Low molecular weight IgM in primary biliary cirrhosis

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Abstract

Low molecular weight IgM is the monomeric subunit of pentameric IgM and is not generally found in the blood of healthy individuals. Using a sensitive immunoblotting technique, low molecular weight IgM was detected in all 17 patients with primary biliary cirrhosis and constituted up to 5% of the total circulating IgM. This low molecular weight IgM moiety correlated significantly with total IgM ($p < 0.01$) but not with the specific biliary cirrhosis mitochondrial autoantibody anti-M2. Furthermore it was not possible to show that a partially purified sample of low molecular weight IgM contained M2 binding activity. Mitogen stimulated peripheral blood mononuclear cells from two of four patients were observed to secrete low molecular weight IgM *in vitro*, a finding seen in only one of six healthy subjects. Immunoblot analysis of patients sera revealed the presence of other oligomers of IgM in addition to low molecular weight IgM. In conclusion this study suggests that during the enhanced IgM synthesis observed in primary biliary cirrhosis a defect occurs in the assembly of the IgM pentamer with release of monomeric and oligomeric IgM into the circulation. The pathogenic significance of these circulating low molecular weight IgM species is unknown.

Primary biliary cirrhosis is characterised by a large variety of immunological disturbances but two of the most consistent serological findings are the raised concentrations of IgM found in over 75% of patients and the almost universal occurrence of autoantibodies to the mitochondrial antigen M2.¹ There is as yet no good explanation for either of these findings and no antigenic or mitogenic stimulus has been identified to account for the prominent IgM response.

The circulating IgM molecule in healthy individuals consists of five monomeric subunits covalently bound together with a single J (Joining) polypeptide chain, the total molecular complex having a mass of approximately 10^6 Daltons.² Low molecular weight IgM, the monomeric subunit whose mass is 2×10^5 Daltons, is not found in the serum of healthy subjects but has been described in a number of autoimmune, infective and B cell lymphoproliferative disorders.³ The reason for the presence of low molecular weight IgM in these conditions is uncertain but its frequent occurrence in immune complex related disorders,⁴ suggests that it may have direct pathogenic significance.

Low molecular weight IgM has been described in one previous study in primary biliary cirrhosis.⁵ Fakunle *et al.*, using a gel diffusion

method found this IgM moiety in one third of their 69 patients particularly in those with high circulating concentrations of IgM and immune complexes.⁵ In the current study we have used a sensitive immunoblotting technique to detect low molecular weight IgM in 17 patients with primary biliary cirrhosis and have compared concentrations of low molecular weight IgM with anti-M2, an autoantibody relatively specific for this condition.¹ We have also attempted to determine whether low molecular weight IgM contained anti-M2 binding activity and whether stimulated peripheral blood mononuclear cells from patients could secrete low molecular weight IgM *in vitro*.

Methods

PATIENTS AND CONTROLS

There were 17 patients with probable or definite primary biliary cirrhosis, 16 women and one man with a mean age of 63.3 years (range 41-87). The diagnosis was made according to standard clinical and laboratory criteria. All patients sera contained raised concentrations of antimitochondrial antibodies and liver biopsy confirmation of the clinical diagnosis was obtained in 13 patients. Two patients also had Sjögrens syndrome and one had systemic sclerosis (CREST). Control subjects consisted of 15 healthy adults (eight women, seven men) five of whom were selected for their high IgM concentrations ($2.5 - 3.9 \text{ g/l}$), six patients (four men, two women) with alcoholic cirrhosis and one male patient with chronic active hepatitis. Serum used as a positive control was obtained from a patient with Waldenstroms macroglobulinaemia. This serum contained large quantities of pentameric, monomeric and oligomeric IgM.

DETECTION AND MEASUREMENT OF LOW MOLECULAR WEIGHT IgM

Low molecular weight IgM was detected in sera by two methods.

FILTRATION CHROMATOGRAPHY

A filtration chromatographic method was used, as described by Roberts-Thomson *et al.*⁶ In brief, serum was separated at room temperature on Sepharose 6B or Sephacryl S300 (Pharmacia, Uppsala, Sweden), equilibrated in phosphate buffered saline, pH 7.4, and the IgM measured in the eluate fraction by laser nephelometry or enzyme linked immunosorbent assay (ELISA).⁷ An IgM profile was then obtained and the detection of a second IgM peak eluting just before the IgG peak, signified the presence of

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low molecular weight IgM. Planimetry was used to determine the quantity of low molecular weight IgM.

IMMUNOBLOT ANALYSIS

The method of Harris *et al*⁸ was used. In brief, serum was separated on sodium dodecyl sulphate/3.6% polyacrylamide gel slabs and the separated serum proteins transferred to nitrocellulose. The IgM bands were developed using a biotin-avidin antihuman μ chain specific system. Sera with and without low molecular weight IgM were included in each run as controls. After immunoblotting, densitometry of bands was performed using a Camag Electrophoresis Scanner. The profile of the band representing monomeric IgM was then traced onto exposed x-ray film and the corresponding weight determined. This value was used to reflect the relative quantity of low molecular weight IgM.

