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Phospholipid epitopes for mouse antibodies against bromelain-treated mouse erythrocytes

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SUMMARY

The reactivity of mouse antibodies against bromelain-treated mouse erythrocytes (BrMRBC) with phospholipid epitopes was assessed by ELISA, using four clones of monoclonal anti-BrMRBC antibodies that had idiotypes distinct from one another. The four antibodies could bind to low-density lipoproteins (LDL) from human and chicken, but not to LDL from mouse and rat. As to liposomes of natural phospholipids, all the clones reacted with liposomes of phosphatidylcholine, and some of them could react with liposomes of sphingomyelin, phosphatidylglycerol, phosphatidylic acid or cardiolipin. For liposomes of synthetic phosphatidylcholine with different fatty acids, the length of carbon chains and the number of unsaturated carbon chains of the fatty acids markedly affected the binding of each monoclonal antibody to the liposomes. The addition of dicetyl phosphate or stearylamine to phosphatidylcholine liposomes changed the reactivity of the liposomes. These results support the view that mouse anti-BrMRBC antibodies can recognize appropriately spaced phosphorylcholine residues on the surface of phospholipid liposomes, LDL and cells. The four clones had similar capacities for binding to LDL as well as to BrMRBC, but they had obviously different capacities for binding to phospholipid liposomes; the epitopes on phospholipid liposomes used in the present study were not so perfect as to react well with every anti-BrMRBC antibody.

INTRODUCTION

Normal mouse sera contain natural antibodies against bromelain-treated mouse erythrocytes (BrMRBC), and normal mouse lymphoid tissues have a large number of anti-BrMRBC B cells that can differentiate to anti-BrMRBC plaque-forming cells (PFC) with polyclonal activation of lipopolysaccharide (LPS) (Cunningham, 1974; Cunningham & Steele, 1981). Phosphatidylcholine, which is one of the membrane phospholipids, has been suggested as the source of molecules that bear the epitopes for anti-BrMRBC antibodies. Linder & Edgington (1973) reported that anti-BrMRBC antibodies reacted with low-density lipoproteins (LDL) from mouse sera. Serban *et al.* (1981) showed that monoclonal anti-BrMRBC antibodies were reactive with trimethylammonium, a moiety present in phosphatidylcholine. Ly-1$^+$ murine B-cell lymphomas with specificity for BrMRBC bound to liposomes of distearoyl phosphatidylcholine (Mercolino, Arnold & Haughton, 1986), and anti-BrMRBC antibodies in mouse sera could bind to dipalmitoyl phosphatidylcholine and phosphatidylcholine from chicken egg yolk (Cox & Hardy, 1985). Every cell has phosphatidylcholine as a basic component of cell membranes, but only a very small portion of cells seem to be reactive with anti-BrMRBC antibodies; the hydrophilic head group of phosphatidylcholine may be reactive only in certain conditions.

We prepared, in a previous investigation, 11 clones of hybridomas secreting anti-BrMRBC antibodies and examined their reactivities with untreated and bromelain-treated erythrocytes from various species of animals (Kawaguchi, Cooper & Kearney, 1986). All the clones showed similar antigen specificities, and it was suggested that mouse anti-BrMRBC antibodies were directed to one epitope and the molecules bearing the epitope were basic components of erythrocyte membranes. Although mouse anti-BrMRBC antibodies exhibited restricted clonal diversities as reported by Poncet *et al.* (1985), we obtained four idiotypically different clones. The idiotypic difference is known to be connected with the difference in fine antigen-binding specificity.

In order to characterize the epitopes further, the reactivities of the four monoclonal anti-BrMRBC antibodies with LDL from various species and liposomes of various phospholipids were assessed in this study. The results show that the epitopes on LDL are so similar to those on BrMRBC as to be almost equally reactive with the four clones, but no phospholipid liposome can bind all the clones equally. It is suggested that the epitopes are composed mainly of appropriately spaced phosphorylcholine residues.

