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GLYCOLIPIDS ARE NOT EXTRACTED FROM PHOSPHOLIPID BILAYERS BY BINDING TO FERRITIN-LECTIN CONJUGATES

RHODERICK E. BROWN [a], MARGARETTA ALLIETTA [b], THOMAS W. TILLACK [b] and THOMAS E. THOMPSON [a]

Departments of [a] Biochemistry and [b] Pathology, University of Virginia School of Medicine, Charlottesville, VA 22908 (U.S.A.)

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A radioactively-labelled glycosphingolipid, asialo-G_{M1}, has been incorporated into phosphatidylcholine multilamellar vesicles. After incubation with ferritin-*Ricinus communis* agglutinin 60 (RCA 60) conjugate at different temperatures, the vesicles were separated from the conjugate by discontinuous density gradient ultracentrifugation. Measurement of the distribution of the radioactively-labelled asialo-G_{M1} in the pelleted conjugate fraction and freeze-etch electron microscopy of the vesicle fraction indicate that the decrease in labelling of asialo-G_{M_1}-containing vesicles by ferritin-RCA 60 conjugate with increasing temperatures (Tillack, T.W., Wong, M., Allietta, M. and Thompson, T.E. (1982) Biochim. Biophys. Acta 691, 261–273) reflects a decrease in apparent binding affinity rather than an ability of the conjugate to extract glycolipid from the phospholipid bilayer after binding.

The morphological organization of biological and model membranes can be readily examined by the freeze-etch technique [1–5]. However, defining the ultrastructural localization of particular components in the plane of the membrane often depends on the binding specificity of electron dense markers [6,7]. For example, an affinity-purified monovalent ferritin conjugate of *Ricinus communis* agglutinin 60 (RCA 60 or ricin) was recently utilized to determine the distribution of the neutral glycosphingolipid, asialo-G_{M_1}, in phosphatidylcholine multilamellar vesicles [8]. During the course of these studies, a correlation was noted between the total amount of ferritin-RCA 60 conjugate bound per unit area of lipid bilayer and the temperature that was utilized during conjugate incubation with the glycolipid-containing vesicles. A greater density of labeling of asialo-G_{M_1} molecules in the phosphatidylcholine vesicles was always observed as temperature decreased. Two different mechanisms of glycolipid-conjugate interaction could produce such a result: (i) The relatively large, water soluble ferritin-RCA 60 conjugate (approx. 100 Å diameter) might extract the glycolipid from the phospholipid bilayer after the binding event. This idea has been suggested as an explanation for the decreased binding of native ricin to human erythrocytes at 20°C compared to 4°C [9]. The facility of this extraction process would be expected to depend upon the physical state and organization of the glycosphingolipid in the phospholipid bilayer. (ii) The apparent binding affinity of the ferritin-RCA 60 conjugate for glycolipid may simply change with temperature. The following experiments were performed to differentiate between the two possibilities mentioned above.

Radioactively-labelled asialo-G_{M_1} (69.6 μCi/μmol) was prepared by treatment of the glycolipid with galactose oxidase and subsequent reduction with tritiated potassium borohydride [10]. Large

Abbreviations: DMPC, dimyristoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; RCA 60, *Ricinus communis* agglutinin 60 or ricin.

