β2-Glycoprotein I Is a Major Protein Associated with Very Rapidly Cleared Liposomes *in Vivo*, Suggesting a Significant Role in the Immune Clearance of "Non-self" Particles*

Arcadio Chonn‡§, Sean C. Semple‡, and Pieter R. Cullis

*From the Department of Biochemistry, The University of British Columbia, Vancouver,
British Columbia V6P 6P2, Canada*

Liposomes recovered from the blood of liposome-treated CD1 mice were previously reported to have a complex protein profile associated with their membranes (Chonn, A., Semple, S. C., and Cullis, P. R. (1992) *J. Biol. Chem.* 267, 18759–18765). In this study, we have further characterized and identified the major proteins associated with very rapidly cleared large unilamellar vesicles. These liposomes contained phosphatidylcholine, cholesterol, and anionic phospholipids (phosphatidylserine, phosphatidic acid, or cardiolipin) that dramatically enhance the clearance rate of liposomes from the circulation. These anionic phospholipids are normally found exclusively in the interior of cells but become expressed when cells undergo apoptosis or programmed cell death, and thus, they are believed to be markers of cell senescence. Analysis of the proteins associated with these liposomes by SDS-polyacrylamide gel electrophoresis revealed that two of the major proteins associated with the liposome membranes are proteins with electrophoretic mobilities corresponding to M_r of 66,000 and 50,000–55,000. The 66-kDa protein was identified to be serum albumin by immunoblot analysis. Using various biochemical and immunological methods, we have identified the 50–55-kDa protein as the murine equivalent of human β2-glycoprotein I. β2-glycoprotein I has a strong affinity for phosphatidylserine, phosphatidic acid, and cardiolipin inasmuch as the levels of β2-glycoprotein I associated with these anionic liposomes approach or even exceed those of serum albumin, which is present in serum at a concentration 200-fold greater than β2-glycoprotein I. Further, we demonstrate that the amount of β2-glycoprotein I associated with liposomes, as quantitated by an enzyme-linked immunosorbent assay, is correlated with their clearance rates; moreover, the circulation residency time of cardiolipin-containing liposomes is extended in mice pretreated with anti-β2-glycoprotein I antibodies. These findings strongly suggest that β2-glycoprotein I plays a primary role in mediating the clearance of liposomes and, by extension, senescent cells and foreign particles.

The clearance of liposomes from the circulation, primarily by

phagocytic cells of the reticuloendothelial system, is markedly affected by the lipid composition of the liposomes (1). Thus, incorporation of normally intracellular occurring phospholipids such as cardiolipin (CL),[1] phosphatidic acid (PA), or phosphatidylserine (PS) markedly enhances the clearance rate of the liposomes. The biochemical and immunological basis for this clearance phenomenon is for the most part poorly understood. However, this clearance is widely believed to involve blood proteins that associate with liposome membranes. To date, few *in vivo* studies have been reported providing evidence that this may in fact be the case. For example, rat perfusion studies have indicated that in order for significant liposome uptake by liver macrophages (Kupffer cells) to occur, the liposome perfusate must contain plasma proteins (2, 3). Further, we have recently shown using an *in vivo* mouse animal model that the ability of liposomes to interact with blood proteins is indeed related to their clearance rate from the circulation (4). Liposomes capable of binding the most blood proteins are cleared very rapidly from blood, whereas liposomes that exhibit reduced blood protein binding abilities are cleared at significantly slower rates.

By analyzing the proteins that associate with the liposomes in blood, we and others (4–8) have shown that a myriad of blood proteins associate with rapidly cleared liposomes. We have demonstrated by using immunoblot analysis that liposomes binding high levels of blood proteins are enriched with those blood proteins that function as opsonins, namely complement component C3 fragments and IgG (5). The complexity of the protein profiles associated with the liposome membranes upon exposure to blood would suggest that other proteins may play important roles in the immune recognition of foreign particles. Here, we report the identification of a protein that appears to have a high affinity for liposomes containing CL, PA, or PS. We employed various biochemical and immunological techniques to show that this protein is the murine equivalent of human β2-glycoprotein I (β2 gpI). The predominance of β2 gpI on rapidly cleared membranes strongly suggests that β2 gpI may be a key blood protein involved in the immunological detection of non-self or apoptotic membranes.

