Effect of Host Lattice on Antigenicity of Glycophorin in Membranes

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The influence of the host lattice on the antigenicity of glycophorin in membranes was confirmed by complement-dependent immune lysis of liposomes with two rabbit antisera, which were prepared by immunization with either human red blood cells or isolated glycophorin A. The immune lysis by either antiserum depended on the kind of phospholipid in the liposomes. Anti-glycophorin antiserum more strongly recognized glycophorin in egg-lecithin membranes than in dipalmitoyl-lecithin membranes, as did anti-red blood cell antiserum. Cholesterol in the liposomal membranes influenced the antigenicity of glycophorin.
The relationship between the state of glycophorin in membranes and recognition by antibody is discussed.

Keywords glycophorin; liposome; membrane; antiserum; antigenicity; complement-dependent immune lysis

The membrane glycoconjugates are considered to be important in cell recognition as receptors for polypeptide hormones, toxins, lectins, viruses and agents of the immune system. It is relatively easy to construct lipid bilayers (liposomes) bearing isolated membrane glycoconjugates, and model membrane studies have contributed significantly to understanding the molecular bases of their functions.[1−3]

It has been reported that the haptenic activity of glycolipid in liposomes was influenced by the physical state of the host lipids.[4,5] These studies suggest that the immune reactions on liposomal membranes depend on the topographical distribution, epitope density and dynamic properties of glycolipids in the membrane. Recently, our group also proposed that rotational motion of a haptenic site should be related to the activity of a lipid hapten.[6] Complement-dependent immune damage to liposomes containing glycolipids is also influenced by the bulk lipid,[7−9] but little is known about the effect of host lipids on the complement-dependent immune lysis of glycoprotein-containing liposomes.

The structure[10] and transmembrane disposition[11] of glycophorin, a major sialoglycoprotein in human erythrocyte membranes and a blood group substance MN antigen,[12] have been established. Liposomes that contain glycophorin serve as useful models to study the effects of glycoprotein on the physical state of the membrane lipid[13−17] and the biological activity of glycoprotein in membranes.[18−21]

In this paper we report on complement-dependent immune damage to glycophorin-containing liposomes. The effects of density of membrane glycophorin and cholesterol on immune lysis were also investigated.

Materials and Methods

Egg yolk phosphatidylcholine (eggPC) was prepared by column chromatography on silicic acid (Mallinckrodt Chemical Works, St. Louis, Mo., USA). L-α-Dipalmitoylphosphatidylcholine (DPPC) was kindly provided by Nihon Seika Co., Ltd., Osaka. Dicetyl phosphate (DCP) was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and cholesterol (Chol) from Wako Pure Chemical Industries, Tokyo. All lipids gave single spots on thin-layer chromatography with a solvent system of chloroform–methanol–water (65:25:5, v/v) and were stocked as chloroform solutions at −20 °C. The concentrations of phospholipids were determined by the method of Gerlach and Deuticke[22] for total phosphorus.

4-Methylumbelliferyl phosphate (UmP) and alkaline phosphatase from *Escherichia coli* were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Glycophorin A (GP) was isolated from human erythrocytes following the general procedure of Tomita *et al.*[10] Following the procedure of Dodge *et al.*[23] red blood cells (RBC) were subjected to hemolysis and washed at 4 °C. Glycoproteins were then isolated by lithium di-iodosalicylate–phenol extraction and precipitation with 100% ethanol.[24] Glycoproteins were further purified by using a Bio-Gel A-1.5 m column with 0.1% of the nonionic detergent, *N,N*-dimethyllaurylamine *N*-oxide (Ammonyx-LO).[25] The preparations showed a typical glycophorin A pattern on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue and Periodic acid-Schiff's (PAS) reagent.

Antisera Anti human red blood cell antisera (Anti-RBC) and anti glycophorin A antisera (Anti-GP) were obtained from New Zealand white rabbits as follows.

