–18 hours. Fixation consisted of acetone for 10 minutes at room temperature.

Serum samples, which had been stored at 4°C, were retrieved from the blood transfusion department (such samples are routinely taken for all patients undergoing biopsy), before being frozen down to –70°C and stored prior to analysis in batches.

The antibody used was the IgG fraction (1.1 mg/ml in 70 mM phosphate buffer at pH 7.4) of rabbit anti-human mannan binding protein, prepared as described previously.¹¹

Staining was carried out by the indirect immunoperoxidase technique using standard protocols, as described in our previous study.¹⁰ Two negative controls (no primary antibody; rabbit anti-human chorionic gonadotrophin (Dako) diluted 1 in 200 as primary antibody) were included on each slide. A known positive case from the previous study was included as a positive control with all the batches of slides stained.

Grading of staining density was assessed on a scale of 0–3, grade 3 being defined as the most intense degree of staining (strong cyto-

plasmic stain) found within this sample. Direct comparisons were made between anti-MBP at 1 in 50 and the hcG control present on each multispot slide.

Microtitre plates (Nunc-Immuno plate, GibCo) were coated at room temperature with rabbit anti-human MBP IgG (100 μl per well of a 10 mg/ml solution) in 15 mM Na_2CO_3 , 35 mM NaHCO_3 , (pH 9.6) for 16 hours and then washed three times with TBS-NTBSA (50 mM TRIS-HCl, 150 mM NaCl, 0.05% (w/v) NaN_3 , 0.05% (v/v) Tween-20 and 1 mg/ml bovine serum albumin, pH 7.4). Unbound sites were blocked with TBS-NTBSA for 2–3 hours at room temperature. After washing in the same buffer the plates were incubated with a 1 in 1000 dilution of mannan-biotin in TBS-NTCa²⁺BSA (20 mM CaCl_2 in TBS-NTBSA) or TBS-NTEBSA, thus providing calcium dependent and calcium independent binding values. The wells were washed with TBS-NTCa²⁺BSA or TBS-NTEBSA and ExtrAvidin-alkaline phosphatase conjugate (Sigma E2636), diluted 1 in 10⁴ in TBS-NTCa²⁺, was added followed by incubation overnight at 4°C. The plates were then washed with TBS-NTCa²⁺BSA and a phosphatase substrate (p-nitrophenyl phosphate disodium; Sigma Chemicals) in 50 mM TRIS-HCl, 5 mM CaCl_2 , 5 mM MgCl_2 and 0.05% (w/v) NaN_3 , pH 9.5, added to give a final concentration of substrate of 1 mg/ml. The plates were read after about 30 minutes of incubation at 37°C on a MultiskanR MCC/340 (Titertek Co Ltd). The assay detected MBP down to a concentration of 1 ng/ml and was standardised by using a preparation of purified MBP, the concentration of which had been determined by amino acid analysis.

Results

The patients were split into six groups on the basis of their liver pathology. These groups were normal (table 1), alcoholic liver disease (table 2), metastatic disease (table 3), biliary disease (table 4), hepatitis (table 5) and miscellaneous (table 6).

Results are given in tables 1–6 for serum MBP, liver MBP, and the patients' liver blood tests performed on the day of the biopsy (aspartate aminotransferase (AST), alkaline phosphatase (AP), γ glutamyl transpeptidase (GGT), albumin and bilirubin) for these six groups of patients.

Table 7 shows the correlation coefficients for the various potential associations: none was significant.

Table 8 shows the 17 patients with serum MBP results of more than 2656 $\mu\text{g/l}$, arranged in ascending order with respect to the serum MBP; correlation coefficient 0.268.

The results expressed in tables 1–6 are summarised as follows:

Normal liver pathology: no significant staining with anti-MBP.