EXPERIMENTAL PROCEDURES

Preparation of Liposomes—Large unilamellar vesicles (LUVs) composed of PC:CH (55:45 mol/mol), PC:CH:egg phosphatidylglycerol (35:45:20), PC:CH:CL (35:45:10), PC:CH:DOPA (35:45:20), or PC:CH:DOPS (35:45:20) were prepared by an extrusion procedure as described in detail elsewhere (5, 9). Liposome suspensions were 20 mM (for *in vivo* experiments) or 50 mM total lipid (for *in vitro* experiments) in isotonic Hepes-buffered saline (20 mM Hepes, pH 7.4, 145 mM NaCl) sterilized

[1] The abbreviations used are: CL, bovine heart cardiolipin; β2 gpI, β2-glycoprotein I; PC, egg phosphatidylcholine; PA, egg phosphatidic acid; PS, bovine liver phosphatidylserine; CH, cholesterol; DO, dioleoyl; LUVs, large unilamellar vesicles; PAGE, polyacrylamide gel electrophoresis.

using Syrfil 0.22-μm filters (Nucleopore, Pleasanton, CA). The concentration of the recovered liposome suspensions was quantitated by using the lipid tracer [^{3}H]cholesterylhexadecyl ether (Amersham) as described previously (4, 10). All phospholipids were purchased from Avanti Polar Lipids (Pelham, AL) and were used without further purification; cholesterol was purchased from Sigma and was used without further purification. Liposome compositions are given in molar ratios.

Recovery of LUVs from Blood Components—CD1 mice (23–25 g, female, Charles River) were treated with 200 μl of liposome suspension. After 2 min, the mice were sacrificed by an overexposure to carbon dioxide, and blood was collected via cardiac puncture into ice-cold 1.5-ml polypropylene micro test tubes (Eppendorf). The blood was immediately cooled to 0 °C using an ice/water bath to prevent coagulation and centrifuged (12,000 rpm, 5 min, 4 °C). For *in vitro* incubations, 120 μl of LUVs suspension was incubated with 480 μl of isolated human serum for 30 min at 37 °C. The incubation mixture was then cooled to 0 °C using an ice/water bath. Aliquots of serum (50 μl) were then loaded onto BioGel A15 m, 200–400 mesh size (Bio-Rad) 1.0-ml spin columns to isolate LUVs from blood components as described previously (5). Alternatively, LUVs were isolated from incubation mixtures of 2 ml of PC:CH:CL LUVs and 8 ml of mouse serum (Cedarlane Laboratories, Hornby, Canada) (37 °C, 30 min) by conventional chromatography on a 2.5 × 90-cm BioGel A15 m, 100–200 mesh column pre-equilibrated with Veronal buffered saline (10 mM sodium barbital, pH 7.4, 145 mM NaCl) at 4 °C.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis—Protein separation was performed by SDS-PAGE using the Mini Protean-II electrophoretic apparatus (Bio-Rad) on precast 4–20% gradient Mini Protean-II gels (Bio-Rad) under non-reducing conditions. The gels were stained with Coomassie Blue to visualize the proteins.

Immunoblot Analysis of Proteins Associated with Liposomes—Proteins separated on 4–20% Mini Protean-II gradient gels were transferred onto nitrocellulose using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad) at a constant current of 350 mA for 60 min according to the manufacturer's instructions, followed by immunoblot analysis employing the enhanced chemiluminescence Western blotting detection system (Amersham). The blocking buffer consisted of phosphate-buffered saline (10 mM phosphate, pH 7.5, 138 mM NaCl, 2.7 mM KCl) containing 5% dried skim milk powder and 1% Tween 20 detergent (Sigma). Rabbit antisera to human β2 gpI (Behring) was used at a dilution of 1/5000, and peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Bar Harbor, ME) was used at a dilution of 1/5000. For control blots, non-immune normal rabbit sera was used at a dilution of 1/5000. Blocking buffer was used as the diluent.