Anti-RBC: Human RBC were washed three times in 0.15 M NaCl solution and 0.2 ml of a 50% suspension was injected intravenously three times at 10-d intervals. To follow the development of antibodies, immunized rabbits were bled at 7-d intervals and the serum obtained on day 42 was used in this study.

Anti-GP: Each rabbit was injected on the first day with 0.3 mg of glycophorin A emulsified in complete Freund's adjuvant (CFA), into the hind footpad. Each immunized rabbit received 2 additional booster injections of 0.3 mg of glycophorin A in CFA subcutaneously on the 7th and 14th days. To follow the development of antibodies, immunized rabbits were bled at 7-d intervals and tested for hemagglutination titer. The serum obtained on the 47th day was used in this study.

The sera were heated at 56 °C for 30 min to inactivate the complement, and were stored at −20 °C.

The hemagglutination titer of anti-RBC and anti-GP was 2^{11} towards human erythrocytes. The presence of anti-glycophorin antibody in both antisera was confirmed by the observation of complement-dependent immune damage to liposomes containing glycophorin as described below.

Complement The source of complement was young guinea pig serum which was stored at −80 °C until used.

Preparation of Liposomes Liposomes containing glycophorin were prepared by the method of MacDonald and MacDonald,[21] slightly modified.[26] Briefly, a mixture of phosphatidylcholine (2.5 μmol), Chol (2.5 μmol), DCP (0.25 μmol) and glycophorin A in chloroform–methanol–water (150:75:1, v/v) was evaporated in a rotatory evaporator and stored for more than 1 h under reduced pressure. The resulting lipid film, containing glycoprotein, was swollen in 250 μl of 100 mM UmP solution above the phase transition temperature of the PC used. Untrapped UmP and unincorporated glycophorin A were removed by centrifugation at 20000 × *g* for 15 min. The liposomes were washed by centrifugation with cold Tris-buffered saline (TBS²⁺) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM MgCl₂ and 0.15 mM CaCl₂ until the untrapped UmP content in liposomes was less than 5% of the total UmP content. The liposomes were dispersed in TBS²⁺ such that the phospholipid was 10 mM with respect to the final suspension.

Measurement of Immune-Specific UmP Release UmP release from liposomes was assayed by enzymatic hydrolysis of the compound with alkaline phosphatase to phosphate and the fluorophor, 4-methylumbelliferone, by the method of Six *et al.*[27] The method for measuring UmP release from liposomes is briefly summarized as follows. The reaction

mixture had a final total volume of 0.1 ml in a cuvette and consisted of liposomes (5 μl), antiserum (25 μl), guinea pig serum (as a complement source, 25 μl) and TBS^{2+}. After 30 min of gentle shaking on a rotating platform at room temperature, 1.9 ml of TBS^{2+} containing 0.1 U of alkaline phosphatase was added. The amount of umbelliferone generated was determined fluorophotometrically with a spectrofluorophotometer (Shimadzu Seisakusho RF-500 apparatus) at 340 nm for excitation and 448 nm for emission. The total amount of UmP trapped in liposomes was determined after adding 20 μl of 10% Triton X-100 above the phase transition temperature of the PC used. Specific UmP release was calculated as follows: percent specific marker release = (experimental release − spontaneous release) × 100/(total release − spontaneous release).

Results

Comparison of the Antibody Activity of Anti-RBC and Anti-GP Sera Figure 1 shows the relationship between the amount of guinea pig serum (as the complement source) and percent release of the marker in the presence of either anti-RBC or anti-GP. The UmP release increased with increase in the amount of guinea pig serum and reached a plateau at about 12.5 μl for both antisera. Twentyfive microliters of guinea pig serum was usually used for the following experiments on antibody-dependent immune lysis. No appreciable UmP release was observed in the absence of anti-GP or -RBC serum (Fig. 1). Further, liposomes lacking glycophorin did not release a significant amount of UmP (Fig. 1). Heating of the native guinea pig serum at 56 °C for 30 min, conditions usually employed to inactivate the hemolytic complement activity, destroyed its ability to produce UmP release in the presence of antiserum. These results indicate that in this assay system the release of UmP from the liposomes is due to complement-dependent immune lysis.