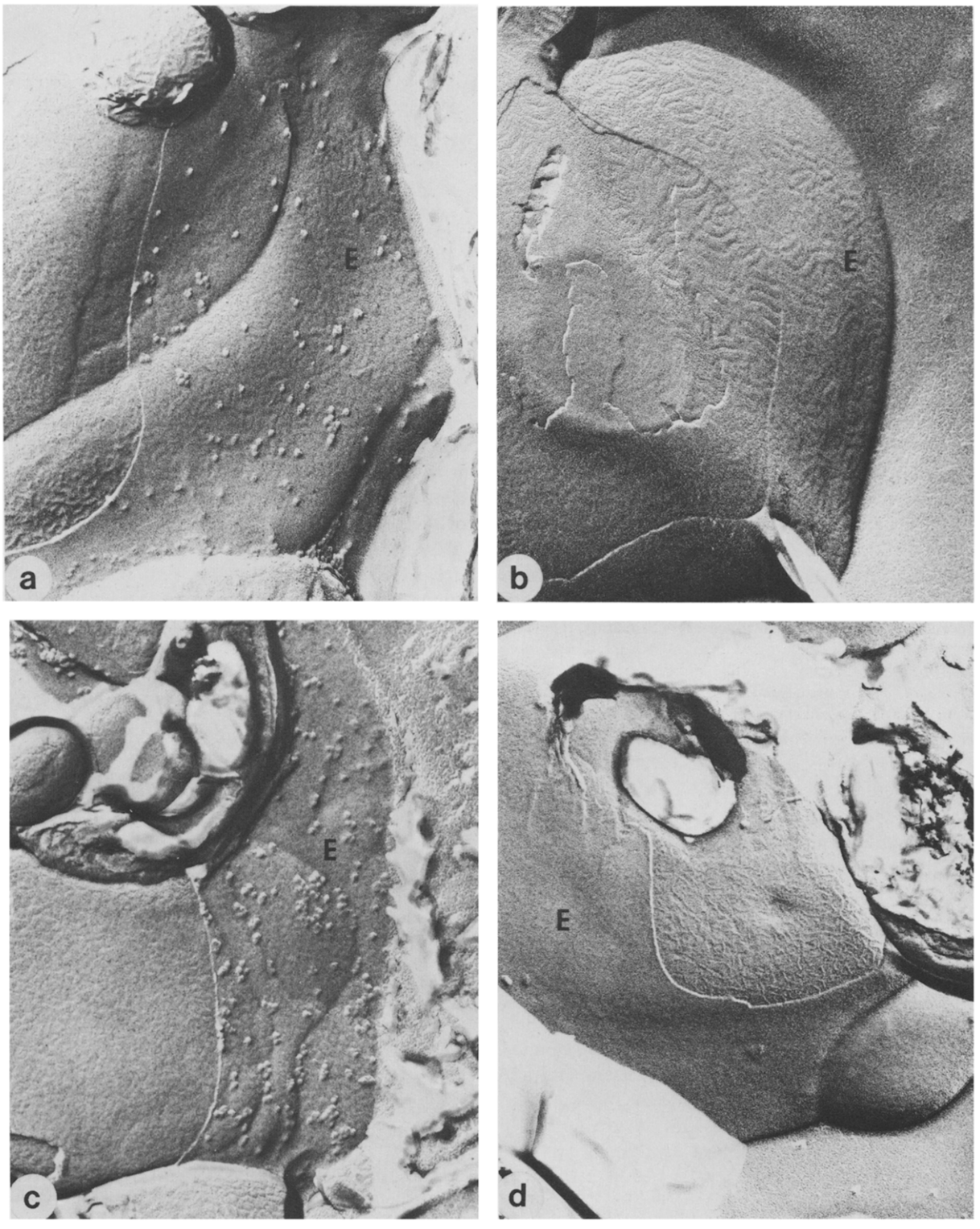
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TABLE I

AMOUNT OF ASIALO-G_{M_1} ASSOCIATED WITH FERRITIN-RCA 60 CONJUGATE AFTER CENTRIFUGATION

The ferritin-RCA 60 conjugate was separated from the [^{3}H]asialo-G_{M_1} containing vesicles (approx. 150000 dpm) after incubation for 30 min at the indicated temperature as described in the text. The ferritin-RCA 60 conjugate was examined for the presence of [^{3}H]asialo-G_{M_1} by liquid scintillation analysis. Control experiments indicated that the ferritin-RCA 60 conjugate produced no changes in the efficiency of tritium counting.

		% vesicles recovered at gradient interface [a] (%)	Conjugate recovered in pellet [b]		Theoretical amount AG_{M_1} that could be bound by pelleted conjugate [c] (nmol)	Observed amount AG_{M_1} bound by pelleted conjugate	
			nmol	%		nmol	%
23°C							
DMPC	(D) [d]	99	3.20	64	–	–	–
DMPC-AG_{M_1}	(M) [d]	99	3.10	62	3.10	0.18	5.8
DMPC-AG_{M_1}	(D)	99	3.25	65	6.50	0.08	1.3
37°C							
DMPC	(D)	99	3.20	64	6.40	–	–
DMPC-AG_{M_1}	(D)	87	3.05	61	6.10	0	0
POPC	(D)	93	3.05	61	6.10	–	–
POPC-AG_{M_1}	(D)	99	2.90	58	5.80	0	0

[a] The recovery of vesicles was calculated from the [^{3}H]asialo-G_{M_1} present. In control vesicles which contained no asialo-G_{M_1}, either [^{3}H]DMPC or [^{14}C]POPC was utilized.

