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GANGLIOSIDE G_{M1} AND HYDROPHILIC POLYMERS INCREASE LIPOSOME CIRCULATION TIMES BY INHIBITING THE ASSOCIATION OF BLOOD PROTEINS

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ABSTRACT

Several agents have been shown to prolong the circulation lifetime of liposomes. These agents, such as ganglioside G_{M1} or phosphatidylethanolamine-derivatives of monomethoxypolyethyleneglycols, provide insight into the mechanism(s) by which liposomes are cleared from the circulation. It is suggested here that the primary mechanism by which these molecules alter the biodistribution of liposomes *in vivo* involves an inhibition of the association of blood proteins to liposomes, resulting in a diminished rate of clearance of liposomes from the circulation.

BACKGROUND

In the past five years, several laboratories have reported that molecules which increase the hydrophilic nature of the liposome surface prolong the circulation lifetime of liposomes. Such molecules include ganglioside G_{M1} [1,2], phosphatidylethanolamine-derivatives of monomethoxypolyethyleneglycols (PE-PEG) [3-6], or polysorbate 80, a nonionic surfactant [7]. The effects of

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ganglioside G_{M1} or PE-PEG* are dependent on their membrane concentration and moreover, are unique in that they are relatively independent of the degree of fatty acyl saturation of the major phospholipid component, or the cholesterol content of liposomes [8]. These findings suggest that these lipids affect the surface properties of the liposomes in such a way that reduces the probability of phagocytic uptake by cells of the reticuloendothelial system (RES). Interestingly, other hydrophilic molecules such as ganglioside G_{D1a} or G_{T1b}, or other glycolipids such as asialoganglioside G_{M1} or globosides do not have this ability to prolong circulation lifetimes [1,2,9].

Hydrogenated phosphatidylinositol (PI) has also been shown to extend the circulation lifetime of liposomes [2]. The effects of hydrogenated PI, however, appear to be dependent on the degree of fatty acyl saturation of not only the PI itself but also of the major phospholipid component [unpublished observations; see also 2].

The mechanism by which these molecules prolong liposome circulation lifetimes is not yet clear; however, three possible mechanisms have been suggested [5,8,10]. The first possibility is that they inhibit liposome interactions with plasma lipoproteins or blood proteins that induce membrane dissolution or membrane permeability. Second, an inhibition of direct liposome/cell interactions has been postulated. Finally, it is possible that molecules which are effective in prolonging the circulation lifetimes of liposomes provide a steric barrier that inhibits the association of blood proteins that enhance receptor-mediated macrophage uptake. This review summarizes the evidence supporting these mechanisms, and incorporates some recent work from our laboratory on the interactions of blood proteins with large unilamellar vesicles (LUVs).

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* PE-PEG, phosphatidylethanolamine-derivatives of monomethoxypolyethyleneglycols; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SM, sphingomyelin; CH, cholesterol; LUVs, large unilamellar vesicles; RES, reticuloendothelial system.

Inhibition of Interactions with Blood Components that Lead to Increased Membrane Permeability or Membrane Dissolution

Net transfer of liposomal lipids to plasma lipoproteins has been shown to destabilize liposome membranes resulting in an increased permeability of entrapped solutes [reviewed extensively in 11]. Although all lipoprotein species may be involved, early studies establish that liposome interactions with primarily high density lipoproteins result in dissolution of the liposome structure and transfer of liposomal phospholipid to high density lipoproteins [12-15]. A role of other plasma lipoproteins in liposome membrane destabilization has also been suggested. With large liposome doses, phospholipids are transferred to low density lipoproteins and probably also very low density lipoproteins, resulting in the formation of larger, less dense particles [13,16]. The increased liposome permeability induced by low density lipoproteins has recently been demonstrated for anionic liposomes at lipid concentrations as low as 40 nM phospholipid [17]. Isolated apolipoproteins have been shown to destabilize liposomes by enhancing lipid movement and leakage of entrapped markers. Several investigators have shown that apolipoproteins A-I, A-II, A-IV, B, C and E are transferred to liposome membranes upon plasma incubation *in vitro* and *in vivo* [12, 18-22]. Incubation of egg PC small unilamellar vesicles with rat apolipoprotein A-I or E resulted in release of entrapped carboxyfluorescein accompanied by structural changes of liposomes to discs [22-24].