Figure 2 shows the influence of anti-RBC concentration on complement–dependent damage to eggPC/Chol or DPPC/Chol liposomes. Release of UmP from liposomes with glycophorin depended on the concentration of anti-RBC in the presence of 25 μl guinea pig serum, and moreover no appreciable difference was observed between eggPC/Chol and DPPC/Chol liposomes.

Figure 3 shows the relation between complement-dependent damage to eggPC/Chol or DPPC/Chol liposomes and anti-GP concentration. Liposomes were the

same preparations as those of Fig. 2. The UmP release by anti-GP was influenced by the liposomal phospholipid composition. The complement-dependent release of UmP from DPPC/Chol liposomes was less than that from eggPC/Chol liposomes.

To compare more precisely the effects of phospholipid composition of liposomes on the antibody activity of each serum, the sigmoid curves were transformed to straight lines by applying the von Krogh equation,[28] and these are
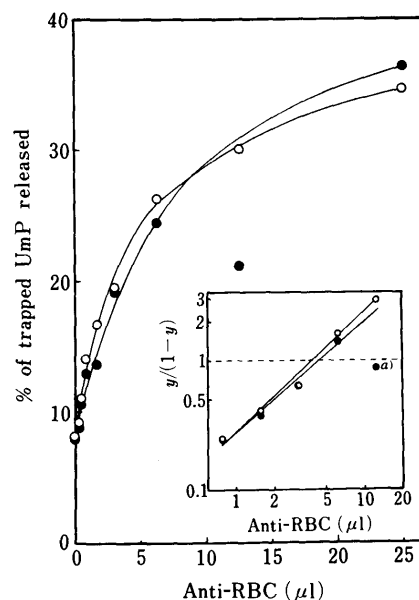
Fig. 2. Effects of Anti-RBC Concentration on Complement–Dependent Damage of eggPC/Chol Liposomes (○) and DPPC/Chol Liposomes (●)

Liposomes consisted of eggPC or DPPC, Chol, DCP (molar ratio, 10:10:1) and glycophorin A (0.06 mol% of total lipid). Complement-dependent UmP release was measured as described in Materials and Methods in the presence of antiserum in the amount indicated. The lines shown in the inset of the figure were calculated from UmP percent release after Krogh transformation as described in the text. a); This point was omitted after the Grubbs–Smirnov test.
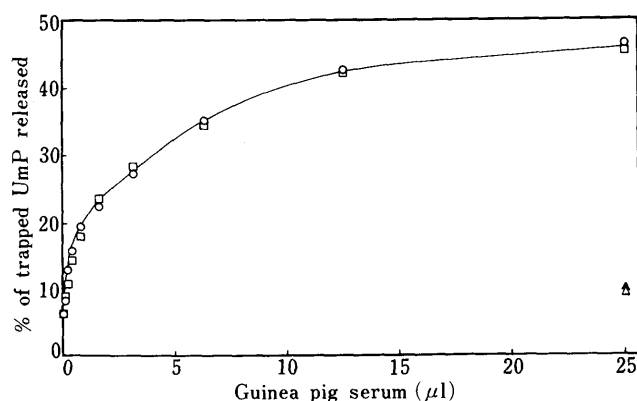

Fig. 1. Comparison of Liposomal Complement Titration Curves Obtained with Either Anti-RBC (○), or Anti-GP (□)