Abbreviations: BrMRBC, bromelain-treated mouse erythrocytes; ELISA, enzyme-linked immunosorbent assay; EyLDL, chicken egg yolk LDL; LDL, low-density lipoprotein; LPS, lipopolysaccharides; PFC, plaque-forming cells.

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MATERIALS AND METHODS

Monoclonal antibodies

Eleven clones of hybridomas secreting anti-BrMRBC antibodies were produced by fusing P3X63-Ag8.653 with LPS-activated spleen and peritoneal cells from unimmunized BALB/c mice (Kawaguchi *et al.*, 1986). The monoclonal antibodies, designated as BrM-1–BrM-11, had an IgMκ isotype and were divided into four groups by rabbit anti-idiotype antibodies. In this study, four idiotypically different clones (BrM-1, BrM-4, BrM-8 and BrM-9) were used. The antibodies were purified from ascites of mice bearing the respective hybridoma.

Preparation of low-density lipoproteins (LDL)

LDL of density between 1·006 and 1·063 g/ml was isolated from fresh mouse, rat, human and chicken serum and chicken egg yolk by the procedure of successive centrifuging at different densities following the method of Hillyard, White & Pangburn (1972).

Materials for phospholipids liposomes

Natural phospholipids from various origins, synthetic L-α-phosphatidylcholine with various fatty acids, cholesterol, dicetyl phosphate and stearylamine were purchased from Sigma (St Louis, MO).

Preparation of liposomes

Five micromoles of phospholipid in either chloroform or chloroform:methanol were transferred to a 15-ml Pyrex conical test tube and the organic solvent was evaporated under a nitrogen stream. Five millilitres of EDTA–saline (0·05% EDTA, 0·9% NaCl) were added to the tube, and lipsomes were created by ultrasonication. In some preparations of liposomes, 5 μmols of choresterol and/or 0·5 μmols of dicetyl phosphate or stearylamine in chloroform were added to the phospholipid solution.

Table 1. Binding of monoclonal anti-BrMRBC antibodies to plates coated with LDL

LDL	Titre (log_2) of binding activity			
	BrM-1	BrM-4	BrM-8	BrM-9
Mouse serum	—	—	—	—
Rat serum	—	—	—	—
Human serum	5	4	6	5
Chicken serum	5	4	6	5
Chicken egg yolk	5	4	6	4

Titration of binding activity of monoclonal anti-BrMRBC antibodies by enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Nunc, Roskilde, Denmark) were coated with 0·1 ml/well of LDL (0·05 mg/ml) or phospholipid liposomes (0·05 mM of phospholipid) in EDTA–saline for 2 hr at room temperature, and unbound sites were blocked with 1% bovine serum albumin in phosphate-buffered saline (pH 7·2). To the wells, 0·1 ml of serial two-fold dilutions of monoclonal antibodies (4 μg/ml) was added. After incubation for 3 hr at 37°, the plates were washed, and 0·1ml/well of peroxidase-labelled anti-mouse μ-chain antibodies was added to detect bound monoclonal antibodies. The plates were incubated at 4° overnight, then washed again and 0·2 ml of substrate solution added. Thirty minutes later the reaction was stopped with 0·05 ml of 1 M NaF solution. The absorbance at 415 nm was measured by an ELISA reader. The maximum of the dilutions (log_2) that produced absorbance at 415 nm over 0·1 was shown as the titre. Peroxidase-labelled anti-mouse μ-chain antibodies were prepared by conjugation of affinity-purified rabbit anti-mouse μ-chain antibodies with peroxidase (Grade I; Boehringer, Mannheim, FRG) according to the method of Nakane & Kawaoi (1974). As a substrate, ABTS (Wako, Osaka) was used.

