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Antibodies to phospholipids and liposomes: binding of antibodies to cells

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Key words: Antibody binding; Monoclonal anti-liposome antibody; Liposome; Phospholipid; Trypsin; Cell surface; Immunofluorescence; Enzyme-linked immunosorbent assay

Binding of two monoclonal anti-liposome antibodies to the surface of cultured murine peritoneal macrophages was investigated by indirect immunofluorescence and enzyme-linked immunosorbent assay. Neither antibody bound to cultures of freshly explanted, nonadherent macrophages, but immunoreactivity was observed following cell adherence to tissue culture plastic. Fluorescent microscopic evaluation revealed heterogeneity in staining patterns of the antibodies on adherent cells. Binding both to viable and fixed adherent macrophages was observed even after a 10000-fold dilution of antibody. Treatment of adherent macrophage cultures with trypsin increased antibody binding. Further treatment of trypsinized-macrophages with alkaline phosphatase or neuraminidase did not affect antibody binding, but phospholipase D and, to a greater extent, phospholipase C resulted in a marked decrease in cellular binding. The data indicate that antibodies produced against liposomes appear to bind to surface phospholipids of macrophages, but binding can be influenced by the physiological state of the macrophage and overlying cell surface proteins.

Introduction

Naturally occurring antibodies that react with liposomes of varied phospholipid composition have been found in the course of experimental trypanosomal infections in rabbits [1], and in normal (nonimmunized) rabbit sera [2,3] and human sera [4,5]. In recent years studies from this laboratory have demonstrated that antibodies against liposomes can be induced by the injection of liposomes containing lipid A into rabbits or mice [6–9]. By using this immunization technique, monoclonal antibodies that have the ability to distinguish between liposomes of distinct phospholipid compositions have been produced [10,11]. Anti-liposome antibodies are experimentally produced only after inclusion of lipid A from endotoxin in the liposomal bilayer used for immunization. However, lipid A in the absence of liposomes also induces antibodies that react with liposomes, presumably because of insertion of lipid A into phospholipids in the immunized animal resulting in autoantibodies against the lipid bilayer [7]. Based on these observations it has been proposed that lipid A characteristically induces unique anti-lipid bilayer antibodies and that these anti-lipid bilayer antibodies are normal autoantibodies that may have important physiological and pathophysiological functions [4,5]. One criterion to support this concept would be the demonstration that

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Abbreviations: CHOL, cholesterol; DCP, dicetyl phosphate; DMPC, dimyristoylphosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; PIP, phosphatidylinositol phosphate.

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these anti-lipid bilayer antibodies can bind to cells. In the present study we have investigated the immunoreactivity of two 'anti-liposome' monoclonal antibodies against murine macrophages by using indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA). The data indicate that each of the monoclonal antibodies can bind to cell surface phospholipids.

Materials and Methods

Monoclonal antibodies

Ascites fluids containing the monoclonal anti-liposome antibodies were induced by hybridomas in pristane-primed BALB/c mice after an initial i.v. immunization with liposomes containing either dimyristoyl phosphatidylcholine (DMPC), cholesterol (CHOL), dicetyl phosphate (DCP), and lipid A (molar ratio 1 : 0.75 : 0.11 : 0.04) [10] or DMPC, CHOL, phosphatidylinositol phosphate (PIP), and lipid A (molar ratio 1 : 1 : 1.5 : 1 : 0.08) [11]. These monoclonal antibodies have been designated, anti-DMPC/CHOL/DCP and anti-PIP, respectively. Both anti-DMPC/CHOL/DCP and anti-PIP are IgM (kappa). Ascites fluid containing a murine monoclonal IgM antibody (32.2A6.1) was produced against an epitope of the variant surface glycoprotein of the WRAT 1 clone of *Trypanosoma brucei rhodesiense* [12] and was a generous gift of Dr. Klaus Esser (Walter Reed Army Institute of Research).

