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MEMBRANE LIPID COMPOSITION MODULATES THE BINDING SPECIFICITY OF A MONOCLONAL ANTIBODY AGAINST LIPOSOMES

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A hybridoma secreting a monoclonal IgM 'anti-liposome' antibody was produced after injecting a mouse with liposomes containing dipalmitoylphosphatidylcholine, cholesterol, dicetyl phosphate, and lipid A. The antibody was selected by assaying for complement-dependent damage to liposomes lacking lipid A. The monoclonal antibody reacted best with liposomes containing the original immunizing mixture of lipids. Deletion of individual lipid consituents from liposomes diminished the ability of the liposomes to bind (adsorb) the antibody. Binding of the antibody was enhanced by including lipid A or galactosylceramide in the lipid bilayer, or by substituting egg phosphatidylcholine for dimyristoyl- (or dipalmitoyl-) phosphatidylcholine. Sphingomyelin could be substituted for dimyristoylphosphatidylcholine without altering the adsorption of antibody. Although the monoclonal anti-liposome antibody was completely inhibited by phosphocholine, it was probably not a conventional anti-phosphocholine antibody. The antibody apparently had a partial specificity for phosphate, and was inhibited by glycerophosphocholine, glycerophosphate, sodium phosphate, sodium sulfate, and inositol hexaphosphate, but not by choline or inositol.

Introduction

In the previous papers in this series, we have demonstrated that liposomes containing lipid A [1–3] or even lipid A in the absence of liposomes [4] induced 'anti-liposome' antibodies having specificity for liposomes containing phosphatidylcholine or sphingomyelin, and inhibitable by high concentrations of phosphocholine. Antibodies against lipid A also were induced in rabbits [1,2,4], but anti-lipid A antibodies have not been found in mice [3]. Low titers of activities against cholesterol and dicetyl phosphate were detected in a solid-phase radioimmunoassay [2].

Several questions have been raised by our studies on anti-liposome antibodies. Are specific antibodies induced against liposomes? Or, is the titer of naturally-occurring anti-phospholipid antibodies nonspecifically increased by mitogenic effects of lipid A? Are the apparent binding specificities of antibodies in anti-liposome antisera due to the presence of a multitude of antibody specificities against individual lipid constituents? Profound effects on complement fixation were caused by inclusion of ceramide, glycosylceramide, or lipid A in the liposomes [4]. Could there be additional effects due to influences of liposomal membrane

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine.

lipids on the expression of 'anti-liposome' specificity? At least partial answers to the above questions were obtained in the present study by examining the specificity of a hybridoma monoclonal anti-liposome antibody.

Materials and Methods

Liposomes. Complete details of sources of lipids and liposome preparation have been presented elsewhere [1,5]. Chloroform-soluble lipid A was employed [6]. The liposomes consisted of dimyristoylphosphatidylcholine (DMPC), cholesterol, and dicetyl phosphate in molar ratios of 2:1.5:0.22. When lipid A was incorporated in the liposomes used for glucose release, it was in a concentration of 12.5 nmol of phosphate per μmol of phosphatidylcholine. When galactosylceramide was incorporated in the liposomes, it was in a concentration of 150 μg per μmol of phosphatidylcholine.

Immunological assays. Complement-dependent immune damage to liposomes resulting in release of trapped glucose has been described elsewhere [5].

The solid-phase radioimmunoassay employed for measuring antibodies against individual lipid antigens has been used previously by us [2] and is based on the techniques described by Zollinger et al. [7]. In brief, lipid antigen was coated on the bottom of the wells of plastic microtiter plates. Dilutions of culture fluid containing hybridoma antibodies were added and washed away, and ^{125}I-labelled anti-mouse globulin was added and removed. The wells were then cut out and counted. Controls consisted of assays run in the absence of culture fluid, or in the presence of culture fluid lacking hybridoma protein. A major drawback of the solid-phase radioimmunoassay from the standpoint of measuring antibodies against lipids is that low affinity antibodies, which are commonly encountered against lipids, are easily removed from the microtiter wells during washing, and are measured poorly or not at all. Because of this, low affinity antibodies that give positive results as measured by the technique of complement-dependent glucose release from liposomes, may give equivocal or even negative results by radioimmunoassay. A similar type of solid-phase analysis for lipids has been described recently by Smolarsky [8].

