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Anti-M9 antibodies in sera from patients with primary biliary cirrhosis recognize an epitope of glycogen phosphorylase

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SUMMARY

Anti-M9 antibodies in sera from patients with primary biliary cirrhosis (PBC) were previously found to recognize two antigenic determinants at 98 and 59 kD, using a purified antigen fraction derived from rat liver mitochondria in the Western blot. Here we show that these antibodies are directed against an epitope of the enzyme glycogen phosphorylase. By Western blotting, a determinant at 98 kD was obtained testing anti-M9 positive sera against phosphorylase from skeletal muscle, and after plasmin treatment a degradation product appeared at 59 kD. Both determinants were identical to the M9-specific determinants 98 and 59 kD as shown by absorption studies. When these antibodies were eluted from the 98 and 59 kD determinants of the M9 antigen after immunoblotting, they again recognized the same epitopes on plasmin-treated phosphorylase. Furthermore, phosphorylase enzyme activity could be also demonstrated in the purified M9 fraction, and anti-M9-positive/anti-M2-negative but not anti-M9-negative/anti-M2-positive sera could be shown to stimulate phosphorylase activity. Testing sera from 1189 patients with different hepatic and non-hepatic disorders against M9 and phosphorylase from skeletal muscle by ELISA, 20% were positive with phosphorylase and only 2% with the M9 fraction. These data indicate that the commercially available phosphorylase from skeletal muscle cannot be recommended as M9 source. It may still contain non-PBC-specific epitopes which are probably recognized by naturally occurring antibodies directed against this highly conserved protein.

Keywords antimitochondrial antibodies anti-M9 glycogen phosphorylase primary biliary cirrhosis

INTRODUCTION

Sera from patients with primary biliary cirhosis (PBS) contain different anti-mitochondrial antibodies (AMA) which react with antigens associated either with inner (M2) or outer mitochondrial membranes (M4, M8, M9) (Berg & Klein, 1987). Anti-M2 antibodies are highly specific for the serological diagnosis of PBC and can be detected in 96% of PBC patients using the ATPase-associated M2 antigen isolated from beef heart mitochondria in ELISA (Berg *et al.*, 1982). Anti-M9 antibodies have been recently described to be a marker for the diagnosis of early stages of PBC (Klein *et al.*, 1988); they can be detected using a purified M9 fraction obtained by ion exchange chromatography of a 100 000 *g* supernatant from rat liver mitochondria (Klein & Berg, 1988).

The M2 antigen consists of five antigenic determinants at molecular weights 70 kD (a), 56 kD (b), 51 kD (c), 45 kD (d), and 36 kD (e) (Lindenborn-Fotinos, Baum & Berg, 1985). All determinants could be identified as subunits of the family of the 2-oxo-acid-dehydrogenase (Coppel *et al.*, 1988; Yeaman *et al.*, 1988; Fussey *et al.*, 1988, 1990; Surh *et al.*, 1989).

The M9 antigen could be also visualized by Western blotting and consists of two determinants at 98 and 59 kD. It co-purifies with outer mitochondrial membranes and is partially organ specific. We were, therefore, interested to see whether this antigen could be also related to any enzyme of the outer mitochondrial membrane or to another cytoplasmic enzyme. We show here that it shares identical epitopes with glycogen phosphorylase, an evolutionary highly conserved enzyme which is enriched in liver and skeletal muscle.

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SUBJECTS AND METHODS

Subjects

Sera from 80 patients with histologically and clinically defined PBC being anti-M2 positive as well as from 393 patients with different hepatic disorders, 261 patients with cardiac diseases, 111 patients with connective tissue disorders, 99 patients with other organ and non-organ specific autoimmune disorders, 325 patients with other disorders and from 100 blood donors were included in the study.

Marker sera

Marker sera with three different constellations of anti-M2 and anti-M9 were used: anti-M9 positive/anti-M2 negative, anti-M9 and anti-M2 positive and anti-M9 negative/anti-M2 positive.

Tests were also performed with sera from patients with other AMA specificities (anti-M1–anti-M8 positive; Berg & Klein, 1989).

Antigens

Preparation of M2 and M9. The M2 antigen was released from beef heart mitochondria by chloroform treatment as described by Lindenborn-Fotinos, Sayers & Berg (1982).