Competitive ELISA of monoclonal anti-BrMRBC antibodies

To 0·15 ml of monoclonal anti-BrMRBC antibodies (1 μg/ml), 0·15 ml of phospholipid liposomes (0·1 mM phospholipid) was added as inhibitor. After incubation at 37° for 2 hr, 0·1 ml of the mixture was added in duplicate to the wells of the plates coated with LDL from chicken egg yolk (EyLDL). The plates were incubated at 37° for 3 hr, and then treated using the same procedures as for the titration. The inhibition of binding of monoclonal antibodies to EyLDL-coated plates by a variety of synthetic phosphatidylcholines at various concentrations (100, 10, 1, 0·1, and 0·01 μM) was also assayed by the above procedures. Percentage inhibition was calculated as follows:

$$100 - \frac{\text{absorbance at 415 nm of the well with the inhibitor}}{\text{absorbance at 415 nm of the well without the inhibitor}} \times 100.$$

RESULTS

Binding of monoclonal anti-BrMRBC antibodies to lipoproteins

The binding of monoclonal anti-BrMRBC antibodies to LDL was assessed by an ELISA in which the ELISA plates were coated with purified LDL (Table 1). None of the clones could bind to mouse and rat serum LDL. All clones could bind to LDL from human serum, chicken serum and chicken egg yolk. There was no great difference in capacity for reacting with anti-BrMRBC antibodies among the three preparations of LDL or in capacity for binding to LDL among the four clones.

Binding of monoclonal anti-BrMRBC antibodies to phospholipid liposomes

Various natural phospholipids were made into liposomes, and the binding of anti-BrMRBC antibodies to the liposome-coated plates was assayed (Table 2). The antibodies could bind to liposomes of choline-bearing phospholipids, phosphatidylcholine from animal cells or sphingomyelin from chicken egg yolk.

The antibodies could also bind to liposomes of synthetic phosphatidylcholine (Table 3). BrM-1 and BrM-9 were highly reactive with liposomes of dipalmitoyl, distearoyl, or diarachidoyl phosphatidylcholine, but barely bound to plates treated with liposomes of phosphatidylcholine with unsaturated fatty acids or saturated fatty acids with shorter carbon chains. BrM-8 bound fairly well to liposomes of dipalmitoyl, distearoyl and

Table 2. Binding of monoclonal anti-BrMRBC antibodies to plates coated with liposomes of natural phospholipids

Phospholipids	Origin	Titre (log_2) of binding activity			
		BrM-1	BrM-4	BrM-8	BrM-9
Phosphatidylcholine	Chicken egg yolk	1	—	3	0
	Bovine heart	3	0	3	2
	Bovine brain	6	0	4	3
	Soybean	—	—	—	—
Phosphatidylglycerol	Chicken egg yolk	—	—	—	—
Phosphatidylic acid	Chicken egg yolk	—	—	—	—
Lysophosphatidylcholine	Chicken egg yolk	—	—	—	—
Sphingomyelin	Chicken egg yolk	6	—	—	4
Cardiolipin	Bovine heart	—	—	—	—

Table 3. Binding of monoclonal anti-BrMRBC antibodies to plates coated with liposomes of synthetic phosphatidylcholine

L-α-phosphatidylcholine	Titre (log_2) of binding activity			
	BrM-1	BrM-4	BrM-8	BrM-9
Didecanoyl (10:0)*	—	—	—	—
Dilauroyl (12:0)	—	—	—	—
Dimyristoyl (14:0)	—	—	—	—
Dipalmitoyl (16:0)	7	—	3	5
Distearoyl (18:0)	8	—	5	7
Diarachidoyl (20:0)	10	—	7	9
Dioleoyl (18:1)	—	—	—	—
Dilinoleoyl (18:2)	—	—	—	—

* The numbers in the parentheses showed the length of carbon chains: the number of unsaturated carbon chains in the fatty acids.

