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REGULATION BY GLYCOPHORIN OF COMPLEMENT ACTIVATION VIA THE ALTERNATIVE PATHWAY

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SUMMARY: The effect of glycophorin on complement activation via the alternative pathway was examined by incorporating it into the liposome membrane with trinitrophenylaminocaproyldipalmitoylphosphatidylethanolamine (TNP-Cap-DPPE). Liposomes having incorporated TNP-Cap-DPPE onto the membrane activate the alternative complement pathway of guinea pig as reported previously, and the additional insertion of glycophorin was found to reduce their activating capacity on the alternative complement pathway. This inhibitory effect was cancelled by pretreatment of the glycophorin-containing liposomes with neuraminidase indicating that the sialic acid in glycophorin is playing a role in the regulation of alternative complement pathway-activation on the biological membrane.

We have previously reported that liposomes containing 2,4,6-trinitrophenylaminocaproyldipalmitoylphosphatidylethanolamine (TNP-Cap-DPPE) in their membrane can activate the alternative complement pathway (ACP) of guinea pig in the absence of antibody reaction (1). More recently, we found that the complement activating capacity of the liposomes containing TNP-Cap-DPPE (TNP-Cap-liposomes) was inhibited by the additional insertion of sialoglycolipids, such as ganglioside GM3, but not by the asialo-type glycolipids (2). This result led us to investigate the effect of glycophorin on the ACP-activating capacity

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Abbreviations: TNP-Cap-DPPE, trinitrophenylaminocaproyldipalmitoylphosphatidylethanolamine; ACP, alternative complement pathway; TNP-Cap-liposomes, liposomes containing TNP-Cap-DPPE; GVB, gelatin veronal buffered saline; GVB^{++}, GVB supplememted with $CaCl_2$ and $MgCl_2$; EGTA, ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetraacetate; Mg-EGTA-GVB, GVB supplemented with $MgCl_2$ and EGTA; EDTA-VBS, veronalbuffered saline containing EDTA; GPS, Hartley guinea pig serum; C4D-GPS, C4 deficient guinea pig serum; DMPC, L-α-dimyristoylphosphatidylcholine; DPPE, L-α-dipalmitoylphosphatidylethanolamine; Chol, cholesterol; CF, carboxyfluorescein.

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of TNP-Cap-liposomes since glycophorin is the major sialoglycoprotein comprising the erythrocyte membrane. This line of study may open a way to better understanding of the phenomenon wherein neuraminidase treated sheep erythrocytes become reactive on the ACP (3,4) from a chemical standpoint. Assessment of ACP activation on the liposome membrane was made by measuring the amount of fluorescent marker release due to liposome lysis as a result of complement reaction according to the method described elsewhere (1).

MATERIALS AND METHODS

Diluents: Gelatin veronal buffered saline (GVB) containing 0.1 % gelatin, 10 mM veronal buffer (pH 7.4) and 148 mM NaCl was used as a base solution for complement reaction. GVB^{++} was GVB supplemented with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Mg-EGTA-GVB was GVB supplemented with 2 mM MgCl and 10 mM ethyleneglycol-bis(β-aminoetyl ether)N,N'-tetraacetate (EGTA). EDTA-GVB was GVB containing 40 mM ethylenediaminetetraacetate (EDTA). EDTA-VBS was a veronal buffered saline containing 10 mM EDTA (pH 7.4).

Serum: Fresh sera from Hartley guinea pigs (GPS) and from C4 deficient guinea pigs (C4D-GPS) were aliquated and stored at -70°C until use.

Materials: L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Calbiochem. Co.,La Jolla, CA. Cholesterol (Chol) was purchased from Sigma Chemicals Co., St. Louis, Mo. TNP-Cap-DPPE was synthesized as described previously (1). Glycophorin preparation from human erythrocytes was prepared according to the method of Marchesi and Andrews (5) with a slight modification (Utsumi et al., in prepartion).

Preparation of liposomes: The standard composition of the loposome membrane was an equimolecular mixture of DMPC and Chol, and when required, TNP-Cap-DPPE was added at a molar ratio of 0.05 to DMPC. The lipid mixture dissolved in chloroform was dried to a film by rotary evaporation, and liposomes containing 0.2 M carboxyfluorescein (CF)(Eastman Kodak, Rochester, N.Y.) were prepared as described previously (1,6). To incorporate glycophorin onto the liposome membrane, the method described by MacDonald and MacDonald (7) was used with slight modification. Briefly, 50 μl of glycophorin dissolved in water at 5 mg/ml was added with 200 μl of methanol and mixed with a lipid mixture (100 μl of 5 mM DMPC, 50 μl of 10 mM Chol and 25 μl of 1 mM TNP-Cap-DPPE, or 100 μl of 5 mM DMPC and 50 μl of 10 mM Chol). Following evaporation of solvent with a rotary evaporator at room temperature , 0.2 M CF was added and processed to prepare liposomes in the same manner as for liposomes without glycophorin (1,6). Liposomes were suspended in GVB at a concentration corresponding to 0.05 mM phosphorus of DMPC in the liposomes.