Enzymes and substrate

Trypsin from bovine pancreas, type III, neuraminidase from *Clostridium perfringens*, type V, alkaline phosphatase from bovine intestine, type VII, phospholipase C from *Clostridium perfringens*, type XII, and phospholipase D from peanut, type III, were purchased from Sigma Chemical Co., St. Louis, MO. [*methyl*-^{3}H] Choline chloride (spec. act. 76 Ci/mmol) was purchased from New England Nuclear, Boston, MA.

Preparation of macrophage cultures

Thioglycollate-stimulated peritoneal macrophages were obtained by previously published methods [13]. Nonadherent cultures of macrophages were established by the addition of $4 \cdot 10^6$ cells into 12 × 75 mm polypropylene culture tubes (Becton Dickinson Labware, Oxnard, CA) at a concentration of $2 \cdot 10^6$ cells/ml of minimal essential medium containing 5% fetal bovine serum (M.A. Bioproducts, Waltersville, MD). After incubation for 60 min at 37°C, the tubes were centrifuged at 250 × *g* and resuspended in either phosphate-buffered saline (PBS) for studies of indirect immunofluorescence as described below or medium and further incubated for 24 h prior to immunofluorescence. Adherent cultures of macrophages were established by aliquoting cells either (a) into 26-well flat-bottom polystyrene tissue culture dishes (Becton Dickinson Labware, Oxnard, CA) at a concentration of $5 \cdot 10^4$ cells/well (for ELISA) or (b) onto 13 mm-diameter coverslip glass placed in tissue culture dishes at a concentration of $2 \cdot 10^5$ cells per coverslip (for indirect immunofluorescence). After incubation for 60 min at 37°C, to allow adherence, the culture vessels were rinsed and fresh medium was added. The cultures were then incubated for 24 h at 37°C in an incubator (5% CO_2/air) prior to further studies).

Indirect immunofluorescence

Indirect immunofluorescence was analysed by modification of previous techniques [14,15]. After fixation the cells were sequentially incubated with a 1 : 50 dilution of anti-DMPC/CHOL/DCP or anti-PIP followed by a 1 : 50 dilution of fluorescein-conjugated affinity purified goat anti-mouse IgM (μ chain specific; Cappel, Malvern, PA). The coverslips or aliquots of nonadherent cells were mounted on microscope slides in 80% glycerol containing 5% *n*-propyl gallate to reduce photobleaching [16] and examined with a Leitz photomicroscope equipped with epi-illumination for fluorescence using a 40 × objective. Photomicrographs were taken on Ilford XPI film with an exposure time of 10 s.

Enzyme-linked immunosorbent assay (ELISA)

Macrophage cultures were washed three times for 5 min each with PBS containing 0.3% gelatin (Difco Laboratories, Detroit, MI) at 20°C. In some experiments macrophage cultures were first fixed as described above prior to addition of primary antibody. 50 μl of ascites fluid containing monoclonal antibodies, diluted in phosphate-buffered saline containing 0.3% gelatin, were ad-

ded to the wells and incubated up to 60 min at 20 °C. The cultures were then washed three times for 5 min each with phosphate-buffered saline containing 0.3% gelatin. After the incubation of primary antibody and cells, the cultures were routinely fixed with paraformaldehyde and rinsed with 0.1 M glycine. 50 μl of goat anti-mouse IgM (μ chain) alkaline phosphatase conjugate (Kirkegaard and Perry Laboratories, Inc, Gaithersburg, MD) at 1 μg/ml in phosphate-buffered saline containing 1% bovine serum albumin (Fraction V, Calbiochem, La Jolla, CA) were then added to the wells and incubated 30 min at 20 °C. The cultures were again washed three times for 5 min each with phosphate-buffered saline containing 0.3% gelatin. 50 μl of *p*-nitrophenyl phosphate 2 mg/ml in diethanolamine buffer (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) were added to the wells and incubated 30 min at 20 °C. Plates were scanned for optical activity at 405 nm using a Titertek Multiscan (Flow Laboratories, McLean, VA). Values reported were adjusted by subtracting values in control wells that lacked primary antibody.

The ELISA procedure to investigate anti-DMPC/CHOL/DCP or anti-PIP binding to purified antigens was developed by modification of previous techniques for analysis of antibody binding to lipid antigens [17–19].