Alcoholic liver disease: virtually all cases showed MBP in the liver sections, with the

Table 3 MBP in patients with metastatic malignant tumours

Case No	Liver biopsy specimen appearance	Serum MBP ($\mu\text{g/l}$)	Tissue MBP	AST	AP	GGT	Albumin	Bilirubin
27	Metastasis	755	1	100	574	715	47	9
28	Metastasis (not in bx)	4235	1	318	596	—	34	84
29	Metastasis	4804	2	695	1874	861	35	145
30	Metastasis	795	3	64	733	149	30	8

Tissue MBP is strength of staining of liver biopsy specimens with anti-MBP on a scale 0–3. See table 1 for normal ranges of liver blood tests.

Cases 27 and 29 represented metastatic neuroendocrine tumours; case 30 was a metastatic adenocarcinoma (origin from large bowel); case 28 was a case of metastatic pancreatic adenocarcinoma, which was present in the formalin fixed specimen but not in the fresh portion (bx) used in this study.

Figure 1 Liver biopsy specimen showing alcoholic cirrhosis. Strong (grade 3) immunoperoxidase staining of hepatocytes with anti-MBP on frozen tissue section.

Table 4 MBP in patients with biliary disease

Case No	Liver biopsy specimen appearance	Serum MBP (µg/l)	Tissue MBP	AST	AP	GGT	Albumin	Bilirubin
31	PSC stage 1	2160	1	189	1906	486	39	28
32	PSC	71	2	26	420	151	47	7
33	PBC stage 2	10113	2	149	492	192	45	10
34	ldo	1018	1	29	745	135	24	7
35	ldo	1972	2	76	1241	763	40	25

Tissue MBP is strength of staining of liver biopsy specimens with anti-MBP on a scale 0–3. See table 1 for liver blood tests units and normal ranges.
PSC = primary sclerosing cholangitis; PBC = primary biliary cirrhosis; ldo = large duct obstruction.

density of staining increasing with severity of liver disease up to grade 3 with the cirrhotics (fig. 1).
Metastasis: all cases showed MBP staining, but only two of the four had grades 2 or 3 staining (fig. 2).
Biliary disease: mild to moderate staining with anti-MBP (grades 1–3).
Hepatitis: either no staining or only grade 1.
Miscellaneous: variable MBP staining in liver: the only ones of grades 2 or 3 were a case of fatty change and a cirrhosis (the latter secondary to chronic active hepatitis).

Discussion

The first conclusions that can be drawn from this study are essentially similar to those of

our previous study,¹⁰ with data from 57 more liver biopsy specimens to add to those from the 71 tested previously.

Broadly speaking, the results confirm a number of features relating to the staining pattern for MBP in the liver:

- 1 Liver biopsy specimens with normal histological features show no significant staining with anti-MBP (13 patients in all now tested).
- 2 The staining pattern with anti-MBP is generally cytoplasmic and diffuse.
- 3 Liver biopsy specimens from patients with alcoholic liver disease tend to show staining with anti-MBP, and the degree of staining seems to be related to the severity and duration of the liver damage—those with fatty change tend only to show minor staining (grade 1); those with cirrhosis show strong staining (grade 3), with the specimens from patients with alcoholic hepatitis or fibrosis falling short of cirrhosis generally showing moderate (grade 2) staining.
- 4 Liver biopsy specimens from patients with biliary disease show mild to moderate MBP staining.
- 5 The miscellaneous hepatitics and others (tables 5 and 6) show generally only mild MBP staining, with only one showing extensive MBP (grade 2/3) staining, a case of cirrhosis secondary to chronic active hepatitis (severe disease).
- 6 The absence of high serum MBP concentrations and detectable MBP in the liver does not exclude the presence of clinically relevant liver disease on morphological or biochemical grounds.