Neuraminidase treatment of liposomes with glycophorin: Liposomes associated with glycophorin prepared as above were suspended in GVB at pH 6.0 (instead of pH 7.4) and 1.0 ml of this liposome suspension was incubated with 0.2 ml of 1 unit/ml neuraminidase from Vibrio comma (Behringwerke, Marburg, W. Germany) at 37°C for 30 min. After incubation, the neuraminidase treated liposomes were washed with, and suspended in GVB (pH 7.4).

Liposome lysis determination: Liposome lysis was determined by the amount of CF released as described previously (1,6). To 50 μl of a serial two-fold dilution of GPS or C4D-GPS in Mg-EGTA-GVB, 5 μl of liposome suspension was added and incubated at 25°C for 60 min. Fluorescent intensity of CF released was determined following addition of 2.0 ml EDTA-VBS by excitation at 490 nm

and emission at 520 nm. The extent of liposome lysis was expressed as the corrected % marker release which was calculated as a percentage of the maximum CF release by antibody and complement-mediated liposome lysis as described elsewhere (1). Briefly, the maximum release was determined by lysing liposomes with a 1/10 dilution of GPS in the presence of heat inactivated rabbit antiserum to TNP in GVB^{++}. GVB^{++} was used as a diluent for the maximum release to permit complement activation via the classical pathway. The maximum release was approximately 60 % of the total releasable CF in the liposomes by Triton X-100 treatment (1).

RESULTS AND DISCUSSION

The reactivity with GPS in Mg-EGTA-GVB of TNP-Cap-liposomes associated with glycophorin (TNP-Cap-liposomes-Gp) was determined for comparison to that of TNP-Cap-liposomes without glycophorin. Mg-EGTA-GVB was used as the diluent in order to inhibit complement reaction via the classical pathway, which requires Ca^{++}, but not the ACP reaction. The reactivity of TNP-Cap-liposomes-Gp was markedly lower than that of liposomes without glycophorin (Fig.1) indicating that glycophorin has an inhibitory effect on ACP activation on the TNP-Cap-liposomes. On the other hand, pretreatment of the TNP-Cap-liposomes-Gp with neuraminidase restored the ACP-activating capacity as shown in Fig. 1.

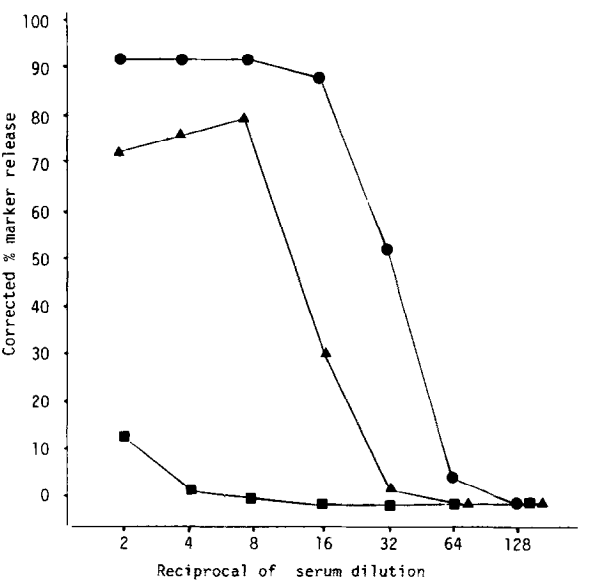

Figure 1. Lysis by GPS in Mg-EGTA-GVB of TNP-Cap-liposomes associated with (■) or without (●) glycophorin. TNP-Cap-liposomes associated with glycophorin treated with neuraminidase (▲) were also examined. TNP-Cap-liposomes with glycophorin were hardly lysed by GPS but neuraminidase treatment of the liposomes partially restored the reactivity with GPS in Mg-EGTA-GVB. The 50 % lysis unit (a reciprocal dilution of serum caused 50 % lysis) of GPS on the neuraminidase treated TNP-Cap-liposomes with glycophorin was 15.3 while that of TNP-Cap-liposomes without glycophorin was 38.6. Similar results were obtained using C4D-GPS in place of GPS.

TABLE I
Effect of glycophorin added to the reaction mixture of TNP-Cap-liposomes and guinea pig complement

TNP-Cap-liposomes mixed with	50 % lysis unit*
-	20.5
Glycophorin 1.0 mg/ml**	17.6
0.3 mg/ml	19.5
0.1 mg/ml	18.9
(TNP-Cap-liposomes-Gp)	4.2

* Reciprocal dilution of serum caused 50 % corrected marker release.
** Final concentration.