The incorporation of as little as 2 mol% ganglioside G_{M1} has been shown to inhibit phospholipid exchange between liposomes and high density lipoproteins [9]. Gangliosides, such as ganglioside G_{M1}, G_D or G_T, have also been shown to reduce the rate of calcein leakage from vesicles [25]. Although the exact mechanism involved is not known, gangliosides are believed to reduce calcein leakage by inhibiting interactions with lipoproteins [25]. Liposomes containing 2 mol% ganglioside G_{M1}, or gangliosides G_{D1a} or G_{T1b}, however, do not prolong the circulation lifetime of liposomes [1,2] and therefore, it is not clear how a reduction in interactions with lipoproteins is related to liposome clearance.

Inhibition of Direct Liposome Interactions with Cells

The reduced rate of liposome clearance by the phagocytic cells of the RES, resulting from the incorporation of ganglioside G_{M1} or PE-PEG into

liposome membranes, has been well documented. There are some indications that these molecules directly inhibit liposome interactions with cells. For instance, the binding of immunoliposomes bearing 34A monoclonal antibody to lung endothelial cells is inhibited by the incorporation of PE-PEG (5000) [10]. PE-PEG (5000) also prevented the agglutination of liposomes containing N-biotinaminocaproyl-PE by streptavidin. This contrasts with the observation that only the rate of agglutination, but not the extent, was retarded by increasing mol% of ganglioside G_{M1}. In turn, this possibly accounts for the observation that ganglioside G_{M1} does not inhibit binding of immunoliposomes to lung [26]. Blume and Cevc [4] have demonstrated that the rate of liposome uptake by human monocyte (THP-1 cells) was reduced for distearoylPC:distearoylPE-PEG (9:1) liposomes compared to distearoylPC liposomes. Similarly, Allen et al. [27] have shown that the uptake of ganglioside G_{M1}- or PE-PEG-containing liposomes by cultured murine bone marrow macrophages was decreased compared to the uptake of PC liposomes. This decreased uptake was dependent on the amount of ganglioside G_{M1} or PE-PEG incorporated. Other gangliosides, such as ganglioside G_{M2}, G_{M3}, G_{D1a}, G_{T1b} or asialoganglioside G_{M1}, failed to inhibit liposome uptake. The presence of serum in these incubation mixtures, however, makes it difficult to rule out the role of serum proteins in mediating liposome uptake.

Other evidence suggests that liposomes containing either ganglioside G_{M1} or PE-PEG are still capable of interacting with cells. For example, ganglioside G_{M1}- or PE-PEG-containing liposomes are taken up by the RES. Although the rate of uptake appears to be much slower, after 24 h the RES uptake appears to be similar to conventional liposomes [2,8]. Furthermore, large liposomes (>600 nm in diameter) containing these lipids appear to be filtered out of the circulation by the spleen and are processed by splenic macrophages [28]. Allen et al. [6] report that higher levels of ganglioside G_{M1}- or PE-PEG-containing liposomes are associated with endothelial cells over time.

Inhibition of Blood Protein Association

The possibility that certain lipid species prolong the circulation lifetime of liposomes by inhibiting the surface adsorption of blood proteins has the central

tenet that particular blood proteins associated with liposomes can promote liposome/cell interactions and consequent liposome elimination. Previous *in vitro* work has shown that upon exposure of multilamellar liposomes to plasma or serum, several proteins are rapidly adsorbed on the liposome surface [29,30]. However, whether these associated blood proteins actually play a role *in vivo* in mediating liposome clearance has not been unambiguously established. Complement, immunoglobulins, and fibronectin have been implicated in mediating liposome clearance because liposomes coated with these purified proteins exhibit enhanced uptake by cultured macrophages [31-33]. However, the role of liposome-associated proteins in enhancing phagocytic uptake in the presence of excess soluble protein is unclear [34]. Furthermore, although liposome uptake by macrophages is widely believed to involve opsonic receptors, the role of scavenger receptors in mediating the uptake of liposomes by mouse or guinea pig peritoneal macrophages has recently been described [35].

Recent advances in isolating LUVs from the circulation of liposome-treated mice by employing a "spin column" procedure [36] have made possible *in vivo* studies on the role of blood proteins in liposome clearance [37]. These studies show that the total protein binding to liposomes is inversely correlated to liposome clearance in a mouse animal model (see Fig. 1), supporting the hypothesis that blood proteins play a significant role in liposome clearance. This is in agreement with other studies employing a rat liver perfusion model to indicate that plasma proteins are required for effective liposomal uptake by rat liver macrophages [38,39].