Heparin-Agarose Chromatography—The proteins associated with the recovered PC:CH:CL LUVs were solubilized with 2% octylglucoside in 50 mM Tris, pH 7.4, 30 mM NaCl and chromatographed on a 1.5 × 7-cm heparin-agarose (Sigma) column as described previously (11).

Immunological Screening for β2 gpI—Column fractions were screened immunologically for β2 gpI using a Bio-Dot apparatus (Bio-Rad). Briefly, a nitrocellulose membrane (Bio-Rad) was soaked in phosphate-buffered saline, followed by the application of 10-μl aliquots of column fractions under vacuum. Immunological screening was performed as described above.

Anti-β2 gpI Affinity Chromatography—Commercially available rabbit anti-human β2 gpI antibodies (Enzyme Research Labs, South Bend, IN) were coupled to cyanogen bromide-activated Sepharose-4B chromatographic gel according to established procedures (12). Normal mouse serum (Cedar Lane Laboratories) was subfractionated using methods previously described for purifying human β2 gpI (11). Briefly, these procedures involved acidifying 40 ml of normal mouse serum with 0.60 ml of 70% perchloric acid with gentle stirring for 15 min at 4 °C, removing the precipitated proteins by centrifugation (3000 rpm, 4 °C, 20 min), adjusting the pH of the isolated supernatant to 7.8 with the dropwise addition of 5 N NaOH, precipitating the β2 gpI-immunoreactive protein with ammonium sulfate (380 g of solid/liter), and redissolving the isolated precipitated proteins in 3 ml of 50 mM Tris, pH 7.5. The proteins were then applied onto a 3-ml β2 gpI immunoaffinity column. The column was incubated for 2 h at 4 °C to enhance binding and washed extensively with 50 mM Tris, pH 7.5. The bound proteins were eluted with 50 mM Tris, pH 7.5, 2.5 M MgCl$_2$. Column fractions (1 ml) were monitored by dot-blot analysis, as well as by SDS-PAGE followed by silver staining according to the procedure of Rabilloud *et al.* (13). Fractions containing anti-human β2 gpI immunoreactive 50–55-kDa proteins were pooled, concentrated, and dialyzed against 10 mM Hepes, pH 7.5, buffer and stored at −20 °C.

N-terminal Region Protein Sequence Analysis—Immunoaffinity-purified murine 50–55-kDa protein was electrophoresed on a homogeneous 12% SDS-PAGE gel as above, blotted onto a polyvinylidene difluoride membrane, and detected by Coomassie staining. The band corresponding to the 50–55-kDa protein was cut from the membrane, and the protein was sequenced using an automated model 477A pulsed liquid phase sequencer (Applied Biosystems) equipped with a model 120A analyzer. A partial N-terminal region protein sequence was obtained and compared to sequences registered in the Swiss-Prot 23 sequence bank.

Quantitation of β2 gpI Associated with LUVs Using an Enzyme-linked Immunosorbent Assay—LUVs were recovered from isolated normal human serum incubations (120 μl of LUVs, 480 μl of normal human serum, 30 min, 37 °C) using spin columns as indicated above. Enzyme-linked immunosorbent assays were performed essentially as described previously (5) with the exception that purified human β2 gpI (1 μg/well, Enzyme Research Labs) in phosphate-buffered saline was bound to 96-well microtitre plates, and rabbit anti-human β2 gpI antibodies (Enzyme Research Labs) were used.

Anti-β2 gpI Antibody Pretreatment of Mice—The rabbit anti-human β2 gpI antibodies obtained commercially (Behring, lot 5506) were dialyzed overnight at 4 °C (Spectra/Por 2 dialysis membranes, molecular weight cutoff 12,000–14,000; Spectrum, Houston, TX) against a 0.9% sodium chloride irrigation solution (sterile, nonpyrogenic; Baxter, Vancouver) to remove azide. The protein concentration of the dialyzed antibody solution was determined using the bicinchoninic acid protein assay (Pierce). Mice were injected with 100 μl of 200 μg/ml (4 mice) or 20 μg/ml (4 mice) or 0.9% sodium chloride solution (4 mice) via the dorsal tail vein at two given times, minus 6 h and minus 2 h. At time 0, all mice received 200 μl of 20 mM PC:CH:CL (35:45:10) LUVs, containing a trace amount of [^{3}H]cholesterylhexadecyl ether, intravenously via the dorsal tail vein. After 30 min, the mice were sacrificed, and plasma samples were collected and analyzed for radioactivity content using standard liquid scintillation methods. The PC:CH:CL liposome recoveries at 30 min in anti-β2 gpI antibody-treated mice were repeated twice.