Inhibitors. Inhibitors were purchased from the following sources: phosphocholine chloride (calcium salt) (Sigma Chemical Co., St. Louis, MO); glycerophosphate (sodium salt) (Calbiochem, La Jolla, CA); choline (chloride) (Mann Research Laboratories, Inc, New York); inositol hexaphosphate (sodium salt), inositol (Sigma Chemical Co., St. Louis, MO). Glycerophosphocholine was obtained as the water soluble de-esterification product of DPPC, obtained by the method of Ballou et al. [9].

Immunization of mouse. A single Balb/c mouse (Jackson Laboratories, Bar Harbor, ME) was injected i.v. with 40 μl of liposomes consisting of DPPC/cholesterol/dicetyl phosphate (molar ratio 1:1.5:0.22). The liposomes also contained lipid A in a concentration of 40 nmol of lipid A phosphate per μmol of DPPC. Three days after injection, a serum sample was taken and the animal was killed and the spleen removed.

Cell fusion. Hybridization of spleen cells to myeloma cell line P3-X63-Ag8 was performed. Preparation of spleen cells for hybridization, cell fusion, cloning, and hybridoma cryopreservation employed the methods given in detail by Kennett et al. [10]. As described in Ref. 10, the culture fluid contained Dulbecco's modification of Eagle's medium, medium NCTC-109, oxalacetate, pyruvate, beef insulin, hypoxanthine, thymidine, gentamycin, and 20% fetal calf serum. Hybridomas were derived from the fusion of $2 \cdot 10^8$ spleen cells with $2 \cdot 10^7$ myeloma cells taken from cultures at a cell density of $6.5 \cdot 10^5$ cell per ml. After fusion, cells were distributed into a 96-well tissue culture plate (Costar) at $4 \cdot 10^5$ cells per well. The hybridoma selected for cloning was identified by assaying the culture fluids of 75 wells for complement-dependent immune damage to liposomes consisting of DMPC/cholesterol/dicetyl phosphate. Out of five possible candidates, one was selected and the rest were discarded. The hybridoma antibody selected was an IgM. The myeloma cell line itself is an IgG secretor, but all of the anti-liposome activity was eliminated by mercaptoethanol treatment. The hybridoma antibody in culture supernatant fluids bound readily to glass surfaces, and loss of activity was diminished when dilution of culture super-

natant was performed in the presence of 1% bovine serum albumin.

Ascites fluids. Ascites fluids were drawn from pristane-primed Balb/c mice that had been intraperitoneally injected with 10^6 cloned hybrid cells, and the ascites fluids were pooled together, heated at 56°C for 30 min, and dialyzed against 0.15 M NaCl. The pooled ascites fluid protein consisted of intraperitoneal proteins, IgG secreted by the myeloma cell line, and IgM anti-liposome antibody, and the total protein concentration (Lowry assay with human Cohn fraction 2-γ-globulin standard) was 24.5 mg/ml.

Results

Anti-liposome activities

Serum from the mouse that was immunized to produce the hybridoma gave the activity shown in Fig. 1. Glucose release clearly occurred from liposomes comprised of DMPC/cholesterol/dicetyl phosphate. Glucose release was higher from liposomes containing lipid A; and as described previously with rabbit anti-liposome serum [4], glucose release was higher when the liposomes contained galactosyl ceramide. The higher glucose release when galactosylceramide was present was not caused by antibodies against galactosylceramide, but rather was due to membrane changes induced by galactosylceramide that influenced complement-dependent glucose release [4].