With regard to the specific nature of the associated proteins which induce clearance, it is clear form studies using particles which require immunologic coating in order to adhere to phagocytic cells that the immune opsonins, IgG and complement component C3, are involved in the adherence step of phagocytosis. Further, various receptors, which specifically recognize portions of these molecules, are the means by which phagocytic cells recognize and attach to their surfaces particles coated with these ligands. It has been shown that liposomes coated with complement or IgG are taken up more readily by cultured macrophages. For example, ingestion of MLVs containing galactosyl ceramide and coated with IgM antibodies direct against galactosyl ceramide by cultured mouse peritoneal macrophages is enhanced five to ten fold by addition of guinea

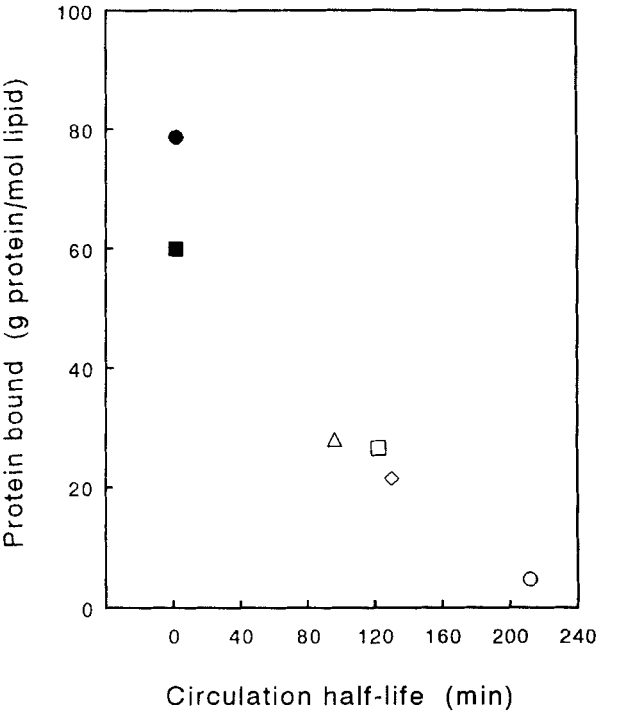

FIG 1. ***Relation of total amount of protein bound to liposomes and circulation half-life.*** [^{3}H]cholesteryl hexadecylether-labeled LUVs, prepared by extrusion through two-stacked 100 nm pore-sized polycarbonate filters (Nuclepore, Pleasanton, CA) according to the method of Hope et al. [53], were administered intravenously via the dorsal tail vein of CD1 mice at an apporximate dose of 20 μmol total lipid per 100 g mouse weight. After 2 min post-injection, LUVs were recovered from blood employing a "spin column" procedure [36]. Aliquots of the recovered liposomes were delipidated according to the method of Wessel and Flugge [54] and the extracted proteins quantitated using the micro BCA protein assay (Pierce, Rockford, IL). The liposomes were composed of (■) PC:CH:CL (35:45:10), (●) PC:CH:DOPA (35:45:20), (□) PC:CH (55:45), (△) PC:CH:plant PI (35:45:20), (◇) SM:PC (4:1) and (○) SM:PC:ganglioside G_{M1} (78:18:10). Liposome compositions are expressed in molar ratios.

pig complement [32]. The covalent attachment of rabbit IgG to LUVs result in a five-fold increase in liposome uptake by rat liver macrophages compared to noncoated liposomes [33]. Specific anti-dinitrophenol antibody/dinitrophenylcaproyl-PE antigen complexes assembled on liposomal surfaces markedly increases the rate and extent of phagocytosis by cultured macrophages [31,40]. Similarly, endocytosis of IgG anti-dinitrophenyl liposomes by Fc receptor positive phagocytic murine tumor cells is enhanced [41].

Until recently, however, the activation of the complement system by liposomes has been described for only a few specific liposome compositions. These compositions include liposomes containing haptenated lipids [42], phosphatidylserine with phosphatidylethanolamine [43], cardiolipin [44], stearylamine in the presence of galactosyl ceramide [45], cerebrosides in dimyristoylphosphatidylethanolamine [46] or saturated phosphatidylethanolamine in saturated phosphatidylcholine liposomes containing cholesterol [47]. A recent study from our laboratory has addressed the question as to whether liposomes, including those commonly used in liposome drug delivery formulations, activate the complement system in the complex biological milieu, and whether this leads to C3b deposition on liposome surfaces [48,49]. Using a complement hemolytic assay to detect complement activation, we have demonstrated that liposomes containing minor lipid constituents which are net negatively or net positively charged activate complement in whole human or guinea pig serum. Furthermore, complement activation resulted in the deposition of activated C3b fragments on the liposome surfaces. The amount of C3 binding to the various liposome compositions containing different anionic phospholipids, such as cardiolipin, phosphatidic acid, phosphatidylserine, phosphatidylinositol, or phosphatidylglycerol differed significantly as measured by a human C3 competitive ELISA [36,50]. The amount of C3 binding to the various liposome compositions indicate a correlation between the amount of C3 bound per liposome and liposome clearance behavior; for example, cardiolipin- and dioleoylphosphatidic acid-containing systems, which bind the most C3, are cleared very rapidly from the circulation. This suggests an important role of C3 in liposome clearance.