Table 4. Binding of monoclonal anti-BrMRBC antibodies to plates coated with liposomes

Lipsomes	Titre (log_2) of binding activity			
	BrM-1	BrM-4	BrM-8	BrM-9
DPPdC*	7	—	3	5
DPPdC+DCP†	7	1	—	4
DPPdC+SA‡	7	1	6	6
DPPdC+Ch§	6	—	4	4
DPPdC+Ch+DCP	5	2	—	3
DPPdC+Ch+SA	6	0	6	6

Dipalmitoyl phosphatidylcholine.
† Dicetyl phosphate.
‡ Stearylamine.
§ Cholesterol.

diarachidoyl phosphatidylcholine. BrM-4 could not bind to any of the liposomes examined.

To make phospholipid liposomes stable or ionic, cholesterol, dicetyl phosphate or stearylamine has often been used. The effects of the addition of cholesterol, dicetyl phosphate or stearylamine to dipalmitoyl phosphatidylcholine are shown in Table 4. Cholesterol reduced or only slightly increased the binding of the antibodies. Dicetyl phosphate reduced to a fair degree the binding of BrM-8, and to a slight extent the binding of BrM-1 and BrM-9, but it augmented the binding BrM-4. Stearylamine increased, more or less, the binding of all the clones. It is noteworthy that BrM-4 and BrM-8 obviously differ from BrM-1 and BrM-9 in their capacity for binding to phospholipid liposomes.

Inhibition of binding of monoclonal anti-BrMRBC antibodies to EyLDL by LDL and phospholipid liposomes

In the above ELISA, a variety of phospholipid liposomes was added to the plates. It was, however, possible that they differed in the efficiency of their adhesion to the plates, and the amounts of some liposomes coating the plates were insufficient for detecting bound anti-BrMRBC antibodies. The reactivities, therefore, of anti-BrMRBC antibodies with phospholipid liposomes were assessed by competitive ELISA using plates coated with EyLDL.

The result of the inhibition by natural phospholipid liposomes is shown in Table 5. The binding of every clone to EyLDL was inhibited by liposomes of phosphatidylcholine regardless of origin. Some of the clones were reactive with liposomes of choline-lacking phospholipids such as phosphatidylglycerol, phosphatidylic acid, and cardiolipin; on the other hand, liposomes of lysophosphatidylcholine could not inhibit the binding of any clone. The results in Table 5 are considerably different from the results in Table 2. This can be explained in terms of the differences in the efficiency of the adhesion to plates among various phospholipid liposomes in Table 2, or in the degree of efficiency to detect antibodies of weak affinity between the techniques used in Table 2 and Table 5.

The detailed profiles of inhibition by liposomes of synthetic phosphatidylcholine with various fatty acids were assessed. No dose of liposomes of phosphatidylcholine with fatty acids with less than 10 carbon chains was able to inhibit the binding of any

Table 5. Inhibition of binding of monoclonal anti-BrMRBC antibodies to EyLDL-coated plates by liposomes of natural phospholipids

Phospholipids	Origin	BrM-1	BrM-4	BrM-8	BrM-9
Phosphatidylcholine	Chicken egg yolk	+*	+	+	+
	Bovine heart	+	+	+	+
	Bovine brain	+	+	+	+
	Soybean	+	+	+	+
Phosphatidylglycerol	Chicken egg yolk	+	−	+	+
Phosphatidylic acid	Chicken egg yolk	−	−	+	+
Lysophosphatidylcholine	Chicken egg yolk	−	−	−	−
Sphingomyelin	Chicken egg yolk	+	−	−	+
Cardiolipin	Bovine heart	+	−	−	+

* More than 50% inhibition is shown as positive inhibition (+) and less than 50% as negative inhibition (−).

