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Specific Rejection of Glycophorin-Reconstituted Liposomes by Human Phagocytes

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The interaction between human phagocytes and glycophorin-reconstituted liposomes was investigated from the view point of internalization efficiency of the liposome by phagocytes. The glycophorin-reconstituted liposome was found to be rejected by phagocytes in the internalization. When the sialic acid residues of glycophorin were partly eliminated by neuraminidase, however, the cell uptake increased about two times.

Saccharide moiety of glycolipids and glycoproteins in cell membranes is well known to play an important role as the recognition site at cell surface in the cell-cell adhesion or the information transfer between cells. Gangliosides were found to be strongly correlated with the cell differentiation and carcinogenesis.[1-4] In particular, when sialic acid moiety on the surface of sheep erythrocytes was removed by neuraminidase, the internarization of erythrocytes by human monocytes increased.[5] Naturally occurring polysaccharides, which were employed to coat the outermost surface of liposomes as an artificial cell wall for an artificial cell, were found to behave as a sensory device of the liposome toward specific cells[6] and a specific tissue.[7] For example, amylopectin or mannan derivative-coated liposomes were shown to be effectively internalized by alveolar macrophages, neutrophiles, and monocytes.[6] Furthermore, they were highly distributed at lung by the receptor-mediated targeting mechanism after intravenous administration.[7]

In this communication, we would like to demonstrate importance of the sialic acid moiety, as assembled on the liposomal surface, in the interaction between the liposomes and human phagocytes, monocytes and neutrophiles. For this purpose, glycophorin has been isolated from human erythrocytes and reconstituted into egg phosphatidylcholine(PC) liposomes according to basically the method of MacDonald,[8] and by employing an artificial boundary lipid, 1,2-dimyristoylamide-1,2-deoxyphosphatidylcholine(DDPC), which has been newly developed in this laboratory.[9,10] Internalization efficiency of the glycophorin-reconstituted

liposomes into human monocytes and neutrophiles was investigated by employing two different methods, flow cytofluorometry[11] and radioactive isotope(RI) technique.[12] For the former method, carboxyfluorescein(CF)-loaded large unilamellar vesicles (LUV)[13] were prepared according to essentially the same method as that employed by Bangham.[14] Uncapsuled CF and glycophorins were removed by centrifugation at 35000 × g and 4 °C for 30 min. The residual pellets were swollen by phosphate buffered saline (PBS). For the RI investigation, multilamellar vesicles (MLV) labelled by [^{14}C]- dipalmitoyl PC (New England Nuclear) were prepared.[15] Human monocytes and neutrophiles were obtained by the following method. Heparinized venous blood from healthy adult was mixed with an equal volume of 3% dextran in isotonic saline and red blood cells were separated by sedimentation at 25 °C for 25 min. Leukocytes-rich supernatants so obtained were centrifuged at 400 × g, 25 °C on Ficoll-Conray density gradients. Neutrophiles were collected as sediments at the bottom layer, while a mixture of monocytes and lymphocytes were obtained from the middle layer. Each separated cells were respectively washed twice with and resuspened in RPMI-1640 medium containing 15% FCS.

First of all, we investigated the internalization efficiency of neutrophiles by flow cytofluorometry on a JASCO FCM-1S.[16] LUV were coincubated with neutrophiles in RPMI-1640 medium containing 15% FCS in 5% CO_2 -95% air incubator by employing a gyratory shaker at 37 °C for 60 min. Number of LUV was kept constant at 4×10^6 vesicles/cell, while that of cells was 2.0×10^6 in 1.0 ml of the medium. After incubation, unphagocyted vesicles were separated by centrifugation at 250 × g and 4 °C for 10 min, cell pellets were resuspended in PBS, and the cell suspensions so obtained were submitted to the measurement. The fluorescence intensity distribution was measured as a function of cell size and the data of the region corresponding to monocyte was analyzed (Table 1).

Table 1. Flow cytofluorometric measurements of the internalization efficiency of liposomes into human neutrophiles.

Liposomes	Internalization efficiency[a] /%
Conventional LUV	43.5
Pig mucin-reconstituted LUV	0.2
Human glycophorin-reconstituted LUV	0.3
Control[b]	0

a) Percent ratio of the liposomes internalized by neutrophiles (within 1h at 37 °C) to the liposomes initially adopted. The ratio of vesicles to a cell was 4.0×10^6. Number of vesicles were estimated on the basis of lipid concentration.[13]
b) An aqueous buffered solution of carboxyfluorecein was adopted.