More recent studies have demonstrated that incorporation of 10 mol% ganglioside G_{M1} into complement-activating PC:CH:PG (35:45:20) LUVs

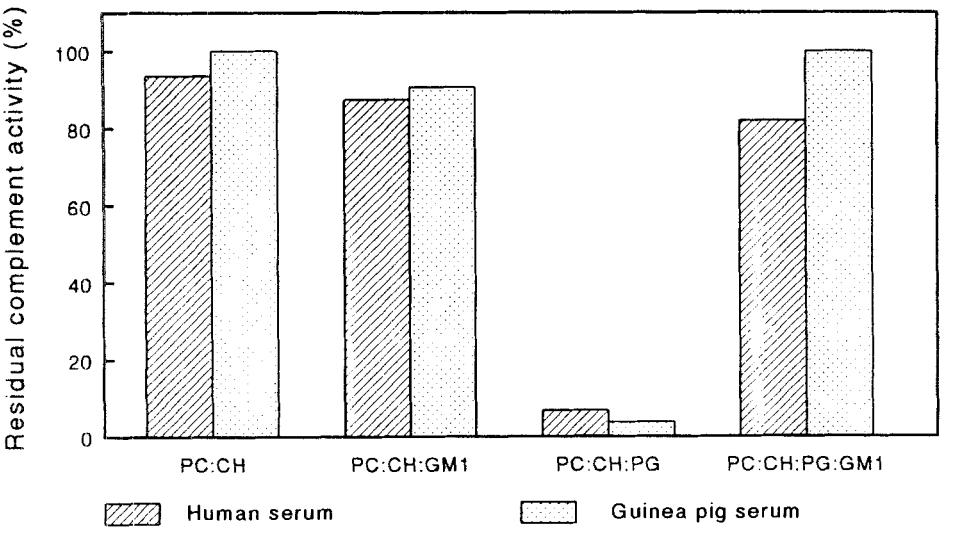

FIG 2. ***Inhibition of complement activation by liposomes containing ganglioside*** G_{M1}. Complement hemolytic assays were performed as described by Chonn et al. [49] to determine the residual complement hemolytic activity of the serum after incubation of the serum with 1 μmol total lipid of liposomes composed of PC:CH (55:45), PC:CH:ganglioside G_{M1} (45:45:10), PC:CH:PG (35:45:20), or PC:CH:PG:ganglioside G_{M1} (25:45:20:10).

transforms these liposomes into non-activators of complement (Fig. 2). Complement hemolytic assays measure the residual total functional complement levels of serum after exposure to liposomes. PC:CH:PG (35:45:20) LUVs reduce the hemolytic ability of the serum, signifying that complement activation by the liposomes had occurred. The incorporation of 10 mol% ganglioside G_{M1} into these liposomes inhibited the consumption of complement by PG-containing LUVs.

Most of the studies describing the role of proteins in macrophage uptake have involved *in vitro* systems employing incubations with isolated plasma or serum, and cultured macrophages. It is important to extend these studies to investigate the *in vivo* blood protein/liposome interactions affecting liposome clearance. Such studies, involving the isolation of LUVs from the circulation of liposome-treated mice after 2 min post-injection followed by analyzing the