M9 was prepared from a 100 000 *g* supernatant of sonicated rat liver mitochondria and further purified by ion exchange chromatography on AH–Sepharose 4B (Pharmacia) by a stepwise elution with a NaCl gradient. The fractions eluted with 1 M NaCl contained highest M9 activity (Klein & Berg, 1988).

In a similar way, antigen fractions were prepared from a 100 000 *g* supernatant from mitochondria of beef heart, pig kidney, rat lung and stomach in order to evaluate the tissue specificity of M9.

Enzymes used as antigens. Different enzymes known to be associated with inner or outer mitochondrial membranes or with other cellular compartments including microsomes (obtained from Sigma Chemicals Co., St Louis MO) were used as antigens in the ELISA and Western blot.

Inner mitochondrial membrane-associated enzymes: beta-hydroxybutyrate dehydrogenase, isocitric dehydrogenase, alpha-glycerophosphate dehydrogenase, glutamic-oxalacetic transaminase, sulphite oxidase, nucleoside-5′-diphosphate kinase, malic dehydrogenase, sarcosine dehydrogenase, aldehyde dehydrogenase, fumarase, pyruvate dehydrogenase, aconitase, myokinase, creatine phosphokinase, cytochrome oxidase.

Matrix-associated enzymes: beta-hydroxy coenzyme A dehydrogenase, lipoamide dehydrogenase, citrate synthase, carnitine acetyltransferase, S-acetyl coenzyme A synthase, pyruvate carboxylase, succinic thiokinase, beta-hydroxyacyl-coenzyme A dehydrogenase.

Outer mitochondrial membrane-associated enzymes: monoamine oxidase, coenzyme A synthetase, phospholipase A2.

Tested was also a group of enzymes associated with other cellular compartments including microsomes: hexokinase, phosphoglucose isomerase, fructose-6-phosphate kinase, aldolase, triosephosphate-isomerase, enolase, lactate dehydrogenase, pyruvate decarboxylase, glycogen phosphorylase a and b, glycogen-synthetase, glucose-6-phosphate dehydrogenase, cytochrom b_5, glucose-6-phosphatase.

Preparation of glycogen phosphorylase from rat liver. Purified glycogen phosphorylase was isolated from rat liver as described by Roesler & Khandelval (1986) and Bahnak & Gold (1982). Briefly, rat liver samples were homogenized, and the crude homogenate was centrifugated at 8000 *g* for 10 min. The supernatant was centifugated at 50 000 *g* for 60 min. The pellet containing glycogen, glycogen synthase and glycogen phosphorylase was dialysed for 16 h against 4 mM Tris (pH 7·4) containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 0·05 M KH_2HPO_4 in order to remove the glycogen. After a second dialysis against 4 mM Tris (pH 7·4) containing 1 mM EDTA, and 1 mM 2-mercaptoethanol to remove KH_2PO_4 the fraction was chromatographed on a DEAE cellulose column, and phosphorylase-activity-containing fractions were concentrated, dialysed against the above buffer, and applied to a 5′ AMP–Sepharose affinity column (Pharmacia). The enzyme which was bound to the column was eluted with 10 mM AMP.

The purified M9 fraction was applied to a 5′ AMP–Sepharose affinity column, and the bound protein was again eluted with 10 mM AMP.

Determination of phosphorylase enzyme activity

The phosphorylase enzyme activity of antigen fractions obtained during the preparation of M9 and phosphorylase was determined according to Bergmeyer, Grassl & Walter (1983).

Method for detection of anti-M2 and anti-M9

ELISA and Western blotting were performed using the purified M2 and M9 fractions as recently described (Klein & Berg, 1988). In the ELISA patients' serum samples were diluted 1/500. Following SDS–PAGE, proteins were transferred from the gels to immobilon transfer membranes (Millipore). After blocking and washing, individual lanes were incubated for 45 min with human sera at a dilution of 1/50. Peroxidase-conjugated anti-human IgG and IgM antibodies from goat (Dakopatts) were used as secondary antibodies at a dilution of 1/200.

After immunoblotting of proteins to immobilon membranes and reaction with patients' sera, the specific bands were cut out of the immobilon sheets and dipped into 0·2 M glycin buffer (pH 2·8) in order to elute specifically bound antibodies. After neutralization, these eluted antibodies were retested by Western blotting.