Liposomes consisted of eggPC, Chol, DCP (molar ratio, 10:10:1) and glycophorin A (0.06 mol% of total lipid). Complement-dependent UmP release was measured as described in "Materials and Methods" by the addition of complement in the amount indicated. Open and closed triangles indicate percent release of UmP from either the liposomes with glycophorin in the absence of antisera, or liposomes lacking glycophorin A, respectively.
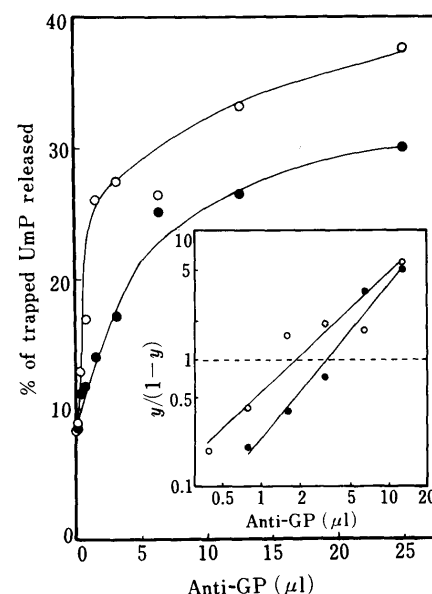

Fig. 3. Effects of Anti-GP Concentration on Complement Damage to eggPC/Chol Liposomes (○) and DPPC/Chol Liposomes (●)

The liposomes used in this experiment were the same as those for Fig. 2. Complement-dependent UmP release was determined as described in the legend to Fig. 2.
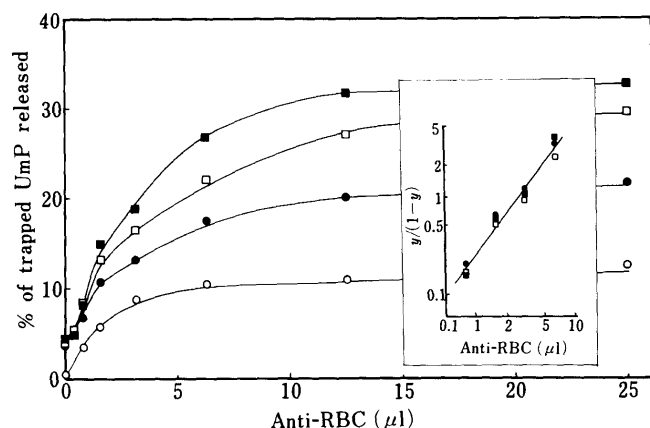
Fig. 4. UmP Percent Release for eggPC/Chol Liposomes with Different Amounts of Glycophorin

Liposomes consisted of eggPC, Chol, DCP (molar ratio, 10:10:1) and various amounts of glycophorin A (mol% to lipid, 0; ○, 0.06; ●, 0.12; □, 0.25; ■). Complement–dependent UmP release was measured as described in the legend to Fig. 2.

Fig. 5. Effect of Cholesterol Content on Complement Immune Damage to Liposomes Containing Glycophorin

Liposomes consisted of eggPC, DCP (molar ratio, 10:1), glycophorin A (0.12 mol% to PC) and various amounts of Chol (mol% to total lipid, 0; ○, 15.4; ●, 26.7; □, 42.1; ■, 59.3; ◇). Complement-dependent UmP release was measured as described in the legend to Fig. 2.

demonstrated in the insets of Figs. 2 and 3. The straight lines indicated in the figures were obtained by the least-squares method. The lines in the case of anti-RBC (Fig. 2) were identical by a test of linear regression, while the lines from anti-GP (Fig. 3) differed distinctly. The difference between the two lines in the case of anti-GP was significant (linear regression test) at $p < 0.05$. The release of UmP in the presence of 25 μl of antiserum was assumed to represent "100%" UmP release. In the case of anti-RBC (Fig. 2), the amounts of antiserum required for half-maximal UmP release (*i.e.*, when $y/(1-y) = 1.0$) were almost identical (4 μl) in both eggPC/Chol and DPPC/Chol liposomes. This suggests that the interaction of glycophorin containing liposomes with anti-RBC may not be affected by the phospholipid composition of the liposomes. On the other hand, anti-GP reacted differently against the two liposomes. Thus, two distinct lines were obtained for the UmP release by anti-GP. The half-maximal UmP release from eggPC/Chol liposomes required 1.8 μl of anti-GP, whereas that from DPPC/Chol liposomes required 3.6 μl (Fig. 3). This suggests that eggPC/Chol liposomes may be more sensitive to immune attack by anti-GP than DPPC/Chol liposomes.