These results indicated that sialic acid residues of the glycophorin associated onto the liposome membrane was responsible for the inhibition of TNP-Cap-mediated ACP activation on the liposomes, and that the removal of the sialic acids by neuraminidase treatment cancelled this inhibitory capacity. However, it was also conceivable that neuraminidase treated glycophorin might have gained the capacity to activate the ACP directly, independent of the TNP-Cap-mediated activation. To examine this possibility, liposomes composed of DMPC, Chol and glycophorin without TNP-Cap-DPPE were prepared and treated with neuraminidase. These liposomes were incubated with GPS or C4D-GPS in Mg-EGTA-GVB to test their reactivity with complement in the absence of TNP-Cap-DPPE. None of the liposomes were lysed by GPS or C4D-GPS in Mg-EGTA-GVB, indicating that the incorporation of neither glycophorin nor of neuraminidase-treated glycophorin alone onto the liposome membrane does not have the capacity to activate the guinea pig ACP.

We have found that the presence of sialoglycolipids was required on the same liposome membrane of TNP-Cap-liposomes for inhibition of ACP activation by the liposomes (1). Sialoglycolipids inserted onto separate liposomes had no inhibitory effect on the ACP activation by TNP-Cap-liposomes coexisting in the same reaction mixture. Glycophorin was also unable to inhibit ACP activation by TNP-Cap-liposomes when it was added separately to the reaction mixture even at a final concentration of as high as 1.0 mg/ml (Table 1).

Since TNP-Cap-liposomes activate the classical complement pathway (CCP) of guinea pig as well as the ACP (1), we also examined the effect of glycophorin-

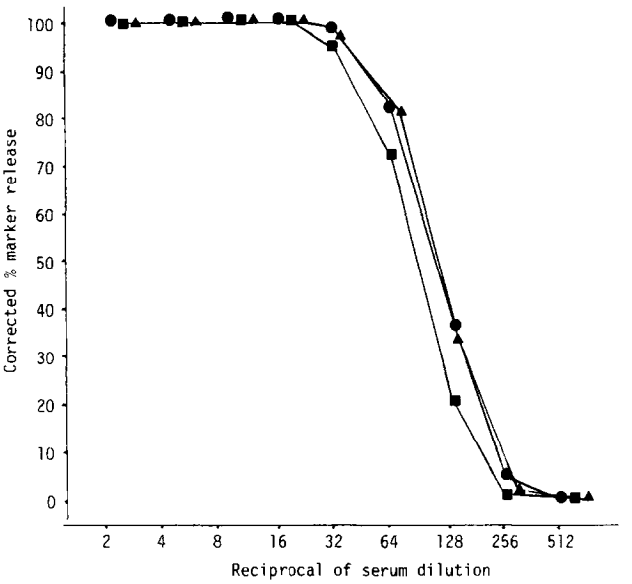

Figure 2. Lysis by GPS in GVB of TNP-Cap-liposomes associated with (■) or without (●) glycophorin, and neuraminidase pretreated TNP-Cap-liposomes containing glycophorin (▲). GVB^{++} was used as a diluent to permit the activation of complement via the classical pathway as well as the alternative pathway. Presence of glycophorin on membrane scarcely, if any, inhibited lysis of TNP-Cap-liposomes by GPS in GVB^{++}. The reciprocal dilution of GPS required to cause 50 % lysis of TNP-Cap-liposomes without glycophorin was 140 while those of TNP-Cap-liposomes with glycophorin and neuraminidase pretreated TNP-Cap-liposomes with glycophorin were 107 and 141, respectively.

incorporation on the lysis of TNP-Cap-liposomes via activation of the CCP. TNP-Cap-liposomes-Gp, TNP-Cap-liposomes-asialo-Gp and TNP-Cap-liposomes were incubated with GPS diluted in GVB^{++}. As shown in Fig. 2, the presence of glycophorin or asialo-glycophorin on TNP-Cap-liposomes did not affect the liposome lysis by guinea pig serum in GVB^{++}. This sharply contrasted to the remarkable inhibitory effect of glycophorin on the ACP activation in GPS on the membrane. Similar results were also noted on sialoglycolipids when they were incorporated onto TNP-Cap-liposomes (2). Taking these evidinces together sialoglycoproteins and sialosylglycolipids are thought to be playing a role in restricting the undesirable amplification reaction of the ACP on cell membranes.

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REFERENCES

1. Okada, N., Yasuda, T., Tsumita,T. and Okada, H. (1982) Immunology 45: 115-124.
2. Okada, N., Yasuda, T., Tsumita,T. and Okada, H. (1982) Immunology,in press
3. Fearon, D.T. (1978) Proc. Natl. Acad. Sci. USA. 75: 1971-1975.
4. Pangburn, M.K. and Müller-Eberhard, H.J. (1978) Proc. Natl. Acad. Sci. USA 75: 2416-2420.
5. Marchesi, V.T. and Andrews, E.P. (1971) Science, 174: 1247-1248.
6. Yasuda, T., Naito, Y., Tsumita, T. and Tadakuma, T. (1981) J. Immunol. Methods, 44: 153-158.
7. MacDonald, R.J. and MacDonald, R.C. (1975) J. Biol. Chem. 250: 9206-9214.