SECRETION OF LOW MOLECULAR WEIGHT IgM IN VITRO

Peripheral blood mononuclear cells were separated from heparinised blood by Ficoll-Hypaque sedimentation using standard techniques. Cultures were established containing 1×10^6 PBMC/ml and pokeweed mitogen (Gibco, Ohio, USA) at a final dilution of 1:800. At days three and seven the culture supernatants were collected (total volume 30-60 ml), concentrated in an Amicon concentrator unit to a final volume of 1-3 ml and analysed for total IgM by ELISA and for low molecular weight IgM by either the immunoblotting technique or by filtration chromatography. Supernatants were also assayed for anti-M2 using a specific ELISA as described below. To confirm active synthesis and secretion of IgM *in vitro*, cycloheximide was added to cultures derived from three healthy subjects to a final concentration of 10 μ g/ml.⁷

ANTI-MITOCHONDRIAL M2 ELISA

Antibodies to the mitochondrial antigen M2 were measured by ELISA according to the instructions issued by the manufacturer (PBC-IgG/IgM - ELISA, Pharmacia, Uppsala,

Sweden). In addition to measuring autoantibodies belonging to both the IgG and IgM isotypes an anti-M conjugated antiserum was also used to quantitate anti-M2 antibodies belonging only to the IgM isotype.

COLUMN CHROMATOGRAPHY

After separation of selected sera on Sepharose 6B columns as described above, fractions containing pentameric IgM and monomeric IgM were pooled respectively, concentrated in an Amicon concentrator unit to a final volume of 1 ml and analysed for antibody binding activity against the mitochondrial antigen M2 using both the anti-IgG/IgM and the IgM conjugate antiserum respectively.

OTHER SEROLOGICAL INVESTIGATIONS

Serum immunoglobulin and rheumatoid factor were measured by rate nephelometry (Beckmans ICS). Antinuclear antibody and antimitochondrial antibody were measured by indirect immunofluorescence using rat liver and rat renal tubules as substrate respectively at an initial screening dilution of 1:10. These methods have been standardised and are in daily use in our laboratory. Low concentrations of IgM in culture supernatants were measured by ELISA.⁷

Results

A summary of the clinical and serological findings for the patients is shown in the Table. Using the immunoblotting techniques dense bands representing low molecular weight IgM were seen in all 17 patients with primary biliary cirrhosis and in the majority of these sera additional weaker staining bands representing oligomeric IgM and other intermediate oligomers were also evident (Fig 1). Low molecular weight IgM bands were not observed in any of the 10 healthy subjects with normal IgM concentrations but faint low molecular weight bands were observed in three or five healthy subjects with high IgM concentrations. Weak staining low molecular weight IgM bands were observed in three of seven patients with other forms of liver disease and all of the positive

TABLE 1 Clinical and serological findings in patients with primary biliary cirrhosis

Patient	Gender	Age	Liver biopsy	Additional diagnosis	AMA	anti-M2	IgM	LMW/IgM†	IgG	IgA	Rheumatoid factor	ANA
1	F	87	+		>1:320	230	2.03	+	14.6	1.38	305	1:10
2	F	68	ND*		>1:320	1150	6.6	+++	14.4	1.45	<60	1:10
3	F	89	+		>1:320	600	8.14	+++	21.4	1.31	<60	neg
4	M	70	+		>1:320	610	6.88	++	23.1	8.65	<60	neg
5	F	60	+	Ulcerative colitis	>1:320	13	3.72	+	10.6	2.17	<60	neg
6	F	52	+		>1:320	250	34.7	+++	14.3	1.57	83	neg
7	F	60	+	Hypothyroid	>1:320	500	5.78	++	11.9	1.17	<60	1:10
8	F	73	+		>1:320	2900	6.52	++	24.3	5.7	<60	neg
9	F	76	+	Sjogrens	>1:320	135	7.49	+++	28.7	2.68	154	>1:2560
10	F	41	ND	Sjogrens		130	2.85	+	11.5	1.19	<60	>1:2560
11	F	48	+		>1:320	34	4.91	++	13.0	3.88	75	neg
12	F	59	+	CREST	>1:320	410	18.0	++	17.9	6.19	177	1:2560
13	F	64	+		>1:320	260	8.54	++	17.9	6.19	<60	neg
14	F	60	+		1:10	160	2.46	+	12.5	4.62	<60	1:10
15	F	52	+		1:160	190	1.65	+	26.0	3.68	<60	neg
16	F	78	ND		1:20	12	3.53	++	9.61	3.52	<60	neg
17	F	69	ND		>1:320	250	2.62	++	17.4	5.38	130	neg
Normal values					neg	<14 IU/l	0.55-2.2g/l		7-19g/l	0.5-4g/l	<60 IU/ml	neg

† graded 0-+++ according to densitometry scans.

*ND=not done.

Figure 1: Specific IgM bands in test sera after immunoblotting. The molecular mass as defined by marker proteins is indicated. Lane 1 – patient with active chronic hepatitis; lane 8 – healthy subject; lane 15 – positive control from patient with macroglobulinaemia; lanes 2–7, 9–13 patients with primary biliary cirrhosis.