Determination of the effect of patients' sera on phosphorylase activity

Patients' serum samples were examined for their ability to inhibit or stimulate enzyme activity. As marker sera we used the anti-M9-positive/anti-M2-negative and the anti-M9-negative/anti-M2-positive serum as well as a anti-M9/anti-M2-negative serum from a healthy blood donor.

The effect of anti-M9 antibodies which had been eluted from the M9 determinants 98 and 59 kD after immunoblotting on phosphorylase activity was also examined. Varying amounts of sera (0–20 μl) were pre-incubated for 30 min at 30°C with 20 μl of purified phosphorylase from skeletal muscle (concentration 0·05 mg/ml; Boehringer, Mannheim), and then total phosphorylase activity was examined.

Absorption studies

The M9 antigen and phosphorylase from skeletal muscle (Sigma) were adsorbed to CNBr-activated Sepharose 4B at a concentration of 10 mg/ml. After extensive washing, patient's sera were added at a dilution of 1/10 and were allowed to react for 16 h at 4°C on a rotating disk. The absorbed sera were retested by ELISA and Western blotting.

Bound antibodies were eluted by 0·2 M glycin buffer containing 0·5 M NaCl (pH 2·8). After pH neutralization, these antibodies were also retested by ELISA and Western blotting.

Protease treatment

M9 and phosphorylase from skeletal muscle (Sigma) were treated with different proteases (trypsin, chymotrypsin, pepsin, carboxypeptidase B, plasmin, endoproteinase Arg-C, endopro-

teinase Glu-C, endoproteinase Cys-C, kallikrein, ancrod, dispase, elastase, collagenase, thrombin, cathepsin B, Cathepsin D; all from Boehringer, Mannheim) at concentrations of 100 and 200 μg protease/mg protein for 30 min at 30°C in a water-bath. The reaction was stopped by adding sample buffer containing tris/glycerol/sodium dodecyl sulphate/mercaptoethanol/bromphenol blue and heating at 100°C for 5 min. These protease-treated antigens were applied to Western blotting and retested against marker sera.

RESULTS

Identification of the M9 antigen as an epitope of glycogen phosphorylase

Three PBC marker sera expressing either anti-M2 or anti-M9 activity were tested by ELISA against different mitochondrial and cytoplasmic enzymes. It could be shown that the anti-M9-positive/anti-M2-negative serum reacted exclusively with glycogen phosphorylase a and b from skeletal muscle but not with any other cytoplasmic or mitochondrial enzyme, as listed in Subjects and Methods. Testing the anti-M2- and anti-M9-positive marker serum, antibody activity could be demonstrated against the pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase derived from the bovine heart belonging to the 2-oxoacid dehydrogenase complex as well as against glycogen phosphorylase while the anti-M2-positive but anti-M9-negative serum reacted only with the M2-related enzymes but not with phosphorylase.

By Western blotting using phosphorylase from skeletal muscle as antigen, anti-M9-positive sera recognized only one band at 98 kD (Fig. 1).

Protein staining of phosphorylase from skeletal muscle also revealed a single band at 98 kD, indicating the purity of this commercially available enzyme.

Furthermore, phosphorylase was isolated and purified from rat liver, and again a single band 98 kD was visualized by protein staining. This band was also recognized by anti-M9-positive PBC sera in the immunoblot.

The association of the M9 antigen with phosphorylase was also proven applying the purified M9 antigen to 5′ AMP–Sepharose affinity column which binds NAD^+-dependent dehydrogenases and ATP-dependent kinases. After elution of the bound protein with 10 mM AMP the 98 and 59 kD, determinants could be detected again by Western blotting using anti-M9-positive sera.

Determination of phosphorylase enzyme activity in antigen fractions obtained in the course of M9 and phosphorylase preparation

As shown in Table 1, the purified M9-fraction clearly showed phosphorylase enzyme activity. However, due to the lower activity of the starting material (mitochondria), the activity was lower than that in the purified phosphorylase fraction. Enzyme activity was also present in the fractions eluted from 5′ AMP–Sepharose.

Distribution of M9 in different organs

In order to substantiate the close association of M9 with phosphorylase, we also tried to prepare the M9 antigen from organs other than liver: heart, brain, skeletal muscle, lung, kidney and stomach.