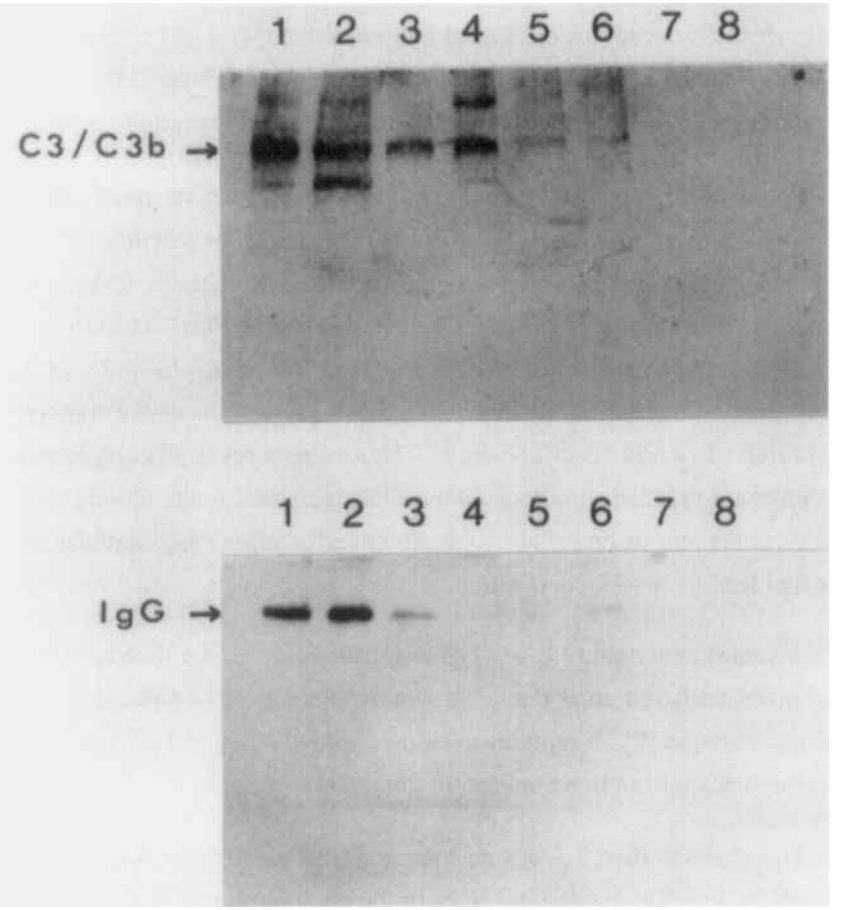

FIG 3. ***Immunoblot analysis of murine opsonins associated with LUVs.*** The proteins associated with LUVs (25 nmol total lipid), recovered from the circulation of mice 2 min post-injection, were separated electrophoretically on 4-20% SDS-PAGE gels and analyzed by immunoblot analysis using the Enhanced Chemiluminescence western blotting detection system (Amersham, Arlington Heights, IL) specific for mouse C3 or mouse IgG. The lanes contain the following liposome compositions: PC:CH:CL (35:45:10; Lane 1), PC:CH:DOPA (35:45:20; Lane 2), PC:CH:DOPS (35:45:20; Lane 3), PC:CH:plant PI (35:45:20; Lane 4), PC:CH:PG (35:45:20; Lane 5), PC:CH (55:45; Lane 6), SM:PC:ganglioside G_{M1} (72:18:10; Lane 7), and SM:PG:ganglioside G_{M1} (72:18:10; Lane 8).

proteins associated with the LUVs by immunoblot analysis specific for murine C3 or IgG, have recently been performed [see Fig. 3; and also 37,50]. These studies show that the relative amounts of C3 and IgG associated with SM:PC:ganglioside G_{M1} LUVs are exceedingly low compared to very rapidly cleared LUVs, such as those containing 10 mol% cardiolipin or 20 mol% dioleoylphosphatidic acid.

The question arises as to whether ganglioside G_{M1} acts to specifically decrease binding of opsonins such as IgG or C3 fragments, or whether the ganglioside G_{M1} effect arises from a non-specific decrease in the binding of all blood proteins. The amount of protein associated with SM:PC (4:1) LUVs is significantly reduced by inclusion of 10 mol% ganglioside G_{M1} [37]. Preliminary results indicate that increasing the mol% ganglioside G_{M1} reduces the association of blood proteins in a non-specific manner. The association of all categories of blood proteins are reduced simultaneously. This decrease in total blood protein association results in a surface that is less effective for supporting immune reactions that lead to opsonin deposition.

Liposomes containing PE-PEG (5000) have been shown to exhibit decreased protein adsorption as demonstrated by an aqueous two-phase partitioning technique [5]. It remains to be determined whether PE-PEG also inhibits protein adsorption in a non-specific manner.

In recent years, there have been some reports that suggest that there are plasma proteins which stabilize liposomes in the circulation [51,52]. It is possible that ganglioside G_{M1} or PE-PEG prolongs circulation lifetimes by enhancing the adsorption of "dysopsonins" to liposomes. Several blood proteins, some of which may represent "dysopsonins", associate with ganglioside G_{M1}-containing liposomes in the circulation [37]. Further studies are required to test this hypothesis.

CONCLUDING REMARKS

In our view it is the total deposition of serum proteins on liposome surfaces which correlates most directly with subsequent clearance. Some of these deposited proteins may be postulated to have opsonic properties and thus, promote clearance. However, available evidence indicates that ganglioside G_{M1},

PE-PEG and other agents leading to extended circulation times do not preferentially inhibit opsonin binding, but rather inhibit binding of all blood proteins, opsonins included.

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