The Effect of Antigen Density In general, the extent of marker release from liposomes containing glycolipids as an antigen depends on the amount of antigen incorporated.[29] We measured complement-dependent UmP release from liposomes containing various amounts of glycophorin. Figure 4 shows the release of UmP from liposomes containing various amounts of glycophorin as a function of the concentration of anti-RBC. As demonstrated in the inset of Fig. 4, the lines evaluated according to the von Krogh equation were almost all the same, suggesting that the susceptibility of eggPC liposomes to immune lysis may not be affected by the amount of glycophorin incorporated.

The Effect of Cholesterol To evaluate the influence of cholesterol content on antigenic activity of membrane glycophorin, eggPC-liposomes containing 0.12 mol% glycophorin and various amounts of cholesterol were prepared and the complement-dependent immune damage determined (Fig. 5). The release of trapped UmP from the
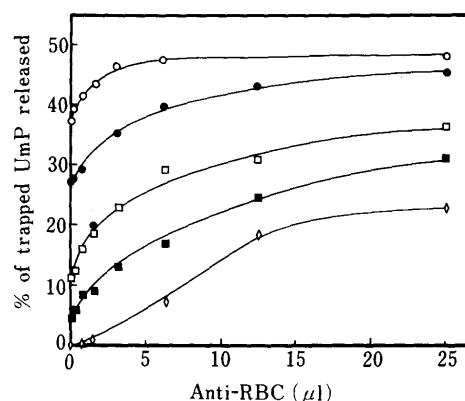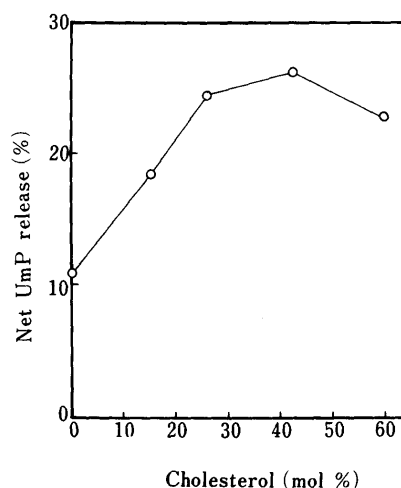

Fig. 6. Effects of Cholesterol Content on Net UmP Release from Liposomes

Net UmP release was calculated from the data shown in Fig. 5 at 25 μl of anti-RBC by subtracting UmP released in the absence of anti-RBC, and was plotted as a function of cholesterol content.

liposomes depended on the concentration of anti-RBC. However, as shown in Fig. 5, the amount of spontaneous liposomal lysis increased with decrease of cholesterol content. This is a well known effect of cholesterol on the permeability of liposomes.[30] Using the data for Fig. 5, the net UmP release, when 25 μl of anti-RBC was added, was plotted as a function of cholesterol content after subtracting the UmP released in the absence of antiserum (Fig. 6). The release of UmP increased with increasing cholesterol content and reached a plateau above 27 mol% cholesterol.