RESULTS

Detection of the Major Proteins Associated with Rapidly Cleared LUVs—To date, the best characterized blood proteins that have been described to enhance the rate of phagocytic uptake of foreign particles involve membrane-bound complement component C3 fragments and IgG. In this study, our principal aim was to characterize other blood proteins that may play an important role in the immune recognition of foreign membranes. Our approach was to use liposomes composed of lipids that are known to enhance their immune clearance from the circulation (typically, those lipids that are normally found exclusively in the interior of cells), to recover these liposomes from the blood of liposome-treated mice, and to analyze and identify the blood proteins that associate with their membranes.

Previously, we have demonstrated that LUVs composed of CL, PA, or PS (20 mol % negative charge) bind blood proteins in amounts exceeding 40 g of protein/mol of lipid immediately upon intravenous administration in mice and are cleared very rapidly from the circulation (half-lives of less than 10 min) (4). This suggests that some of the proteins associated with the liposome membranes enhance immune recognition and clearance. Fig. 1 depicts the major proteins associated with liposomes recovered from the blood of CD1 mice 2 min post-injection. It is immediately clear from Fig. 1 that there are at least two major proteins, having electrophoretic mobilities corresponding to M_r of approximately 66,000 and 50,000–55,000 that are associated with these anionic membranes. Identification of these proteins was carried out. Based on the apparent M_r and the fact that albumin is the most abundant protein in plasma (concentration approximately 40 mg/ml), we suspected immediately that the 66,000-Da protein band corresponded to serum albumin. This was confirmed by immunoblot analysis (results not shown).

Identification of the Murine 50–55-kDa Protein—We have observed that one of the most predominant proteins associated with
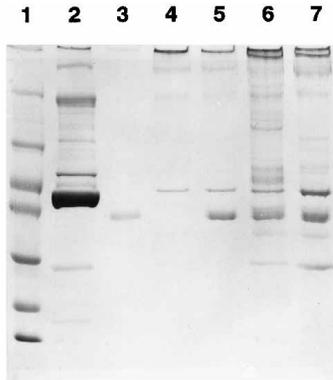
FIG. 1. **Major proteins associated with LUVs recovered from the circulation of mice.** The proteins associated with recovered LUVs (400 nmol total lipid) were separated on a 4–20% SDS-PAGE gel under non-reducing conditions and visualized by Coomassie staining. *Lane 1* represents molecular weight standards (Bio-Rad; myosin, 200,000; phosphorylase B, 97,400; albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; hen egg lysozyme, 14,400); *lane 2*, 10 μl of normal mouse serum (1:25 dilution); *lane 3*, 1 μg of purified mouse β2-glycoprotein I. *Lanes 4–7* represent the proteins associated with recovered PC:CH (55:45), PC:CH:DOPS (35:45:20), PC:CH:DOPA (35:45:20), and PC:CH:CL (35:45:10) LUVs, respectively.
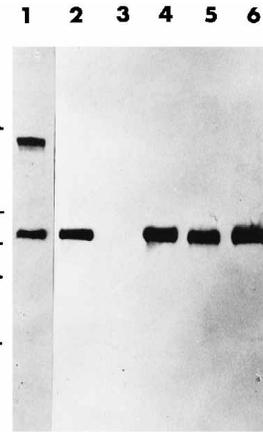
FIG. 2. **Immunoblot detection of β2-glycoprotein I associated with rapidly cleared LUVs.** The proteins associated with LUVs (25 nmol total lipid) recovered from mice were separated on a 4–20% SDS-PAGE gel under non-reducing conditions and were subsequently immunoblotted for β2-glycoprotein I using commercially available rabbit antisera to human β2-glycoprotein I. *Lane 1*, 10 μl of normal mouse serum (1:25 dilution); *lane 2*, 1 μg of purified mouse β2-glycoprotein I. *Lanes 3–6* represent the proteins associated with recovered PC:CH (55:45), PC:CH:DOPS (35:45:20), PC:CH:DOPA (35:45:20), and PC:CH:CL (35:45:10) LUVs, respectively.