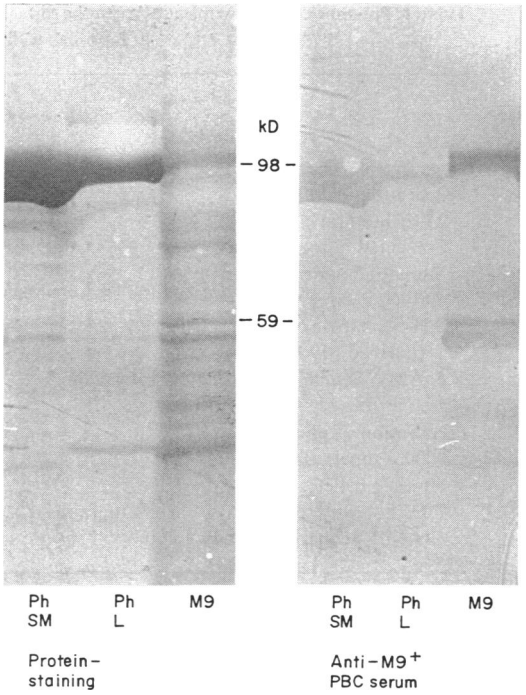

Fig. 1. Demonstration of the reaction of an anti-M9-positive/anti-M2-negative PBC marker serum with phosphorylase from skeletal muscle (Ph SM) and rat liver (Ph L) by Western blotting with the reaction with the purified M9 fraction expressing the typical bands at 98 and 59 kD. Using the two types of phosphorylases, the serum also recognizes a determinant at 98 kD. Protein staining revealed one major band with phosphorylase from skeletal muscle and rat liver and multiple bands with the M9 fraction.

Applying the 100 000 ***g*** supernatants from skeletal muscle, kidney, heart, lung and stomach to AH–Sepharose and eluting proteins with a step-wise NaCl gradient (0–1 M), the M9 antigen was detected in the 1 M NaCl fractions of skeletal muscle and kidney, and in the 0–0·25 M NaCl fractions from the heart. All these M9-positive fractions also contained phosphorylase enzyme activity. M9 could not detected in any of the fractions obtained from lung and stomach, and this finding is in accordance with the known fact that phosphorylase is nearly absent in these organs.

Effect of patients' sera on phosphorylase activity

Incubation of phosphorylase b from skeletal muscle with the anti-M9-positive/anti-M2-negative marker serum markedly increased the enzyme activity (Fig. 2), while the anti-M9-negative/anti-M2-positive and a negative control serum had no effect.

Similar results were obtained using the 98- and 59-kD-specific anti-M9 antibodies which had been eluted from the immobilon sheets after immunoblotting.

Enzyme treatment of M9 and phosphorylase

The purified M9 fraction was treated with different proteases and then tested by Western blotting against the anti-M9-positive/anti-M2-negative marker serum. The 98 kD determinant was destroyed by trypsin treatment, while the 59 kD determinant was resistant. Treatment with papain destroyed

Table 1. Phosphorylase enzyme activity in the different antigen fractions obtained during the preparation of the purified M9 fraction as well as of phosphorylase from rat liver

Step	Protein amount (total mg)	Protein concentration (mg/ml)	Units (total)	Specific activity (U/ml)
Preparation of M9				
Mitochondria	1550	10	4·65	0·003
Sonicated mitochondria	1550	10	4·65	0·003
Submitochondrial particles	232	58	0·928	0·004
100000 *g* supernatant	1000	10	3·0	0·003
AH-Sepharose chromatography (purified M9 fraction)	4	0·4	2·88	0·72
5′ AMP–Sepharose chromatography eluate*	0·3	0·1	0·096	0·32
Preparation of phosphorylase				
8000 *g* supernatant	950	10	47·5	0·05
50000 *g* pellet	60	12	6	0·1
DEAE–cellulose sepharose chromatography	1·8	0·3	0·59	0·33
5′ AMP–Sepharose affinity column	0·044	0·022	0·61	13·9

* Prepared from 1 ml of the purified M9 fraction (0·4 mg).