Discussion

Recombination of glycophorin with lipids according to the method of MacDonald and MacDonald,[21] which avoids the use of detergents, leads to the formation of mostly unilamellar vesicles with the protein incorporated in the bilayer. In addition, it has been demonstrated that glycophorin spans the lipid bilayer of this reconstituted system, and is predominantly oriented with its sugar-containing N-terminal portion to the exterior of the ves-

icles.[16] Thus, it is reasonable to use the antibody for the N-terminal portion which bears the sugar residues of the molecule. In the present paper, complement-dependent membrane damage to liposomes containing glycophorin was investigated with two different antisera, anti-RBC and anti-GP, which were prepared by immunization with human RBC and isolated glycophorin A, respectively. The interaction of membranous glycophorin with anti-GP depended significantly on the kind of phospholipid, while the interaction with anti-RBC did not; nevertheless both antisera have the same hemagglutinating ability. Lines obtained by Krogh transformation demonstrated that the amount of anti-GP required for half-maximal UmP release from eggPC/Chol liposomes is one-half that from DPPC/Chol liposomes (Fig. 3), while the lines for anti-RBC were identical within experimental error for both eggPC- and DPPC-liposomes. It is noteworthy that there was no appreciable difference in the lines obtained by Krogh transformation among anti-GP-DPPC/Chol interaction and anti-RBC-eggPC/Chol or DPPC/Chol interaction (Figs. 2 and 3). These facts suggest that only anti-GP recognizes the state of glycophorin in the membranes. There are several possible explanations for the difference in recognition of glycophorin by anti-GP. One possibility is that glycophorin in eggPC/Chol liposomes has a higher affinity for anti-GP than glycophorin in DPPC/Chol liposomes. This could be due to favorable topographical distribution or exposure of the antigenic site of glycophorin. Another possible factor is the population of antibodies in anti-GP, since we used polyclonal antibodies in the present experiment. It might be possible that there was enough antibody to induce only complement-dependent immune lysis but not hemagglutination, since both antisera, anti-GP and anti-RBC have the same hemagglutination titer, 2^{11}.

The relationship between the affinity of membranous antigen and the kind of phospholipid may depend on the glycolipid antigen (galactosyl ceramide and Forssman) in the bilayer.[4,5,9] Alving and Richards[9] suggested that exposure of hapten molecules may be subject to interference by surrounding phospholipids. Our group previously reported that the haptenic activity of galactosyl ceramide in liposomes was fundamentally parallel to the topographical distribution of hapten molecules.[5] Recently, this hypothesis was also supported by Grant et al.[3]

Rueppel et al.[13] observed that glycophorin was not distributed randomly in liposomal membranes, and linear alignment of glycophorin in clusters was proposed by them. Cluster formation of glycophorin in membranes was also suggested by van Zoelen et al.,[16] Sharom et al.[20] and MacDonald and MacDonald.[21] In fluid membranes, glycophorin may be distributed rather randomly, resulting in an increase of glycophorin molecules accessible to antibody. Electron spin resonance (ESR) studies of the dynamics of the oligosaccharide-bearing segment of the glycophorin molecule in bilayers[14] indicate that its motion is largely unrestricted and there is no strong evidence for interaction with phospholipid head groups. Therefore, we do not favor Alving and Richards'hypothesis that the effect of liposomal phospholipid composition may be related to steric hindrance to the antigenic site by surrounding phospholipids.

Anti-RBC could not recognize the difference in glyco-phorin distribution on the two liposomal membranes. This contradiction might be due to a difference in the recognition site for antigen between anti-RBC and anti-GP. It can be assumed that the recognition site of glycophorin by anti-GP is located closer to the membrane surface than that by anti-RBC.

Cholesterol in the lecithin membranes influenced the antigenicity of glycophorin. It has been reported that the antigenic activity of lipid haptens on the liposomal membranes changes with cholesterol content.[31] Our group found that cholesterol regulated both the topographical distribution of the hapten molecules and the rotational motion of the haptenic site, resulting in enhancement of the immune reaction by cholesterol.[6] The distribution of glycophorin on eggPC liposomes might be influenced by the presence of cholesterol. It is tempting to speculate that the ability of lipid rigidification to increase antigenic activity was, in this case, due to the known tendency of glycophorin to phaseseparate out of the rigid region of the membrane,[17,19,20,32] since this would increase local antigen density in the plane of the membrane. However, it should be mentioned that the effects of host lipids on glycophorin were much less than on glycolipid haptens.

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