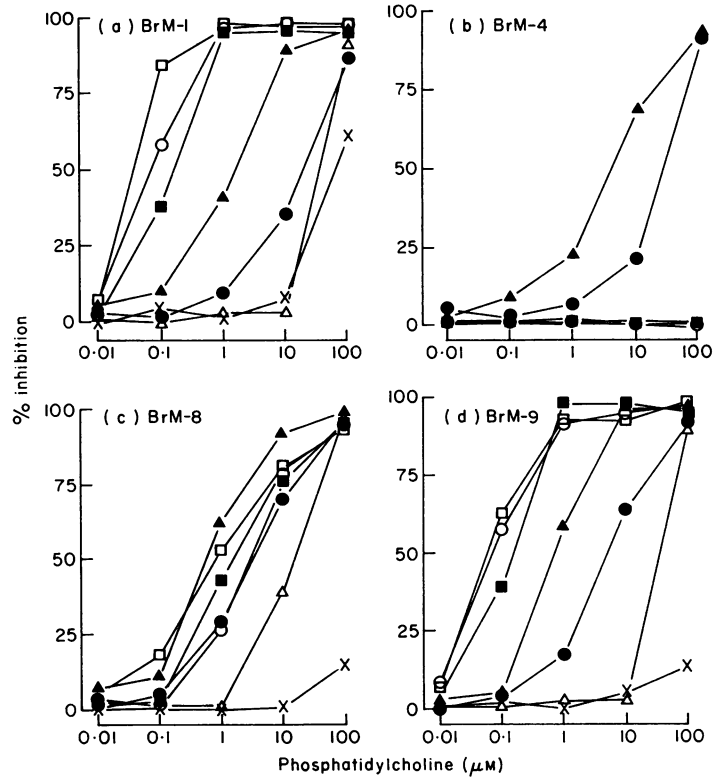

Figure 1. Inhibition of binding of monoclonal anti-BrMRBC antibodies to EyLDL-coated plates by liposomes of synthetic phosphatidylcholine: L-α-phosphatidylcholine, dilauroyl, (X); dimyristoyl, (△); dipalmitoyl, (○); distearoyl, (□); diarachidoyl, (■); dioleoyl, (▲); and dilinoleoyl, (●).

clones (data not shown). Figure 1 clearly demonstrates the differences in the reactivity among BrM-1, BrM-4 and BrM-8, although BrM-1 and BrM-9 showed a rather similar reactivity. BrM-1 and BrM-9 could react strongly with liposomes of phosphatidylcholine with fatty acids with longer saturated carbon chains, and they could react moderately with liposomes of phosphatidylcholine with fatty acids with unsaturated carbon chains. BrM-8 could react almost equally with liposomes of a variety of phosphatidylcholines with fatty acids with saturated or unsaturated longer carbon chains. BrM-4 was reactive only with liposomes of phosphatidylcholine with unsaturated carbon chains. The four clones are similar in their profiles of inhibition by liposomes of dioleoyl or dilinoleoyl phosphatidylcholine.

DISCUSSION

Linder & Edgington (1973) reported that anti-BrMRBC antibodies from the serum of NZB mice made a precipitin line with mouse LDL. They suspected that the antigen molecules on the mouse LDL were probably not lipid or aproprotein, but

substances lipophilic in the serum or molecules on a small population of LDL. Our monoclonal anti-BrMRBC antibodies, which were produced by hybridomas of LPS-activated cells from healthy BALB/c mice, also made a precipitin line with mouse LDL at 4°, but the line disappeared at room temperature (unpublished observation). Further, their binding to mouse LDL could not be detected by ELISA (Table 1) and mouse LDL could not inhibit the binding of the four monoclonal antibodies to EyLDL (data not shown). They may have weak affinities to mouse LDL.

All or none of the four clones could react with each of the LDL from various species (Table 1), as well as each of the untreated and bromelain-treated erythrocytes from various species (Kawaguchi *et al.*, 1986). These findings suggest that all of the four clones are directed to the same epitope, and the epitope on the LDL is identical to that on the erythrocytes. The similarity of the identity of the epitope on EyLDL with that on BrMRBC is also supported by the following observations: the plaque formation by anti-BrMRBC PFC in normal, LPS-activated mice can be inhibited completely by the addition of EyLDL, and the injection of EyLDL can induce a specifically anti-BrMRBC PFC response in mice (S. Kawaguchi, manuscript in preparation). The molecules bearing the epitope are probably basic components of erythrocyte membranes and LDL, suggesting that they may be phospholipids.