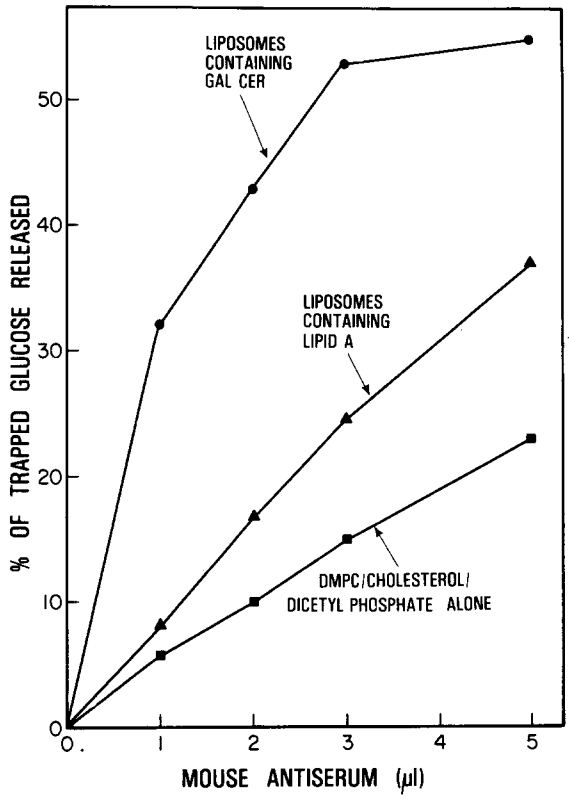

Fig. 1. Immunological activities of mouse anti-liposome serum. Serum was obtained from the mouse three days after immunization, and tested against liposomes having the indicated lipid compositions, Gal Cer, galactosylceramide.

The pattern shown in Fig. 1 was duplicated by the hybridoma immunoglobulin (Fig. 2), and all of the activity was inhibited by phosphocholine. Absence of a separate anti-lipid A specificity in the ascites fluid is demonstrated by the observation that the activity against liposomes containing lipid A was removed by adsorbing the ascites fluid with liposomes lacking lipid A (Fig. 3) (also, vida infra).

Inhibition by phosphocholine

The inhibiting effect of phosphocholine mentioned above (Fig. 2) is shown in more detail in Fig. 4. In the concentrations shown, the original mouse antiserum was not inhibited by phosphocholine, and the antiserum activity actually was enhanced. The observation of enhanced activity in the presence of phosphocholine has been made previously both with mouse and with rabbit anti-liposome antisera [3,4]. The influence of phosphocholine (i.e., inhibition or enhancement) differs, depending on several factors, including the individual animal, the concentration of antiserum, and the concentration of phosphocholine [4]. Enhancement, when it occurred, presumably was due to nonspecific binding of phosphocholine to liposomes, resulting in an increased hapten density and increased antibody binding [4], while inhibition was due to fluid phase blocking of antibody. The antiserum activity represents an average of numerous individual antibodies, some of which may be enhanced, and some of which may be inhibited by phosphocholine. As noted in Fig. 2 and shown in more detail in Fig. 4, under the conditions used the monoclonal antibody was partially inhibited by as little as 0.2 mM phosphocholine, and was completely inhibited by 1.5 mM phosphocholine.