The group of biopsy specimens with tumours was smaller than in our first study,¹⁰ where four of five liver biopsy specimens containing metastatic tumour had shown grade 2 or 3 staining, most noticeable in the tissue adjacent to the tumour. In the current study two showed grade 2 or 3 staining, and two showed only grade 1. One of these did not contain the tumour within the fresh sample of liver, and thus may not be representative—that is, the much raised MBP concentration in hepatocytes might be only a local effect seen adjacent to the tumour and not a general effect throughout the liver.

The main question which the study set out to investigate was whether there was any association between the amount of MBP in the liver (its site of synthesis), as detectable by immunohistochemistry, and the serum concentration.

There was no obvious correlation between serum and liver MBP concentrations. The correlation coefficient (–0.076) is given in table 7—virtually random. There is a wide range for serum MBP concentrations in the general population, with 90% lying between 33–2656 µg/l.¹¹ In our sample of patients with liver disease, however, 17 of 57 (30%) lay above this “normal” range. Table 8 shows these top 17 arranged in ascending order of serum MBP, and again there was no significant correlation between serum and tissue MBP (correlation coefficient 0.268).

Table 5 MBP in patients with hepatitis of varied aetiology

Case No	Liver biopsy specimen appearance	Serum MBP (µg/l)	Tissue MBP	AST	AP	GGT	Albumin	Bilirubin
36	Reactive hepatitis	574	0	26	267	67	44	5
37	Reactive hepatitis	5801	0/1	21	303	200	42	11
38	Reactive hepatitis	1472	1	50	246	65	50	15
39	Reactive hepatitis	5212	1	15	916	—	38	2
40	Reactive hepatitis	2324	1	34	288	94	41	20
41	Reactive hepatitis	6073	1	16	617	111	44	3
42	Reactive hepatitis	1563	1	29	510	75	40	3
43	Granulomatous hepatitis	1091	0	61	655	279	35	6
44	Cholestatic hepatitis ?NANB	275	1	57	784	42	26	206
45	Hepatitis ?NANB	82	1	21	434	58	43	9
46	Mild CPH (NANB)	287	1	384	150	—	43	11

Tissue MBP is strength of staining of liver biopsy specimens with anti-MBP on a scale 0–3. See table 1 for liver blood tests and normal ranges.
CPH = chronic persistent hepatitis; NANB = non-A, non-B viral hepatitis (including hepatitis C, *et al.*).

Figure 2 Hepatocytes showing moderate (grade 2) staining with anti-MBP on frozen tissue section from patient with metastasis in the liver (tumour not seen in this field).

Table 6 MBP in patients with abnormal liver biopsy specimens of varied aetiology

Case No	Liver biopsy specimen appearance	Serum MBP (µg/l)	Tissue MBP	AST	AP	GGT	Albumin	Bilirubin
47	Fatty change (Crohn's)	7160	1	35	418	52	44	11
48	Fatty change	10468	2	39	201	—	43	30
49	Sinusoidal dilatation	1511	1	18	400	247	35	8
50	Mild fibrosis ?drug	1705	0/1	50	368	1035	40	6
51	Mild fibrosis	900	1	41	459	—	41	8
52	Mild fibrosis	1643	1	37	344	152	41	15
53	Cholestasis (drug)	2224	0	48	840	41	43	294
54	Cholestasis ?drug	1418	1	33	394	189	42	79
55	Minor abnormalities (methotrexate)	1133	1	30	156	—	—	7
56	Haemochromatosis with cirrhosis	2385	1	106	413	—	25	55
57	Cirrhosis (CAH)	201	2/3	837	1118	79	39	161

Tissue MBP is expressed as strength of staining of liver biopsy specimens with anti-MBP on a scale 0–3. See table 1 for units and normal ranges of liver blood tests.
Drugs (case 50) antiepileptics including phenytoin and phenobarbitone; case 53 unknown drug (took many cardiac/antihypertensive drugs); case 54 azathioprine.
CAH = chronic active hepatitis

The results for liver blood tests are also included in tables 1–6, these samples being taken on the day of the biopsy. Again there was no significant correlation between any of these figures and the serum or tissue MBP (see table 7 for correlation coefficients).