Results

Immunofluorescent detection of antibody binding to cultured murine macrophages

Immunoreactivities of the two IgM anti-liposome monoclonal antibodies were initially examined on paraformaldehyde-fixed cultures of nonadherent or adherent murine peritoneal exudate macrophages. The cells were observed by indirect immunofluorescence following incubation at 20 °C for 30 min with a 1:50 dilution of anti-DMPC/CHOL/DCP or anti-PIP. Neither of the anti-liposome antibodies demonstrated immunoreactivity with either freshly explanted, nonadherent macrophages or macrophages that were maintained in a nonadherent state by constant mild swirling for 24 h.

The absence of antibody binding to nonadherent cells was in marked contrast to results observed with cultures of adherent macrophages. Extensive binding of each antibody occurred with adherent cells. The phase-contrast and corresponding fluorescence images of adherent cells are shown in Figs. 1A and 1A′, and Figs. 1B and 1B′ for anti-DMPC/CHOL/DCP and anti-PIP, respectively. The pattern of reactivity for each monoclonal antibody was similar and consisted of heterogenous stained areas restricted to the cell surface. Certain cells, particularly less flat ones, often exhibited bright surface label; but certain other cells were not stained at all. Control cultures of macrophages yielded essentially no detectable labeling when they were either (a) incubated with nonimmune ascites fluid, (b) incubated in the absence of primary antibody, or (c) incubated with a murine monoclonal IgM antibody that did not have anti-liposome activity (32.2A6.1 against the variant surface glycoprotein of the WRAT-1 clone of *Trypanosoma brucei rhodesiense*). These experiments demonstrate that the anti-liposome monoclonal antibodies can bind to the surface of adherent macrophages but not to nonadherent macrophages.

ELISA detection of antibody binding to adherent macrophages

The binding of antibody to adherent cultures of paraformaldehyde-fixed or viable cells was further investigated by utilizing whole cells as antigens in ELISA. The cells were first incubated at 20 °C for 15, 30, or 60 min with a 1:100 dilution of anti-DMPC/CHOL/DCP or anti-PIP, followed by processing for ELISA. The data in Fig. 2 support and further quantify the microscopic evidence presented in Fig. 1 that demonstrated that both of the antibodies could bind to the cell surface of adherent macrophages. Immunoreactivity of either monoclonal antibody to $5 \cdot 10^4$ fixed or viable murine macrophages was essentially identical over the time sequence studied. Under identical conditions 32.2A6.1 did not bind significantly to macrophages (absorbance < 0.100, Fig. 2). The data validate the concept that whole macrophages used as antigens in an ELISA do not adsorb IgM antibody having an irrelevant specificity. This control therefore illustrates the feasibility of using ELISA to study specific anti-liposome antibody interactions with macrophages.