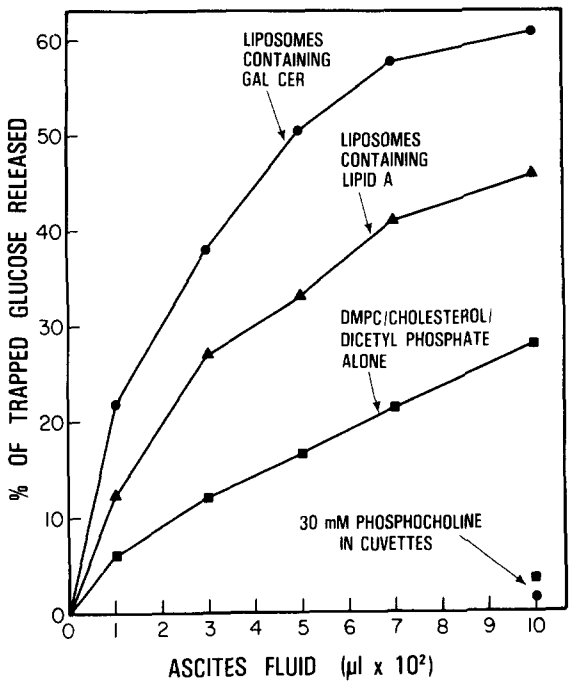

Fig. 2. Immunologic activities of the hybridoma anti-liposome antibody. Ascites fluid containing the hybridoma antibody originally derived from the mouse shown in Fig. 1 was tested against liposomes containing the indicated lipid compositions. For convenience, the ascites fluid was diluted 1:100 with 0.15 M NaCl before assay, and the values shown are corrected for dilution. Gal Cer, galactosylceramide.

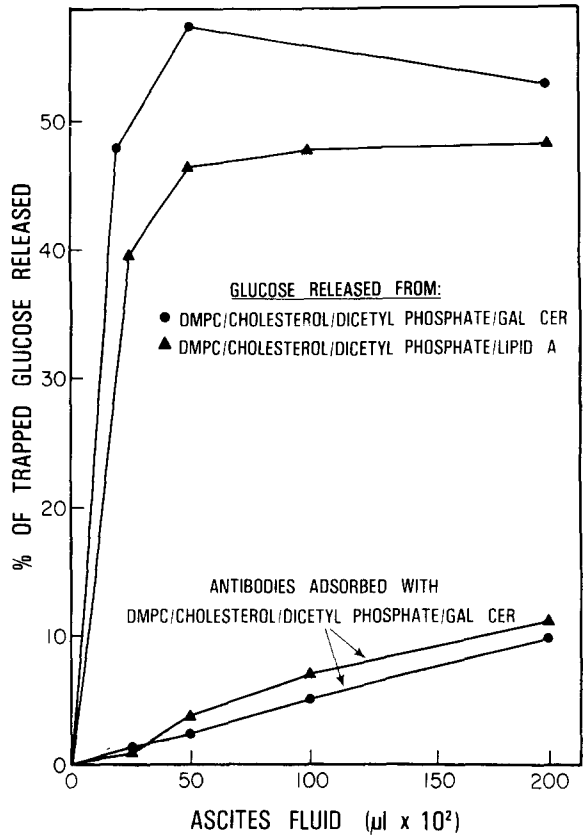

Fig. 3. Adsorption of hybridoma antibodies by liposomes lacking lipid A. Adsorption was performed by incubating 100 µl of a 1:100 dilution (in 1% bovine serum albumin in 0.15 M NaCl) of ascites fluid with 50 µl of liposomes for 30 min at room temperature. The liposomes were removed by centrifuging at 27000× *g* for 10 min. 50 µl of fresh liposomes were added to the supernatant, and the process was repeated. The second supernatant was diluted by adding 300 µl of bovine serum albumin in saline. The unadsorbed ascites fluid was diluted with an appropriate quantity of bovine serum albumin in saline. All the data shown are corrected for dilution. Gal Cer, galactosylceramide.

Influence of lipid composition on binding of the hybridoma immunoglobulin

In previous work, we found that deletion of one or more individual lipid constituents diminished the ability of liposomes to serve as immunoadsorbents of anti-liposome sera [1–3]. This could have been due to the presence of antibodies in the antiserum that recognized individual liposome constituents. Alternatively, deletion of individual lipids may have altered the 'membrane pattern' and led to decreased recognition and binding affinity of anti-liposome antibodies. Although multiple anti-lipid specificities due to different antibodies in anti-liposome serum cannot be excluded, and in fact have been observed [2], deletion of lipid constituents did diminish binding to liposomes by the monoclonal hybridoma anti-liposome antibody. As shown in Fig. 5, DMPC, DMPC/cholesterol, or DMPC/dicetyl phosphate adsorbed only a slight amount of hybridoma antibody, while much more was adsorbed by DMPC/cholesterol/dicetyl phosphate and much more yet by DMPC(or DPPC)/cholesterol/dicetyl phosphate/lipid A. The latter liposomes contained the lipid composition used for immunizing the mouse. Galactosylceramide, which was not a constituent of the immunizing liposomes, also enhanced antibody binding, although not as much as lipid A (Fig. 5).