As the hepatocytes are considered to be the major site of synthesis of MBP, it was rather unexpected to find no correlation between serum concentrations of MBP and staining for MBP in hepatocytes. It would perhaps have been very useful in the clinical assessment of liver disease to find higher concentrations of serum MBP in patients with

increased MBP detectable in the liver (which in turn appears to be related to severity of liver damage in certain clinical settings). As it is, the correlation coefficient is virtually 0. However, with there being such a wide “normal” range for serum MBP concentrations, and with there being no way of determining the concentration of MBP in the serum before the onset of liver disease, it is always possible that those patients with high intensity of staining in the liver for MBP had in fact also shown a substantial proportionate increase in serum MBP from the level prior to the onset of disease, but that this new level was still within the “normal” range. To investigate this would require measurement of serum MBP perhaps several years before a biopsy specimen was taken as a base-line level, which would obviously be impossible unless MBP was widely measured on a routine basis, which is unlikely for the foreseeable future.

This lack of correlation between blood concentrations and apparent tissue presence of a compound is not unprecedented when considering synthesis and release of substances from cells. In endocrine cells and tumours it is well known that there is not always a direct correlation between what is found in the serum and what is detectable in the cells of origin. In a study of 95 patients with insulinoma (an endocrine tumour of pancreatic islet cells), all of whom had documented hyperinsulinaemia, tumour cells uniformly stained weaker for insulin than corresponding normal cells in the same pancreas, and in a few cases insulin was not detectable at all by immunohistochemistry.¹² Conversely, in the liver patients with α -1-antitrypsin deficiency in the serum have large amounts of it in the liver.¹³

These examples may not be directly com-

Table 7 Spearman rank correlation coefficient between MBP in serum or liver and other recorded variables (possible range –1.0 to +1.0).

	Serum MBP	Tissue MBP	AST	AP	GGT	Albumin	Bilirubin
Serum MBP		–0.076	–0.030	–0.098	0.052	0.119	0.020
Tissue MBP	–0.076		0.385	0.096	0.290	–0.318	0.260

Table 8 Patients with serum MBP outside range 33–2656 µg/l arranged in ascending order of serum MBP, and liver MBP included for comparison

Case No	Histology	Serum MBP	Tissue MBP
4	Fatty (ALD)	2832	0/1
18	Hepatitis/fibrosis (ALD)	3021	2/3
11	Hepatitis (ALD)	3407	1/2
28	Metastasis	4235	1
2	Normal	4532	0/1
17	Hepatitis/fibrosis (ALD)	4532	2
29	Metastasis	4804	2
39	Reactive hepatitis	5212	1
37	Reactive hepatitis	5801	0/1
41	Reactive hepatitis	6073	1
10	Hepatitis (ALD)	6122	1/2
47	Fatty (Crohn's)	7160	1
22	Cirrhosis (ALD)	7558	2
33	PBC 2	10113	2
48	Fatty change	10468	2
15	Hepatitis/fibrosis (ALD)	13197	2
14	Hepatitis/fibrosis (ALD)	17306	1/2

Spearman rank correlation coefficient = 0.268.

ALD = alcoholic liver disease.

parable with the present situation, but abnormalities in storage capacity (as in endocrine tumours), or structural variations in the secreted product (as for α -1-antitrypsin), among others, could be relevant.

In conclusion, there does seem to be an association between pathological changes in the liver and staining for MBP, most pronounced in alcoholic liver disease where there seems to be a direct correlation between the severity of the damage and detectable MBP. A greater proportion of patients with liver disease have raised serum MBP concentrations compared with the general population, but this does not correlate with either the severity of the hepatic disease or the amount of MBP detectable in the liver by immunohistochem-

istry. And finally, there was no significant correlation between MBP in serum or liver and other tests of liver function or damage.

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