It is clear that anti-DMPC/CHOL/DCP and

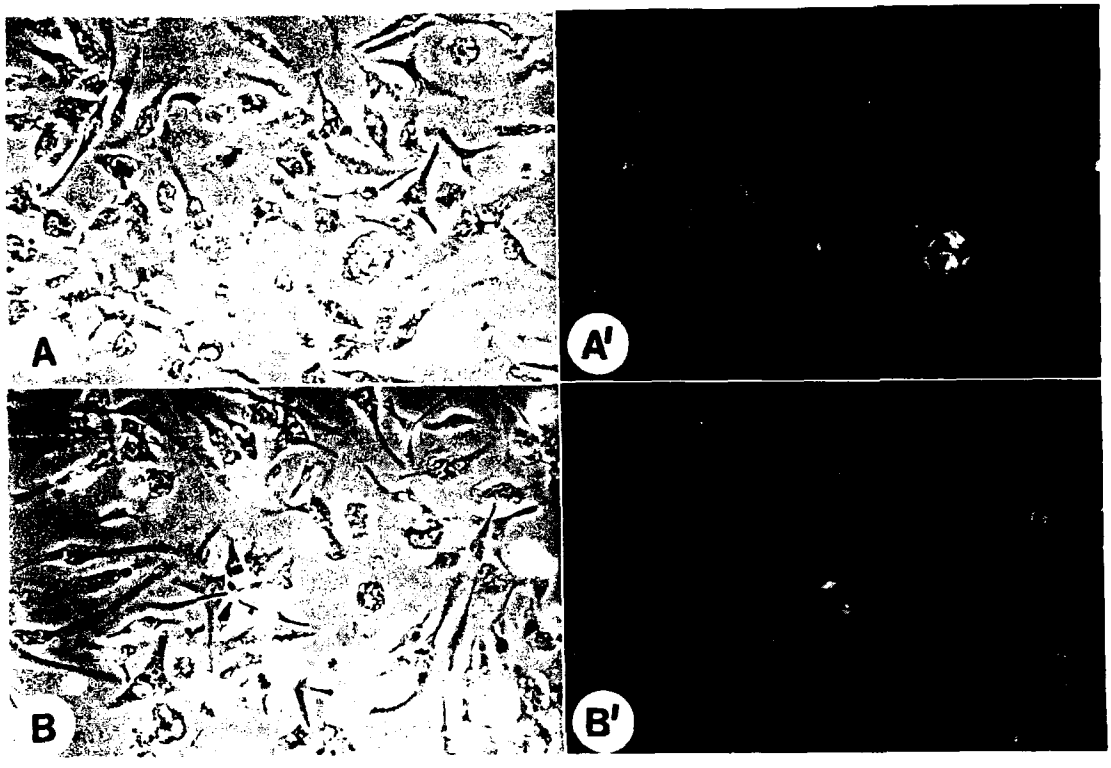

Fig. 1. Indirect immunofluorescence detection of cell-surface bound anti-DMPC/CHOL/DCP and anti-PIP. Macrophage cultures were processed as described in Materials and Methods. (A) Phase-contrast micrograph of macrophage cultures incubated with anti-DMPC/CHOL/DCP; (A′) immunofluorescence micrograph of field under (A); (B) phase-contrast micrograph of macrophage cultures incubated with anti-PIP; (B′), immunofluorescence micrograph of field under (B) (×350).

anti-PIP each recognized and bound antigenic determinants on the cell surface of adherent macrophages. As shown in Fig. 3, immunoreactivity with viable cells could still be detected following a 1 : 10000 dilution of each ascites fluid.

Ineffectiveness of ELISA for predicting macrophage antigens

In the above studies it was presumed that the anti-liposome monoclonal antibodies were binding to cell surface phospholipids with a specificity determined by the respective immunizing liposomes. However, it is well-known that antibodies to liposomes can show considerable cross-reactivities with soluble haptens, and even with a wide range of phospholipids other than those used for immunization [20]. Such cross-reactivities are maximized when purified antigens are spread out on a solid support as in an ELISA and minimized in the natural bilayer configuration found in liposomes and cells [20]. Indeed, when we tested the antibodies by solid-phase ELISA we found no basis for predicting reactivity with liposomes and cells because the antibodies reacted strongly with nearly every purified phospholipid, phosphoprotein, sialoprotein, and ganglioside examined. One interesting observation was that the antibodies could bind to one phosphoprotein (casein) but not to another phosphoprotein (ovalbumin). However, lack of ELISA reactivity with purified anionic substances was the exception rather than the rule. The ELISA results with purified lipids were therefore not effective as a means to predict the actual chemical structure of antigens being bound by antibodies on the macrophage surface.