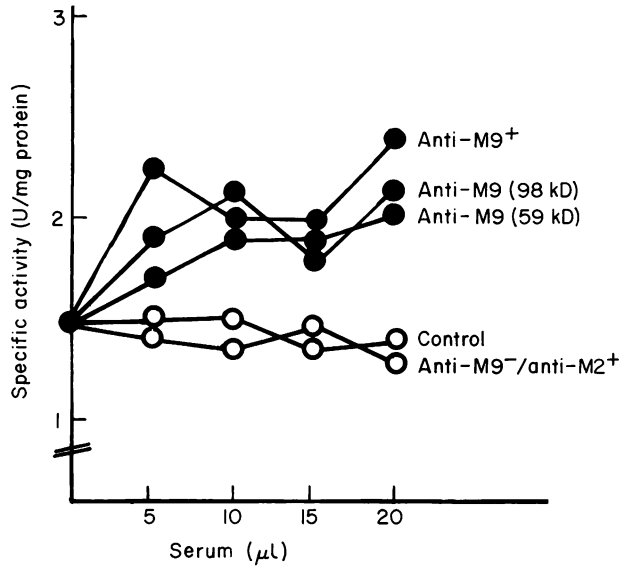

Fig. 2. Effects of anti-M9 antibodies on phosphorylase activity. An anti-M9 positive/anti-M2 negative serum as well as anti-98 kD and anti-59 kD antibodies which had been eluted from immobilon sheets after immunoblotting were incubated with phosphorylase b from skeletal muscle in varying amounts. The activity was markedly increased while the anti-M9-negative/anti-M2-positive as well as the antibody-negative serum had no effect.

both epitopes. Other proteases had no effect on the M9-specific reaction.

Treating phosphorylase from skeletal muscle with trypsin or papain, the 98 kD epitope was completely destroyed. After treatment with plasmin a degradation product at 59 kD appeared, as shown by protein staining, and this polypeptide was also recognized by anti-M9-positive sera in the blot (Fig. 3).

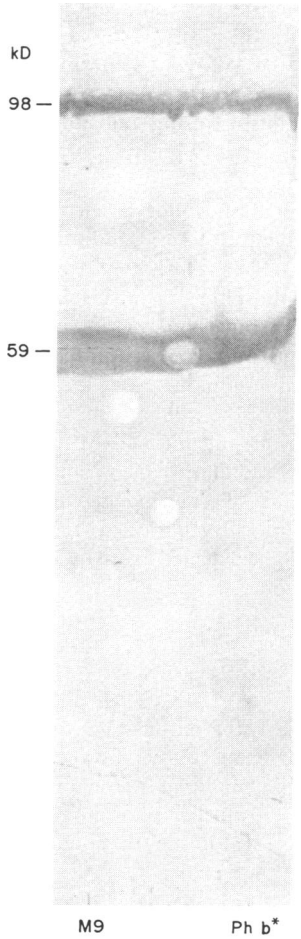

Fig. 3. Demonstration of a degradation fragment at 59 kD which appeared after plasmin treatment of phosphorylase b from skeletal muscle (Ph b). The anti-M9-positive marker serum was tested against the purified M9 fraction and plasmin-treated phosphorylase b (Ph b*).

Studies proving the presence of the M9 epitope on glycogen phosphorylase

In these experiments the anti-M9-positive/anti-M2-negative and the anti-M9-negative/anti-M2-positive marker sera were absorbed with phosphorylase from skeletal muscle as well as with the purified M9 antigen, both being coupled to CNBr-activated Sepharose 4B.

This procedure abolished completely the anti-M9 and anti-phosphorylase activity but did not affect the anti-M2 reaction as shown by Western blotting using M9, phosphorylase and M2 as antigens (Fig. 4a).

Eluting the antibodies from the coupled M9 antigen as well as from the phosphorylase and retesting them by Western blotting again the specific M9 determinants at 98 and 59 kD as well as the 98 kD band with phosphorylase could be detected.

Identical results were obtained when the 98- and 59-kD specific anti-M9 and the 98-kD related anti-phosphorylase antibodies were eluted from the immobilon sheets after Western blotting and retested against the two antigens.

In order to show that the 59 kD determinant obtained after plasmin treatment of phosphorylase has a common epitope with the M9-specific 59 kD determinant, the antibodies were eluted from this band and retested against the purified M9 antigen by Western blotting. This antibody recognized both the 59 and the 98 kD determinants, indicating that the M9-specific 59 kD determinant is a degradation product of the 98 kD polypeptide and that M9 and phosphorylase share identical epitopes (Fig. 4b).