Lipid A also greatly enhanced the binding ability of liposomes when sphingomyelin was sub-

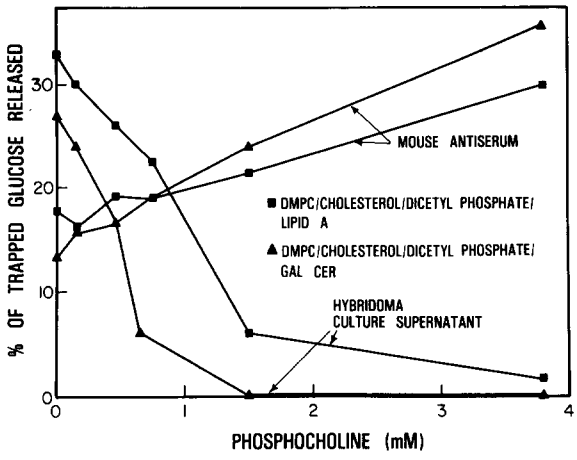

Fig. 4. Inhibition by phosphocholine. 5 μl or 7 μl of cloned hybridoma culture supernatant were present at each assay point, respectively for liposomes containing lipid A or galactosylceramide (Gal Cer).

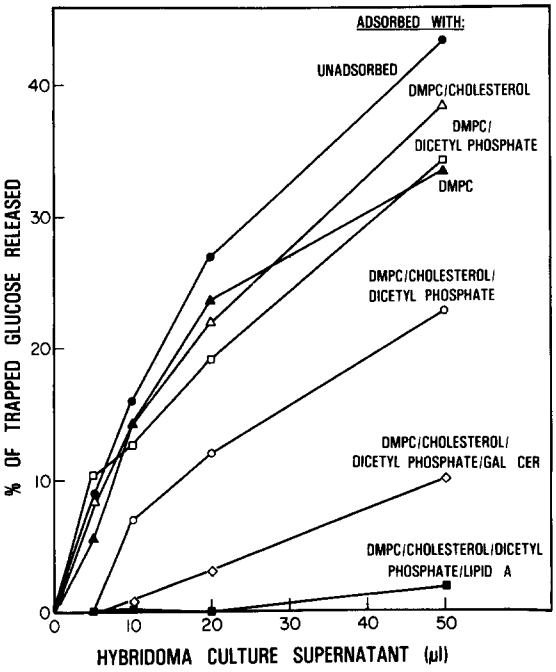

Fig. 5. Influence of liposomal lipid composition on binding of hybridoma antibody. The cloned hybridoma culture supernatant was adsorbed once by incubating 100 μl of culture fluid with 50 μl of liposomes (or 0.15 M NaCl for the unadsorbed control) for 30 min at room temperature. The liposomes were removed by centrifuging at 27000$\times g$ for 10 min. For convenience, the supernatant was diluted with 350 μl of 0.15 M NaCl. The values shown are corrected for dilution. Gal Cer, galactosylceramide.