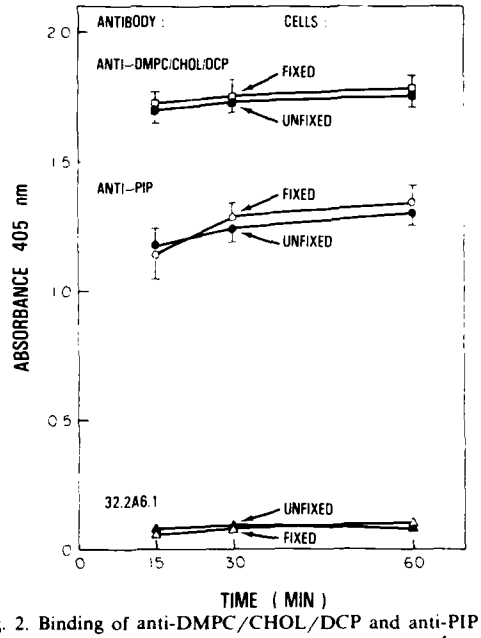

Fig. 2. Binding of anti-DMPC/CHOL/DCP and anti-PIP to macrophage cultures as determined by ELISA. $5 \cdot 10^4$ paraformaldehyde-fixed or viable macrophages were incubated at 20 °C for the indicated times with a 100-fold dilution of either anti-DMPC/CHOL/DCP, anti-PIP, or 32.2A61 and ELISA was performed. Data shown represent means ± S.D. of triplicate cultures.

TABLE I

BINDING OF anti-DMPC/CHOL/DCP AND anti-PIP ANTIBODY TO CULTURED MACROPHAGES FOLLOWING CELL SURFACE ALTERATION

$5 \cdot 10^4$ cultured macrophages were incubated for 30 min at 37 °C with 40 μg/ml trypsin in serum-free minimum essential medium. The cultures were washed and incubated an additional 30 min at 37 °C either with 10 U/ml alkaline phosphatase, 5 U/ml neuraminidase, 50 U/ml phospholipase D, or 5 U/ml phospholipase C. Following an additional wash, cultures were then incubated for 30 min at 20 °C with a 100-fold dilution of ascites fluid and ELISA was performed. Values shown are mean ± S.D. of triplicate cultures.

Macrophage treatment	Antibody binding (A_{405})	
	anti-DMPC/CHOL/DCP	anti-PIP
Control, no treatment	1.643 ± 0.108	1.201 ± 0.039
Trypsin	2.119 ± 0.131	2.087 ± 0.086
Trypsin + alkaline phosphatase	2.063 ± 0.092	1.923 ± 0.041
Trypsin + neuraminidase	1.927 ± 0.011	1.911 ± 0.040
Trypsin + phospholipase D	1.001 ± 0.027	0.988 ± 0.004
Trypsin + phospholipase C	0.619 ± 0.032	0.794 ± 0.019

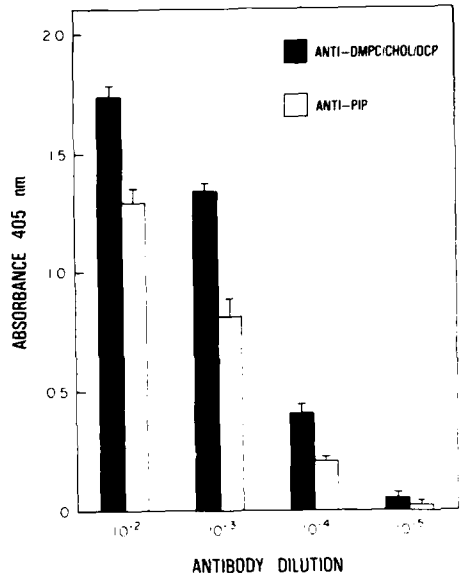

Fig. 3. Titration of the cellular immunoreactivity of anti-DMPC/CHOL/DCP of anti-PIP. $5 \cdot 10^4$ macrophages were incubated at 20 °C for 30 min with the indicated dilutions of ascites fluid and ELISA was performed. Data shown represent means ± S.D. of triplicate cultures.

The data demonstrating lack of binding of antibodies to nonadherent cells (see above) suggested that specific binding to cells may contrast with nonspecific binding to purified anionic substances under solid-phase assay conditions where bilayers are not present. It was therefore evident that identification of the reactive antigens on adherent cells could only be determined by examination of the cells directly.

Effects of enzyme-treatment on binding of anti-DMPC/CHOL/DCP or anti-PIP to adherent macrophages

Adherent macrophage cultures were treated with specific enzymes to examine the subsequent effects on monoclonal antibody immunoreactivity (Table I). Under the conditions employed, enzyme treatments did not alter cellular morphology and did not cause detachment of adherent macrophages from the substratum as determined by cell count. The binding of anti-DMPC/CHOL/DCP and anti-PIP antibodies to macrophages was increased following a 30 min incubation of the cell monolayer with trypsin. Sequential incubation of

trypsin-treated macrophages with alkaline phosphatase or neuraminidase did not influence the binding of the monoclonal antibodies. However, when trypsinized macrophages were further treated with phospholipase D or, even more revealing, with phospholipase C, the immunoreactivities of anti-DMPC/CHOL/DCP and anti-PIP antibodies were decreased. Treatment of cells with alkaline phosphatase, neuraminidase, phospholipase C, or phospholipase D without pretreating with trypsin did not cause any change in antibody binding.