with these rapidly cleared liposomes is a protein that migrates with an electrophoretic mobility corresponding to a molecular weight of approximately 50,000–55,000. Earlier work by Sommerman (37), employing *in vitro* incubations of multilamellar vesicles with human serum, demonstrated that a similar protein was associated in large amounts with PS-containing liposomes; that under reducing conditions, this protein migrates with a similar M_r as that under non-reducing conditions; and that by using two-dimensional gel electrophoresis, this protein has several charged forms characteristic of sialoglycosylated or sulfated proteins.

We were not able to identify this protein by immunoblot analysis using antisera to several of the major blood proteins (the so-called "Big Twelve" group) (14). However, by comparing the observed properties of this protein to the reported properties of minor protein components of blood, one possibility for the identity of this protein was β2 gpI. Immunoblot analysis of the proteins associated with the circulating LUVs indicated that this protein cross-reacted with an antibody to human β2 gpI (Fig. 2) and suggested that the 50–55-kDa protein was the murine homologue of human β2 gpI.

In subsequent studies, we determined whether this 50–55-kDa protein shared similar biochemical properties to human or rat β2 gpI. Human and rat β2 gpI have been previously reported to be heparin-binding proteins (11, 15–18). To test whether the murine 50–55-kDa protein shared this property, the proteins associated with PC:CH:CL LUVs were solubilized with octylglucoside and chromatographed on a heparin-agarose column. As demonstrated by retention on heparin-agarose (Fig. 3), the murine 50–55-kDa protein also has an affinity for heparin. The 50–55-kDa protein from mouse serum was found to be soluble in 1.0–3.0% perchloric acid, which is in agreement with previous findings for human and rat β2 gpI (11, 15–18).

Finally, to determine conclusively whether 50–55-kDa protein cross-reacting with the anti-human β2 gpI antibody was the murine homologue of human β2 gpI, the murine 50–55-kDa protein was purified using an anti-human β2 gpI affinity column (Fig. 4) and subjected to N-terminal region protein sequence analysis. As shown in Fig. 5, the amino acid sequence in the N-terminal region of the murine 50–55-kDa protein shares a high degree of homology with that of human β2 gpI and furthermore is identical to the recently published amino acid
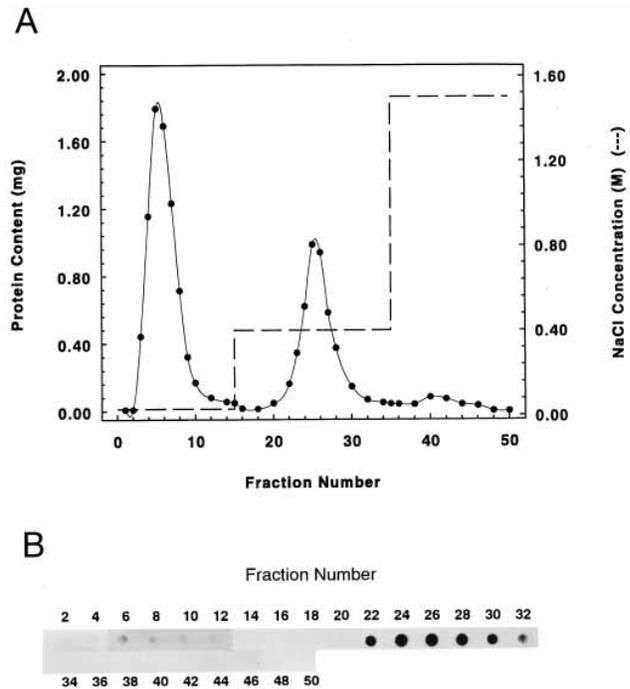