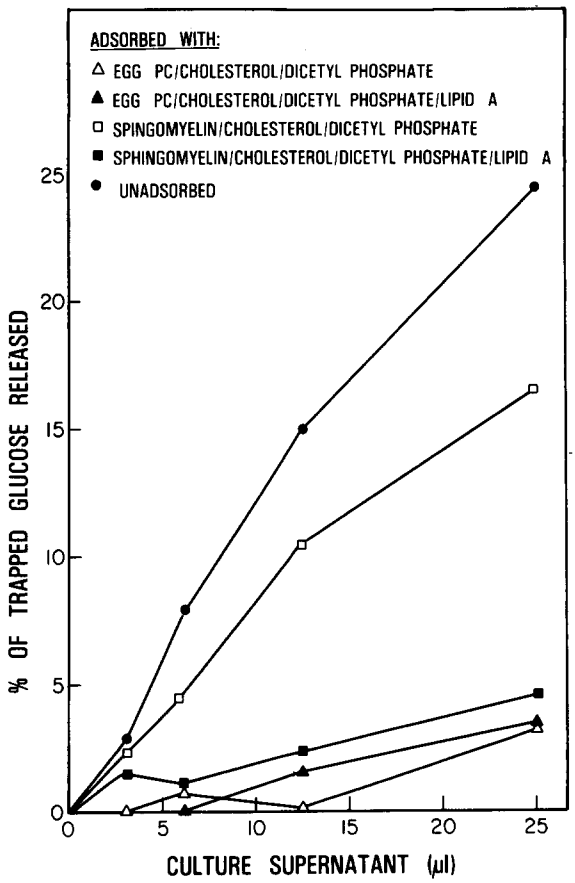

Fig. 6. Influence of phospholipid and lipid A on binding of hybridoma anti-liposome antibody. Adsorption was performed as described in the legend of Fig. 5, except that 50 μl of culture fluid and 50 μl of 0.15 M NaCl were incubated with 50 μl of liposomes.

stituted for DMPC in liposomes used for adsorption (Fig. 6). Fig. 6 also shows that liposomes containing egg PC, even without lipid A, were approximately equivalent in binding capacity to those containing either DMPC plus lipid A, or sphingomyelin plus lipid A. These results suggest that the effects of lipid A might have been due at least partially to changes of membrane fluidity.

Although anti-cholesterol and anti-dicetyl phosphate antibodies have been observed by us previously in rabbit anti-liposome serum [2], such activities were not found in the anti-liposome hybridoma (Table I). The solid phase radioimmunoassay failed to show any activity against DPPC, cholesterol, dicetyl phosphate or lipid A

TABLE I

SOLID-PHASE RADIOIMMUNOASSAY OF BINDING OF HYBRIDOMA ANTIBODY TO LIPOSOMES AND INDIVIDUAL LIPOSOME CONSTITUENTS

Microtiter wells were coated, as appropriate, with: 1.25 nmol of either DPPC, cholesterol, or dicetyl phosphate; or 1.25 nmol of lipid A phosphate; or with liposomes containing 1.25 nmol of DPPC, 0.94 nmol of cholesterol, 0.14 nmol of dicetyl phosphate, and 0.025 nmol of lipid A phosphate. The latter liposomes had the same lipid composition as those used for immunizing the mouse from which the hybridoma was derived. The culture fluid containing the hybridoma antibody was compared with an identical culture fluid (control) lacking the hybridoma protein.

Antigen	Culture fluid	cpm		
		No culture fluid	Reciprocal of culture fluid dilution	
			125	5
DPPC	Control	210	125	310
	Hybridoma	92	109	197
Cholesterol	Control	73	150	145
	Hybridoma	72	78	235
Dicetyl phosphate	Control	321	200	313
	Hybridoma	268	269	308
Lipid A	Control	281	209	252
	Hybridoma	283	417	324
DPPC/cholesterol/dicetyl phosphate/lipid A	Control	134	136	102
	Hybridoma	159	5293	9327

separately, but activity was observed when all of these constituents were combined in liposomes (Table I). It should be pointed out that any low affinity antibody binding might be missed in the solid phase radioimmunoassay technique [2,8]. The observations in Table I are compatible either with the concept that the antibody combining site is sufficiently large to include all of the lipid constituents in a 'membrane pattern', or with the concept that binding of antibody to DPPC is strongly influenced by other liposomal lipids.