Figure 2: Densitometric scans of immunoblots: upper panel – normal serum; middle panel – serum from patient with primary biliary cirrhosis; lower panel – culture supernatant from same patient with primary biliary cirrhosis.

patients had raised IgM concentrations. In the biliary cirrhotic sera the low molecular weight IgM bands were still clearly evident despite diluting the sera to achieve IgM levels in the normal range. Filtration chromatographic analysis was performed on six sera from patients with primary biliary cirrhosis and 10 healthy control sera. This technique is less sensitive than the immunoblot technique but a late emerging IgM peak was observed in three of the biliary cirrhosis sera and represented up to 5.1% of the total IgM profile. None of the healthy sera contained low molecular weight by this technique.

Fifteen of the primary biliary cirrhotic sera contained raised concentrations of IgG/IgM antibodies to the mitochondrial antigen M2 whilst all healthy subjects and seven patients with other forms of liver disease contained levels less than 14 IU/l (the upper limit of normal as quoted by the ELISA manufacturer). When the concentrations were quantified using the anti-IgM specific conjugate antiserum only marginal binding activity was observed in 10 biliary cirrhotic sera suggesting that antibodies of the IgG isotype was primarily responsible for the M2 binding in the patients sera.

Low molecular weight IgM (as assessed by planimetry) in the 17 patients with primary biliary cirrhosis correlated significantly with the total IgM concentrations ($r=0.71$, $p<0.01$), but not with IgG/IgM anti-M2 binding ($r=0.003$, $p=NS$).

Column fractions containing pentameric IgM and LMW IgM respectively from one patient with high serum concentrations of anti-M2 were tested for M2 binding. M2 binding was detected only in the low molecular weight fraction using the IgG/IgM conjugate but not with the IgM conjugate. This suggested the presence of IgG anti-M2 antibodies only in this fraction. No binding was detected in the high molecular weight fraction using either of the conjugate antisera.

Concentrated peripheral blood mononuclear

cells culture supernatants from four biliary cirrhotic patients and six healthy subjects were analysed for total IgM by ELISA and for low molecular weight IgM by either the immunoblot technique or the filtration chromatographic technique depending on the total quantity of IgM secreted. Patients supernatants contained less IgM than supernatants for healthy subjects (mean (SE) of 3147 (2119) ng/ml compared with 5173 (1916) ng/ml respectively). Low molecular weight IgM was detected in supernatants from two patients and in one healthy subject (Fig 2). Other low molecular weight IgM oligomers were also faintly visible in the supernatant immunoblots. Antibodies to mitochondrial antigen M2 were detected in two of four of the patients culture supernatants (levels of 19 and 20 IU/l respectively) but not in any of three healthy subjects. Cycloheximide inhibited synthesis and secretion of IgM by greater than 90% in all cultures tested.

Discussion

Low molecular weight IgM was found in the sera of all 17 patients with primary biliary cirrhosis and a significant correlation was noted with the circulating IgM concentrations but not with concentrations of antibodies against the mitochondrial M2 antigen. Peripheral blood mononuclear cells from two of four patients when stimulated with pokeweed mitogen secreted low molecular weight IgM *in vitro*, an unusual finding with cells obtained from healthy subjects.⁷ Finally it was not possible to show that low molecular weight IgM from one patient would bind specifically to the M2 antigen.

The finding of low molecular weight IgM in all our patients as compared with one third reported by Fakunle *et al*⁵ is attributed to the greater sensitivity of the immunoblot technique. It was also of interest to observe dimeric IgM and other low molecular weight IgM species in many of the sera although always in lesser quantities than

monomeric IgM. This observation, together with the significant correlation between low molecular weight IgM and the total IgM concentrations suggests that the presence of low molecular weight IgM might be closely linked with enhanced IgM secretion rates seen in these conditions. In primary biliary cirrhosis, however, low molecular weight IgM made up only a relatively small proportion of the total circulating IgM (<5%) in contrast with a mean of approximately 10% seen in rheumatoid arthritis.⁶ In this later condition the IgM is raised to only minor levels as compared with the large increase seen in primary biliary cirrhosis. The reason for the differences in the proportion of low molecular weight IgM between the two disorders is still unclear.

The pathogenic significance of low molecular weight IgM in primary biliary cirrhosis is still obscure. Low molecular weight IgM has weak agglutinating and precipitating activity in comparison with the pentameric molecule³ and appears to have a valency of only one if the evidence obtained from experimental reduction of purified pentameric IgM antibodies to the monomeric form is correct.⁹ This low molecular weight IgM might thus form small immune complexes which could persist in the circulation for prolonged periods or possibly block the effector activities of the larger pentameric molecule.

In conclusion we have noted the universal occurrence of low molecular weight IgM in primary biliary cirrhosis and have observed its significant correlation with the total IgM concentrations. The most logical explanation for its occurrence is that it is secreted during the assembly of the pentameric molecule in the enhanced IgM response seen in this condition. Its pathogenic significance is still unknown.

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