FIG. 3. **Heparin affinity of the 50–55-kDa protein associated with rapidly cleared LUVs.** PC:CH:CL LUVs were incubated with normal mouse serum and isolated from unbound components on a 2.5 × 90-cm BioGel A15, 100–200 mesh column as described under "Experimental Procedures." The isolated liposomes were solubilized in 2% octylglucoside and applied onto a 1.5 × 7-cm heparin-agarose affinity column (*A*). The column was eluted with a step gradient of NaCl in 50 mM Tris, pH 7.4, and 2-ml fractions were collected. Eluted proteins were analyzed by dot-blot analysis for β2-glycoprotein I (*B*).

sequence of murine β2 gpI.

Level of β2 gpI Associated with LUVs in Vitro Correlates with Circulation Half-lives—As we have noted previously (4), *in vitro* total serum protein binding values of LUVs are generally predictive of their clearance behavior *in vivo*. To determine whether a similar relation holds true for β2 gpI binding levels and clearance, the amount of β2 gpI associated with the LUVs recovered from human serum incubations was quantitated us-

ing an enzyme-linked immunosorbent assay specific for human β2 gpI and related to the observed liposome circulation half-life in mice. As indicated in Table I, LUVs that exhibit the most rapid clearance kinetics in mice also bind the highest levels of β2 gpI *in vitro*.

Recovery of PC:CH:CL LUVs in Anti-β2 gpI Antibody-treated Mice—The relation between β2 gpI binding levels and clearance noted above is highly suggestive of a role of β2 gpI in mediating liposome clearance. To further investigate this role, we studied the effect of pretreating the mice with varying doses of anti-β2 gpI antibodies (to depress the circulating levels of β2 gpI) on the clearance properties of PC:CH:CL LUVs. CL-containing LUVs were of particular interest because of the recent reports describing a role of β2 gpI in the binding of anticardiolipin antibodies to cardiolipin (17, 18). As depicted in Fig. 6, the amount of liposomes recovered at 30 min was significantly enhanced in mice treated with 100 μl of 200 μg/ml anti-β2 gpI antibody solution at 6 and 2 h prior to administration of LUVs.

DISCUSSION

LUVs represent ideal model systems to study the blood proteins that mediate the clearance of foreign particles. First, the clearance properties of liposomes are markedly dependent on their lipid composition. Second, the lipid compositions of the liposomes are readily altered and defined; thus, factors such as surface charge, lipid head group specificity, and membrane fluidity can be considered. Third, the extrusion procedure through 100-nm pore-sized filters generates stable vesicles that are essentially unilamellar, allowing the quantitation of the amount of protein associated with the vesicles (4, 5, 9). Fourth,

recent advances in the isolation of LUVs from the blood components have made possible the rapid isolation of LUVs from the blood of liposome-treated mice in the absence of coagulation inhibitors; thus, the stable blood protein-liposome interactions that occur *in vivo* can be analyzed (5). Finally, analysis of the proteins that associate with the LUVs is greatly simplified due to the absence of interfering membrane proteins that exist for systems such as bacteria or senescent cells. An analysis, therefore, of the various proteins that associate with very rapidly cleared liposomes compared to circulation-stable compositions should yield clues as to which blood proteins play a role in mediating the clearance of foreign particles. In this study, we have employed various biochemical and immunological methods to identify one of the major proteins associated with very rapidly cleared liposomes, and not with slowly cleared liposomes, as being β2-glycoprotein I.

As shown qualitatively in Fig. 1, the levels of β2 gpI binding to CL-, PA-, or PS-containing PC:CH LUVs corresponds to similar or even greater levels than those for albumin. This is significant inasmuch as the reported values for the concentration of β2 gpI in rats and humans is approximately 0.2 mg/ml plasma (60% is found in the lipoprotein-free δ > 1.21 g/ml bottom fraction after ultracentrifugation, and the remaining 40% is associated with triglyceride-rich lipoproteins) (19, 20). By direct comparison, the reported values for the concentration of albumin in rats and humans is approximately 40 mg/ml, 200-fold greater than the plasma concentration of β2 gpI. If one assumes that the association of albumin to these vesicles is nonspecific (14), then this finding would indicate that β2 gpI is greatly concentrated on these anionic membranes, suggesting that β2 gpI has a high affinity for CL, PA, and PS. Interestingly, apolipoprotein J, which shares some structural similarities to β2 gpI (also known as apolipoprotein H), has no affinity for these anionic LUVs as determined by immunoblot analysis using antibodies to apolipoprotein J.[2] This indicates that not all apolipoproteins have an affinity for CL-, PA-, or PS-containing LUVs.