Specificity for inhibitors

What is the minimal chemical structure that can inhibit the hybridoma anti-liposome antibody? Fig. 7 shows that the antibody was inhibited by every phosphorylated or sulfated molecule tested, including Na_2HPO_4 and Na_2SO_4. The best inhibition occurred with phosphocholine. Glycerophosphocholine, although not as effective as phosphocholine, was superior to glycerophosphate and phosphate alone (Fig. 7). Phosphocholine inhibition was more than twenty times greater than that caused by phosphate alone (Fig. 7). Although not

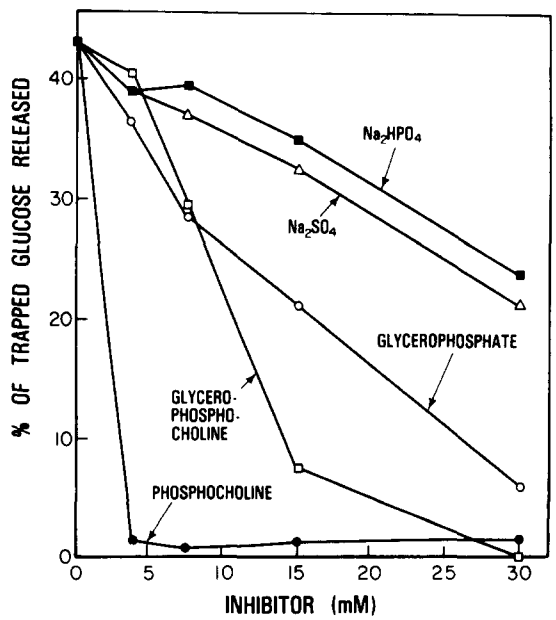

Fig. 7. Effects of inhibitors on complement-dependent glucose release in the presence of the hybridoma anti-liposome antibody. 5 μl of 1 : 100 diluted ascites fluids were present at each point. Isotonic solutions of inhibitors were used, and each replaced an identical volume of 0.15 M NaCl in the glucose assay reagents.

shown, inhibition was not observed with choline; and inhibition was observed with inositol hexaphosphate, but not with inositol.

Discussion

Our results demonstrate that a hybridoma monoclonal anti-liposome antibody had nearly all of the specificity and binding characteristics previously displayed by rabbit anti-liposome sera antibodies [1,2,4]. With regard to specificity, the activities of rabbit and mouse antibodies [1,3,4] and the activity of the mouse hybridoma antibody (Figs. 2, 4 and 7) were completely inhibited by phosphocholine. It should be noted that the antiserum of the mouse from which the hybridoma was derived was not inhibited, and in fact was enhanced, by phosphocholine (Fig. 4). Lack of inhibition, or enhancement, by phosphocholine has been observed previously by us with serum from certain individual rabbits or mice immunized with liposomes containing lipid A [3,4]. Enhancement of anti-liposome activity, when it occurred, presumably was due to binding of phosphocholine to liposomes, resulting in increased hapten density and increased anti-liposome binding [4]. Our observation that the hybridoma antibody was inhibited by phosphocholine while the antiserum was enhanced by phosphocholine confirms our previous suggestion that the antiserum activity is an average of numerous anti-phosphocholine antibodies having a variety of affinities for phosphocholine and for liposomes [4].

It should be pointed out that the adsorption data that were used as evidence of antibody specificity did not reflect a general nonspecific binding capability of immunoglobulins to liposomes. In previous studies (see, for example, Ref. 11) with recognized antigens in liposomes, antibody binding was tested by leaving the antigen out of the liposomes, and nonspecific binding was not observed. In other experiments in which mice were immunized with liposomes containing lipid A we found that IgM anti-liposome antibodies had a high degree of specificity [3]. The mouse IgM antibodies were absorbed by liposomes containing DMPC(or DPPC)/cholesterol/dicetyl phosphate, but were not adsorbed by dipalmitoylphosphatidylethanolamine/cholesterol/dicetyl phosphate [3].