Very interestingly, both the human glycophorin and pig mucin[17]-reconstituted liposomes, which are bearing sialic acid residues on the liposomal surface, were significantly rejected by neutrophile and the internalization efficiency was decreased compared with the conventional liposome. In our previous investigation on the polysaccharide-coated liposomes, mannan-coated liposome was more

effectively internalized by phagocytes than conventional liposome without polysaccharide coat. This increased internalization efficiency was attributable to the receptor-mediated uptake by phagocytes.[18] Judging from these previous results, it seems reasonable to consider that the rejection observed in this work may be responsible for the existence of sialic acid moieties, on the liposomal surface, recognizable by neutrophiles.

Secondly, a liposomal suspension (50.0 μl, 1.5 × 10^{11} vesicles) was added to a monocytes suspention (1.0 ml, 1.5 × 10^6 cells/ml) in RPMI-1640 containing 10% FCS. After the incubation at 37 °C for 30 min, the cell suspension was centrifuged at 300 × g for 10 min at 25 °C. In order to completely eliminate liposomes attached on the cell surface, velocity-gradient centrifugation was carried out in silicon oil; namely, cell pellets obtained above were resuspended in PBS (0.5ml) and the resulting cell suspension was tenderly transfered to a silicon oil-containing micro test tube (1ml vol.). The density of silicon oil was correctly adjusted by mixing six parts of No. DC500(d=1.07) and five parts of No. DC556(d=0.980) by vol. The tubes were centrifuged at 12000 × g and 25 °C for 3 min. Cells were collected as sediments in a silicon oil layer at the bottom of the tube and the upper layer was containing both culture medium and free liposomes. The tubes were frozen at -40 °C, and cut by knife. They were respectively dissolved in ACS II and submitted to scintilation counting on a ALOKA-550.[16] Numbers of liposomes phagocyted by human monocytes within 30 min at 37 °C are given in Fig. 1. MLV-0 stands for the conventional MLV and MLV-2 refers the glycophorin-reconstituted one. Clearly, the internalization efficiency of liposomes by monocytes were decreased by the reconstitution of glycophorin.

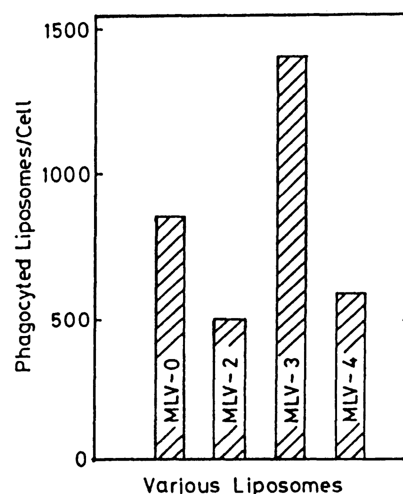

Fig. 1. Internalization Efficiency of Various Liposomes into Human Monocytes at 37 °C for 30 min (see detail in the text).

Another interesting finding in this work is the effect of structure of the terminal saccharide moiety of glycophorin. The neuraminidase treatment of glycophorin alters the terminal saccharide residue of the protein from sialic acid to galactose and the acetylgalactosaminidase treatment eliminates all the saccharide moieties from the protein.[10] When the MLV-2 was treated by neuraminidase (EC.3.2.1.18, Seikagaku Kogyou, 0.05U) at 25 °C for 2 h, nevertheless only approx. 25% of sialic acid residues were eliminated, the internalization of the obtained liposomes (denoted as MLV-3 in Fig. 1) into monocyte drastically increased. When the MLV-2 was treated with α-N-acetylgalactosaminidase (EC.3.2.1.49, Seikagaku Kogyou, 0.01U) at 25 °C for 2 h to afford MLV-4 (54% of saccharide moieties bearing sialic acid terminals were remained and 46% of saccharide moieties were eliminated off), the monocyte uptake of MLV-4 by decreased compared with MLV-3. The increased internalization of the

MLV-3 must be responsible for molecular recognition of galactose moiety on the liposomal surface by a specific receptor in the monocyte membrane.[10] This concept is supported by the evidence that the MLV-4 was also rejected in the monocyte uptake. The results obtained in this work are closely related with those obtained in our previous investigation on the specific lectin-induced aggregation of these glycophorin-reconstituted liposomes.[10]

These results suggest us that the saccharide moiety of glycophorin is important in cell-cell recognition, especially between red blood cells and white cells. In addition, the present study will provide a strategy for making liposome more cell specific on the basis of molecular recognition mechanism.

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15) One MLV (for the RI study) bears total 8.0×10^{-9} mol of glycophorin in the outermost leaflet of the vesicle. These were estimated from determinations of both protein contents[a] and sialic acid analysis[b] ; a) O. H. Lowrey, N. J. Rowebrough, A. L. Farr, and R. T. Randall, J. Biol. Chem., 193, 265 (1951). and P. Bohlen, S. Stein, W. Dairman, and S. Udenfriend, Arch. Biochem. Biophys., 155, 213 (1973); b) G. W. Jourdain, L. Dean, and S. Roseman, J. Biol. Chem., 246, 430 (1971).
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