As suggested by these findings, β2 gpI may play a significant role in the clearance of foreign membranes by phagocytic cells of the reticuloendothelial system. To this effect, there appears to be a correlation between the amount of β2 gpI associated with liposomes and their clearance rate. β2 gpI is associated in relatively high amounts with CL-, PA-, or PS-containing liposomes, all of which possess very rapid clearance kinetics; low amounts with phosphatidylglycerol- or phosphatidylinositol-containing or PC:CH (55:45) LUVs, all of which possess moderately slow clearance kinetics; or in very low levels with ganglioside G_{M1}-containing liposomes, which are capable of extended circulation lifetimes (4). Further substantiating a possible role of β2 gpI in vesicle clearance is the finding that β2 gpI exerts a significant effect on triglyceride clearance in rats (21). Perhaps the most suggestive evidence that supports a role of β2 gpI in the clearance of liposomes is our preliminary observation showing that we are able to significantly prolong the circulation half-life of CL-containing LUVs in mice that were pretreated with anti-human β2 gpI antibodies to depress
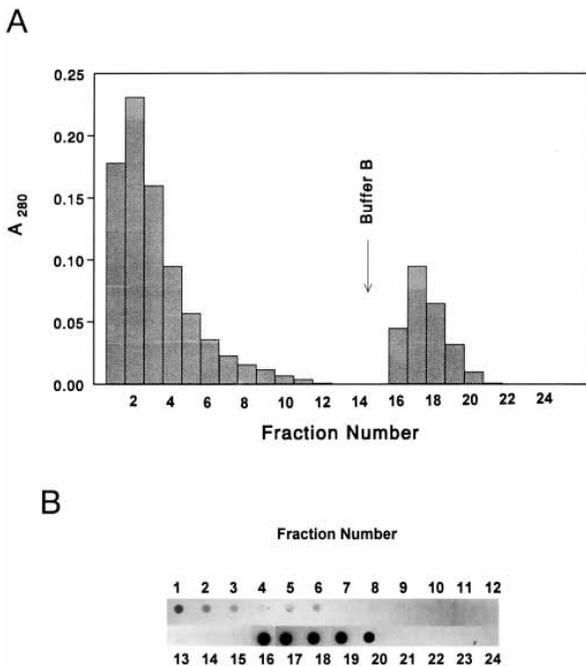

FIG. 4. **Anti-human β2-glycoprotein I affinity chromatography.** *A*, column elution profile of subfractionated murine 50–55-kDa protein (see "Experimental Procedures" for details). The bound protein was eluted from the column with buffer B (50 mM Tris, pH 7.5, 2.5 M MgCl₂). *B*, dot-blot analysis of column fractions.

[2] A. Chonn, S. C. Semple, P. R. Cullis, and J. Tschopp, unpublished data.

								5						10					15					20					25
p50-55	G	R	I	C	P	K	P	D	D	L	P	F	A	T	V	V	P	L	K	T	S	Y	D	P	G
Murine	G	R	I	C	P	K	P	D	D	L	P	F	A	T	V	V	P	L	K	T	S	Y	D	P	G
Human	G	R	T	C	P	K	P	D	D	L	P	F	S	T	V	V	P	L	K	T	F	Y	E	P	G

FIG. 5. **Partial N-terminal region sequence analysis of immunoaffinity-purified 50–55-kDa protein.** The obtained sequence was compared to the previously reported amino acid sequences of human (35) and murine (36) β2-glycoprotein I.