The adsorption data in this study also did not reflect general nonspecific binding properties of monoclonal IgM molecules. We previously showed that a monoclonal human IgM (Waldenström macroglobulin) having specificity for Forssman glycolipid in liposomes did not react with liposomes lacking Forssman [12]. In addition, in separate experiments, we have also examined five mouse IgM monoclonal antibodies against irrelevant (i.e., nonliposomal) parasitic and bacterial antigens, and we failed to find nonspecific activity against liposomes.

In previous studies, a glycosylceramide, such as galactosylceramide, which was not a constituent of the immunizing liposomes, markedly enhanced complement-dependent glucose release in the presence of sera from immunized rabbits [4]. The same enhancing effect of galactosylceramide was observed on the activities of both mouse antiserum (Fig. 1) and the hybridoma antibody (Fig. 2). The glycosylceramide exerted its effect, at least partially, by increasing the binding of the hybridoma antibody (Fig. 5). Previously, ceramide itself did not increase rabbit antibody binding, but did enhance complement-dependent damage in the presence of rabbit anti-liposome antibodies [3]. It therefore appears that ceramide, or glycosylceramide, can influence either binding or complement damage initiated by anti-liposome antibodies. The specificity of the hybridoma antibody strongly suggests that it arose as a result of immunization, rather than by nonspecific stimulation of natural antibodies. The relative degree of activity was greatest against liposomes containing all of the lipids that comprised the liposomes used for immunization. Activity diminished markedly when individual lipid constituents were omitted from liposomes used for adsorption (Fig. 5).

By using a solid-phase radioimmunoassay, we demonstrated previously that rabbit anti-liposome serum contained detectable, but very low level, antibody activities against cholesterol and dicetyl phosphate [2]. Such anti-cholesterol or anti-dicetyl phosphate specificities did not exist in the hybridoma anti-liposome antibody (Table I). This could be interpreted to indicate that the rabbit antiserum was immunologically heterogeneous and

contained a few antibodies having predominately (or exclusively) anti-cholesterol or anti-dicetyl phosphate specificities. In the present study, cholesterol and dicetyl phosphate, although not recognized (or not strongly recognized) individually, each was important in influencing the binding of the hybridoma antibody. It is possible that the hybridoma exclusively recognized phosphatidylcholine, but did so only when the phosphatidylcholine was held in the correct configuration by appropriate quantities of cholesterol and dicetyl phosphate. In like manner, lipid A did not react with the hybridoma antibody (Table I), but it did markedly enhance the binding of the hybridoma antibody (Figs. 5 and 6). Lipid A does influence the fluidity of lipid bilayers [13], and it is possible that the lipid A also helped to hold the phosphatidylcholine in the exact configuration required for optimum antibody binding. The role of membrane fluidity in anti-liposome antibody binding is supported by the observation that liposomes containing egg PC (high fluidity) bound much more hybridoma antibody than did liposomes containing DMPC (low fluidity), and that these differences were overcome by inclusion of lipid A in the DMPC liposomes (Fig. 6).

Of those studied, the only soluble molecular species that strongly inhibited the hybridoma antibody, was phosphocholine. Choline by itself was not inhibitory, but other phosphorylated compounds, such as glycerophosphocholine, glycerophosphate, or even inorganic phosphate or sulfate, were inhibitory. This latter finding would suggest that part of the antigen binding site consists of a sequence of cationic amino acids that can bind phosphate or sulfate.

The preponderance of data in this paper suggests that the hybridoma anti-liposome antibody recognized phosphatidylcholine as an immunodominant group. However, the pattern of fluid phase inhibition by several small phosphorylated species (Fig. 7) was similar to that observed by us with a hybridoma anti-liposome antibody having specificity for phosphatidylinositol phosphate [14]. The latter antibody was inhibited by phosphocholine, inositol hexaphosphate, AMP, and ATP, but not by choline or inositol [14].

Acknowledgement

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