Since Serban *et al.* (1981) observed a weak reactivity of monoclonal anti-BrMRBC antibodies with choline, phosphatidylcholines on the surface of cell membranes have been considered to be molecules bearing the epitope for anti-BrMRBC antibodies (Mercolino *et al.*, 1985; Cox & Hardy, 1985). Our anti-BrMRBC antibodies could best bind to liposomes of phosphatidylcholine (Table 2). They could not, however, react with liposomes of phosphatidylcholine with fatty acids with shorter carbon chains (Fig. 1). The binding of anti-BrMRBC antibodies was greatly affected by the number and saturation of carbon chains of fatty acids of phosphatidylcholine. The distances between the head groups on the surface of liposomes of phosphatidylcholine depend on the carbon chains of fatty acids (Janiak, Small & Shipley, 1979; Cornell & Separovic, 1983). Therefore, the anti-BrMRBC antibodies are probably reactive with phosphorylcholine residues present at definite intervals on the surface of phospholipid liposomes.

As to the antibodies reactive with phospholipids, the cross-reactivity between DNA and cardiolipin is known (Guarnieri & Eisner, 1974). Lafer *et al.* (1981) reported that mouse monoclonal anti-DSNA autoantibodies were reactive with liposomes of various phospholipids. Their antibodies could react strongly with liposomes of cardiolipin and phosphatidylic acid, and weakly with liposomes of phosphatidylglycerol, but barely at all with liposomes of phosphatidylcholine. They suggested that their antibodies were directed to the regularly spaced phosphate groups, and that the positively charged groups of phosphatidylcholine may interfere with the binding of the antibodies to the phosphate groups. Some clones of our monoclonal anti-BrMRBC antibodies were also reactive with liposomes of phosphatidylglycerol, phosphatidylic acid and cardiolipin (Table 5). None of them, however, could react with plates coated with double- or single-stranded DNA, and neither of these DNA preparations could inhibit the binding of any clone to EyLDL (data not shown). It might be reasonable to suggest that both anti-DNA and anti-BrMRBC antibodies recognize spaced phosphate residues, but only when they are at distinct intervals. Another close relationship between both groups of antibodies was reported by Hayakawa *et al.* (1984); they found that both were produced by Ly-1$^+$ B cells.

We have 11 monoclonal anti-BrMRBC antibodies, and they are idiotypically grouped as [(BrM-1), (BrM-9)] [(BrM-4), (BrM-2, BrM-3, Brm-4, BrM-5, BrM-6, BrM-7, BrM-8, BrM-10, BrM-11)] (Kawaguchi *et al.*, 1986). BrM-9 shares a cross-reactive idiotype with BrM-1 and has another idiotype distinct from other antibodies. BrM-4 shares a cross-reactive idiotype with another eight antibodies and has another idiotype distinct from them. The seven clones that have identical idiotypes with BrM-8 were similar to BrM-8 in their reactivity with erythrocytes (Kawaguchi *et al.*, 1986) and LDL and phospholipid liposomes (data not shown). The four clones, which have idiotypes distinct from one another, could react almost equally with LDL (Table 1) and erythrocytes (Kawaguchi *et al.*, 1986) from various species, but none of the phospholipid liposomes used in this study could bind the four clones equally well. These findings reveal the difference in fine antigen-binding specificity among the four clones, and suggest that the epitopes on the phospholipid liposomes are not sufficiently perfect to bind every clone. Thus, it can be proposed that anti-BrMRBC antibodies are somewhat heterogeneous in their fine antigen-binding specificity. All of them recognize an identical epitope on BrMRBC and EyLDL; the epitope is mainly composed of phosphorylcholine groups at definite intervals.

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