Comparison of the diagnostic relevance of anti-M9 antibodies using glycogen phosphorylase or the purified M9 fraction in the ELISA

Testing sera from 80 anti-M2-positive patients with clinically and histologically defined PBC against phosphorylase from skeletal muscle and the purified M9 fraction by ELISA, 55% were positive with the phosphorylase and 44% with the M9 fraction at a serum dilution of 1/500 and antigen concentrations of 10 μg/ml. The antibodies were of the IgG and/or IgM type. There was a high correlation between the antibody titres obtained with phosphorylase and M9, and the correlation coefficient for both the antibodies of the IgG and IgM type was 0·8.

None of the sera from patients with other AMA specificities reacted with phosphorylase by ELISA. Testing sera from 1189 patients with different hepatic and non-hepatic disorders against phosphorylase from skeletal muscle and the M9 fraction by ELISA, 20% were positive with phosphorylase and only 2% with the M9 fraction (Table 2). Sera from 15 out of 100 healthy blood donors reacted with phosphorylase but not with M9.

DISCUSSION

We have shown that the anti-M9 antibodies are directed against an epitope present on glycogen phosphorylase. Thus, testing anti-M9-positive marker sera against a wide spectrum of mitochondrial and other cytoplasmic enzymes, the commercially available phosphorylase a and b from skeletal muscle were the only enzymes which gave a positive reaction by ELISA.

Phosphorylase a (active form) and b (inactive form, requires A-5-MP for activity) consist of four respectively two identical subunits at molecular weights 98 kD (Fletterick & Madsen,

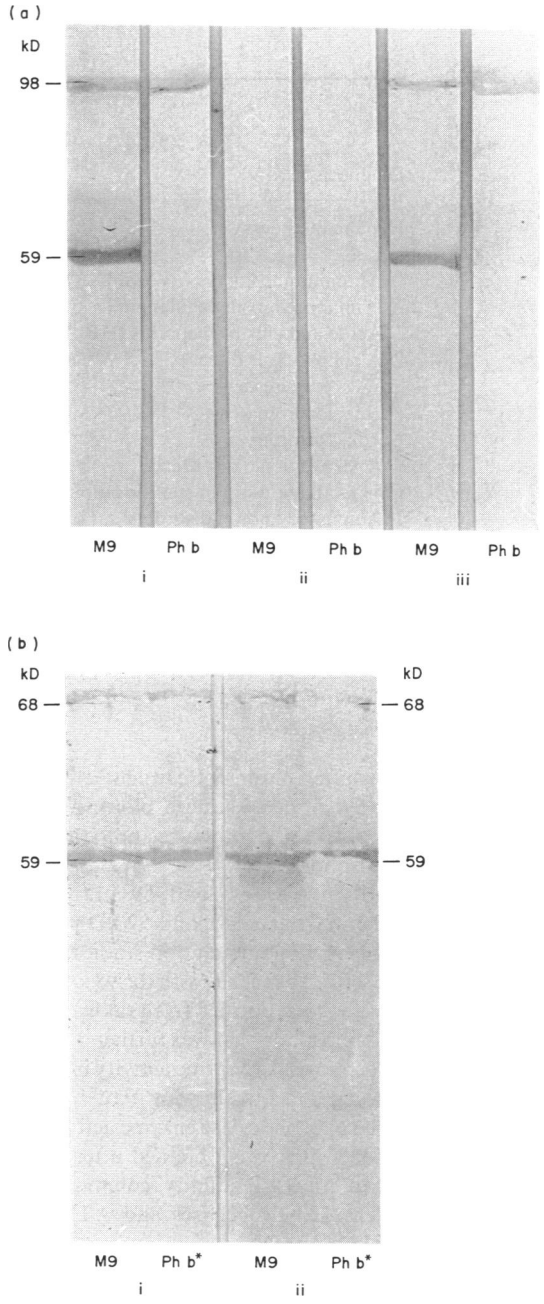

Fig. 4. (a) Demonstration of the identity of the epitopes recognized by the anti-M9-positive marker serum in the M9 fraction and on phosphorylase b (Ph b) by absorbing the serum with phosphorylase b. (i) Reaction before absorption; (ii) absorption with phosphorylase b completely abolished the reaction with the M9 fraction and phosphorylase b; and (iii) antibodies that had been eluted from phosphorylase b coupled to CNBr-activated Sepharose 4B again recognized the PBC specific M9 epitopes 98 and 59 kD as well as the 98 kD determinant of phosphorylase b. (b) Demonstration of the identity of the 98 and 59 kD determinant of the M9-antigen and plasmin treated phosphorylase b. After immunoblotting antibodies bound to the 98 (i) and the 59 kD determinant (ii) of the M9 antigen were eluted from the immobilon sheet and retested against the M9 fraction and plasmin-treated phosphorylase b (Ph b*). The eluted antibodies again reacted with the 98 and 59 kD epitopes of these two antigens.