The ability of phospholipase C to decrease the binding of anti-liposome antibodies to macrophages was correlated with removal of radiolabelled choline. [^{3}H]Choline-labeled cells were treated with trypsin alone or trypsin followed by phospholipase C and the remaining cell-associated radioactivity was measured (Table II). Trypsin caused a mild reduction (22%) of cell-associated radioactivity, probably due to removal of proteins having tightly bound phospholipids. Antibody binding increased sharply following trypsin treatment, suggesting the unmasking of underlying phospholipids. When trypsin-treated cells were treated with phospholipase C, choline specific activity associated with the macrophages was reduced by 49%, and binding of the anti-DMPC/CHOL/DCP or anti-PIP antibody was reduced by 66% and 43%, respectively.

When compared with trypsinized cells alone, calculations from the data of Table II show that further treatment of trypsinized cells with phospholipase C caused a 35% drop in cell-associated radioactivity, and this was associated with a 74% drop in binding of anti-DMPC/CHOL/DCP and 66% drop in binding of anti-PIP antibodies.

Discussion

Naturally occurring antibodies that react with liposomes of varied phospholipid composition are found in various sera [1–5], and antibodies against liposomes can be induced by the injection of lipid A [7] or liposomes containing lipid A [6–9]. Based on these observations it has been proposed both that lipid A characteristically induces unique anti-lipid bilayer antibodies and that these antibodies are normal autoantibodies that may have important physiological effects [4,5]. However, direct demonstration that these antilipid bilayer antibodies can bind to cells has been lacking. Prior studies with cultured Chinese hamster ovary cells presented indirect evidence that antibodies to liposomes could bind to the cell surface [21]. The present study shows direct evidence for the binding of anti-liposome antibodies to cells and also presents evidence for the phospholipid nature of the reactive antigen.

Two monoclonal anti-liposome antibodies, anti-DMPC/CHOL/DCP and anti-PIP, were demonstrated to bind to adherent cultures of macrophages. Indirect immunofluorescence revealed similar cellular patterns of antibody binding and also revealed that the immunoreactive sites were present at the cell surface. By utilizing adherent

TABLE II

CORRELATION BETWEEN REMOVAL OF [^{3}H]CHOLINE FROM MURINE MACROPHAGES BY PHOSPHOLIPASE C AND THE DECREASE IN BINDING OF anti-DMPC/CHOL/DCP AND anti-PIP ANTIBODIES

$5 \cdot 10^4$ cultured macrophages were labeled for 24 h at 37 °C with 5 μCi/ml [^{3}H]choline chloride. The cultures were washed and treated with the enzymes as reported in Table I. Cultures were then incubated for 30 min at 20 °C with a 100-fold dilution of ascites fluid. Cell-associated radiolabel was determined and the ELISA performed. Values shown are mean ± S.D. of triplicate cultures. Numbers in parentheses indicate percent inhibition (−) or stimulation (+) as compared to respective controls.

Macrophage treatment	Cell-associated radioactivity (dpm/$5 \cdot 10^4$ cells)	Antibody binding (A_{405})	
		anti-DMPC/CHOL/DCP	anti-PIP
Control, no treatment	25893 ± 1237	1.592 ± 0.097	1.254 ± 0.112
Trypsin	20086 ± 846 (−22)	2.129 ± 0.134 (+34)	2.101 ± 0.014 (+68)
Trypsin + phopholipase C	13012 ± 417 (−49)	0.547 ± 0.041 (−66)	0.714 ± 0.056 (−43)

cells as antigens in ELISA, the immunoreactivity of the two anti-liposome antibodies was marked, and could be observed following a 10000-fold dilution of the ascites fluid. The cellular binding of anti-DMPC/CHOL/DCP and anti-PIP antibodies was specific in that an antibody of irrelevant specificity (32.2A6.1) did not bind to cells. Collectively, these results represent the first direct demonstration showing that antibodies produced against liposomes can bind to cells.