TABLE I
Amount of β2-glycoprotein I bound to LUVs recovered from in vitro incubations with isolated human serum and the relation to liposome circulation half-life

Composition of LUVs	β2g pI bound[a]	Circulation half-life[b]
	g/mol total lipid	min
PC:CH (55:45)	0.03, 0.02	120
PC:CH:PG (35:45:20)[c]	1.2, 1.6	32
PC:CH:PS (35:45:20)	3.4, 4.0	6–8
PC:CH:PA (35:45:20)	7.8, 8.1	<2
PC:CH:CL (35:45:10)	9.1, 8.4	<2

[a] Values from enzyme-linked immunosorbent assays performed on two independently isolated vesicle preparations.
[b] Values were estimated from previously published results in Ref. 4.
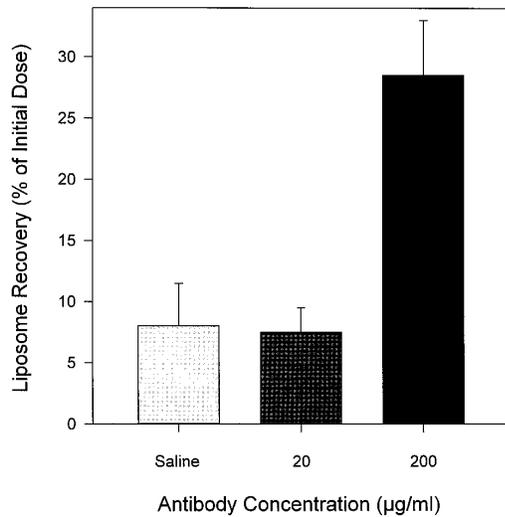[c] PG, egg phosphatidylglycerol.

FIG. 6. **Plasma recovery of CL-containing LUVs from anti-β2 gpI antibody-treated mice.** CD1 mice were pretreated with two different doses of anti-β2 gpI antibody or 0.9% sodium chloride solution at 6 and 2 h prior to administering a dose of ~100 mg/kg of PC:CH:CL (35:45:10) LUVs as described under "Experimental Procedures." The *bars* represent the average recoveries (plus standard deviation) from eight mice.

the circulating levels of β2 gpI.

Our finding that β2 gpI has an affinity for negatively charged phospholipids is consistent with a previous report on the lipid specificity of β2 gpI (16). We have extended these studies here to show that β2 gpI binds to higher levels to liposomes containing specifically CL, PA, or PS. Inasmuch as these phospholipids are not normally expressed on the exterior surfaces of cells, it is interesting to speculate that β2 gpI plays a role in the detection of these "foreign" phospholipids, which are expressed when cells undergo apoptosis or senescence. A recent study has indeed demonstrated a direct relation among PS exposure in the outer leaflet of human red blood cells, cell age, and the propensity for clearance by mononuclear cells (22). As well, PS expression on B cells undergoing apoptosis is enhanced due to loss of membrane phospholipid asymmetry (23). Several investigators have suggested that PS expression is a direct signal for macrophage adhesion and/or internalization via PS or scavenger receptors (24–28). Recognition via the PS receptors has been proposed to be an important phagocyte recognition system of cells undergoing apoptosis (29, 30).

A recent study, however, has challenged the role of scavenger receptors in the phagocytic uptake of PS-containing liposomes (31) and implied that another, yet unidentified, mechanism was involved. Here, we propose that inasmuch as β2 gpI is one of the major proteins coating PS-containing LUVs, perhaps β2 gpI mediates the phagocytic uptake of these vesicles by macro-

phages, either directly via β2 gpI receptors or via a multimeric complex with other blood proteins similar to the C1-IgG complex of the initiation complex of the classical activation pathway of complement. With regard to the latter possibility, β2 gpI has recently been described to function as a cofactor for the binding of anti-phospholipid antibodies to membranes containing anionic phospholipids (17, 18). Anti-phospholipid antibodies have been shown to be expressed in high titres in autoimmune diseased states, human immunodeficiency-infected serum, as well as in some normal serum (32–34). The exact role of β2 gpI in the formation of the anti-phospholipid-anionic phospholipid complex is not known. Whether this anionic phospholipid(s)-β2 gpI-anti-phospholipid antibody complex leads to the activation of the classical pathway of complement is not known. The role of β2 gpI in immune clearance warrants further investigation.

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