Table 2. Comparison of the specificity of the purified M9 fraction and phosphorylase b for the diagnosis of PBC

	Phosphorylase b			M9-fraction	
Diagnosis	(*n*)	(*n*)	(%)	(*n*)	(%)
Hepatic disorders					
PBC	80	44	55	35	44
Autoimmune CAH (ANA/SMA$^+$)	133	24	18	13	10
Primary sclerosing cholangitis	10	2	20	0	
Acute and chronic hepatitis (HBsAg$^+$)	72	13	18	2	3
Alcoholic liver disease	170	33	19	0	
Wilson's disease	5	1	20	0	
Haemochromatosis	3	0		0	
Cardiac disorder	261	46	18	0	
Connective tissue disorders	111	20	18	4	4
Organ- and non-organ-specific autoimmune disorders	99	22	22	4	4
Inflammatory bowel disorders	115	27	23	0	
Other disorders	210	50	24	0	
Blood donors	100	15	15	0	

Tested were sera from 1269 patients with different hepatic and non-hepatic disorders by ELISA.

1980). We found that after plasmin treatment a degradation product appeared at 59 kD. By Western blotting it could be shown that anti-M9-positive sera recognized both the 98 and the 59 kD epitopes of phosphorylase.

The shared identity of the 98 and 59 kD epitopes of phosphorylase and the M9-related 98 and 59 kD polypeptides could be proven by absorption and elution studies.

Anti-M9-positive sera also reacted with the 98 kD subunit of phosphorylase which had been purified from rat liver. The close association of M9 and phosphorylase was further demonstrated by the presence of phosphorylase enzyme activity in the purified M9 fraction and by experiments showing that both the M9 antigen activity and the phosphorylase enzyme activity could be recovered in a fraction which was obtained after application of the M9 fraction to 5′ AMP affinity column (known to bind NAD$^+$-dependent dehydrogenases and ATP-dependent kinases) and elution with AMP.

The fact that incubation of phosphorylase b with both anti-M9 eluted from the 98 kD and the 59 kD band stimulated the enzyme activity probably indicates that these antibodies have an affinity to the A-5-MP binding site hereby increasing the conversion of inactive phosphorylase b to active phosphorylase a. The fact that the M9 antigen is present in high amounts in liver tissue (Klein & Berg, 1988) also fits well with the postulated association of this antigen with phosphorylase. Thus, glycogen phosphorylase is an enzyme which is involved in intracellular degradation of glycogen and is therefore enriched in liver but also present in skeletal and heart muscle and only in small amounts also in kidney (Proux & Dreyfus, 1973; Proux *et al.*, 1974). The data presented in this paper with respect to the distribution of M9 in different organs are in accordance with the above findings.

Glycogen phosphorylase is a cytoplasmic enzyme which easily binds to particular structures, and this would explain why we could measure phosphorylase enzyme activity in mitochondrial preparations and why M9 copurified with outer mitochondrial membranes (Klein & Berg, 1988). There was also a good correlation between both antigens in detecting anti-M9 antibodies by ELISA. However, 15–20% of sera from healthy controls as well as from patients with non-hepatic disorders reacted with phosphorylase, while only 2% were positive with the M9 fraction. Furthermore, by Western blotting all sera from healthy controls recognized the 98 kD band of phosphorylase from skeletal muscle and also the 59 kD degradation product after plasmin treatment. In contrast, using the M9 fraction, only 10% of normal sera also reacted with an epitope present on the 98 kD determinant but were negative for 59 kD band (data not shown). These data indicate that this 98 kD epitope can be recognized also by naturally occurring antibodies. For the detection of PBC-specific anti-M9 antibodies either by ELISA or Western blotting, the commercially available phosphorylase from skeletal muscle can therefore not be recommended.

These findings strongly indicate that additional epitopes are expressed on the phosphorylase determinants. Taking in account that phosphorylase is an evolutionary highly preserved antigen (Fletterick & Madsen, 1980), it is likely that natural antibodies are continuously produced against this large molecule even in normal individuals (Holmberg & Coutinho, 1985).

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