The binding of anti-DMPC/CHOL/DCP and anti-PIP antibodies to macrophages apparently occurred following an alteration of the cell surface membrane. This conclusion is based on several lines of evidence. (a) Neither antibody bound to freshly explanted cultures (or 24-h-old cultures) of nonadherent macrophages. (b) Anti-DMPC/CHOL/DCP and anti-PIP antibody each bound to cultures of adherent macrophages in a heterogeneous fashion. (c) Binding of each antibody was greatly enhanced following trypsin treatment of cell monolayers. (d) Antibody binding was sensitive to the action of phospholipase D and to a greater extent to phospholipase C.

Cell surface phospholipid probably represents the major antigenic determinant for anti-DMPC/CHOL/DCP and anti-PIP antibody binding. Each monoclonal antibody reacted strongly with nearly every purified anionic moiety examined in ELISA. However, the conclusions derived from the in vitro ELISA were contrasted with the conclusions based on cellular binding of antibody following enzymatic treatment of adherent macrophages. The inability of trypsin, neuraminidase, or alkaline phosphatase to decrease anti-DMPC/CHOL/DCP and anti-PIP antibody binding precludes that proteins in general (phosphoproteins and sialoproteins, specifically) and gangliosides represent significant cell surface immunodeterminants for these antibodies. Indeed, trypsin treatment of adherent macrophages increased the binding of each monoclonal antibody, suggesting the unmasking of cryptic antigens. The cellular binding of anti-DMPC/CHOL/DCP and anti-PIP was strongly inhibited following treatment of adherent cells with phospholipase D, and to a greater extent phospholipase C. Because of potential cross-reacting properties of the antibodies, these results cannot delineate the specific phospholipid antigens being bound at the surface of the macrophage. Nonetheless the data do reveal that cell surface phospholipids probably represent the major antigenic determinant of antibody binding.

The observed inability of anti-DMPC/CHOL/DCP and anti-PIP antibodies to bind to numerous cells, including freshly explanted cells, was expected. The inability of antibodies to lipid bilayers to bind to most cells under normal conditions was predicted from the fact that naturally-occurring antibodies to lipid bilayers are widespread and it would be difficult to explain how this could occur without pathological consequences [4]. Therefore it was not surprising that freshly explanted cells did not bind the antibodies. Furthermore, separate preliminary experiments with these two anti-liposome antibodies demonstrated lack of reactivity to another cell, human erythrocytes from freshly drawn blood.

It is well-known that overlying proteins can sterically block the binding of antibodies to lipid bilayer antigens [22–24]. Previous studies from this laboratory even showed that a glycolipid, ceramide tetrahexoside (globoside), when present in the glycolipid-to-phospholipid ratio normally found in human erythrocytes, completely suppressed the binding of anti-DMPC/CHOL/DCP antibody to DMPC/CHOL/DCP liposomes [25]. Examination of space-filling models showed that the theoretical open area on the liposome surface between adjacent globoside oligosaccharides at the inhibitory concentration approached the molecular dimensions of a 7S immunoglobulin molecule [25]. Therefore the constrained space at the surface of the lipid bilayer can sometimes pose unique physical barriers to antibody binding. Based on these previous studies, it was expected that antibody binding to membrane phospholipids might be strongly influenced by adjacent membrane-associated molecules that exert steric hindrance.

It is not known whether the proposed hindrance caused by large polar groups such as membrane proteins adjacent to the target phospholipid antigen was solely responsible for the inability of anti-DMPC/CHOL/DCP or anti-PIP antibody to react with certain cells. However, in the present study removal of potential hindrance groups by enzymatic treatment, and possibly by membrane alterations that occurred during the process lead-

ing to adherence of cells, may have allowed the anti-liposome monoclonal antibodies to bind to the underlying membrane phospholipids.

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