[b] The recovery of conjugate was estimated from absorbance measurements (molar extinction coefficient of ferritin at 440 nm is 1.35 $cm^{-1} \cdot mg^{-1} \cdot ml$). The total amount of conjugate mixed with the vesicles was 5 nmol.

[c] Approx. 17.5 nmol of asialo-G_{M_1} was present in the outer monolayer of the vesicles and accessible to conjugate binding. However, the maximum amount of AG_{M_1} that could be bound was limited by the amount of conjugate added to (5 nmol) and recovered from (column II) the gradients.

[d] (D) refers to divalent and (M) to monovalent ferritin-RCA 60 conjugate.

multilamellar vesicles containing 7 mol% asialo-G_{M_1} and [^{3}H]asialo-G_{M_1} (approx. 0.1 μCi) were prepared from dimyristoylphosphatidylcholine (DMPC) or palmitoyloleoylphosphatidylcholine (POPC) as described previously [8]. The vesicles were allowed to equilibrate at temperatures of 23°C or 37°C for 24 h. After addition of ferritin-RCA 60 conjugate [8], incubations were continued for 30 min at 23°C or 37°C. The absolute amount of conjugate added was 70-fold less than the amount of glycolipid present. However, if one assumes that only 5% of the glycolipid present in multilamellar vesicles is accessible to the ferritin-RCA 60 conjugate [11], a 3.5-fold excess of conjugate binding sites was present. Therefore, all of the ferritin-RCA 60 conjugate had the opportunity to bind to glycolipid during the incubation. The conjugate-vesicle preparations were then layered onto discontinuous sucrose gradients (10%/54% (w/v)) and centrifuged for 18 h at 23°C or 37°C (110000 × g). The vesicles were recovered from the 10%/54% gradient interface; whereas, the ferritin-RCA 60 conjugate pelleted to the bottom of the tubes. After overnight dialysis to remove the

Fig. 1. Freeze-etch electron micrographs of multilamellar liposomes containing 7 mol% [^{3}H]asialo-G_{M_1} labelled with ferritin-RCA 60 and quenched from 37°C: (a) DMPC liposome before sucrose-gradient centrifugation; (b) DMPC liposome after sucrose-gradient centrifugation; (c) POPC liposome before sucrose-gradient centrifugation; (d) POPC liposome after sucrose-gradient centrifugation. E indicates the external surface of the liposome revealed by deep-etching. The ferritin-RCA 60 conjugate present on the external surface before sucrose gradient centrifugation is completely removed as a result of centrifugation. Magnification is approx. 70000×.

sucrose, aliquots of the vesicle and conjugate fractions were prepared for liquid scintillation and freeze-etch analyses [8].

Table I shows the amount of asialo-G_{M_1} that is bound to the ferritin-RCA 60 conjugate after the incubation and centrifugation procedures. The actual amount of asialo-G_{M_1} bound to the conjugate is almost zero compared to the theoretical maximum amount of asialo-G_{M_1} that could be bound by the known amount of conjugate present. From Table I, it is evident that the ferritin-RCA 60 conjugate is not capable of removing the asialo-G_{M_1} from phosphatidylcholine vesicles at 23°C or 37°C. Furthermore, use of divalent ferritin-RCA 60 conjugate did not change the result initially obtained with monovalent ferritin-RCA 60 conjugate. However, since only 58–65% of the conjugate was recovered in the pellet, the vesicles were examined by electron microscopy to determine how much of the unaccounted for conjugate remained bound to the vesicles. Freeze-etch replicas of the glycolipid containing vesicles recovered at the gradient interface (Fig. 1) showed no ferritin-RCA 60 conjugate remaining bound. Furthermore, the recovery of conjugate in the pellet from control vesicles which contained no asialo-G_{M_1} also ranged from 61–64% (Table I). These facts indicated that the unaccounted for conjugate was either distributed throughout the 54% sucrose of the density gradient or remained adsorbed to the bottom of the cellulose nitrate centrifugation tubes and the cellulose dialysis tubing. Control experiments indicated that the latter possibility was most likely.

The poor binding of conjugate observed at 37°C compared to the binding observed at lower temperatures [8] cannot be explained by removal of binding sites (asialo-G_{M_1}) from the vesicle bilayer by the conjugate molecule. Rather, the binding affinity of the ferritin-RCA 60 conjugate for asialo-G_{M_1} must be dependent on temperature. It is this temperature dependent change in conjugate binding affinity that causes the total amount of ferritin-RCA 60 conjugate bound on asialo-G_{M_1} containing vesicles to change as a function of temperature [8]. Additional support for this finding is provided by recent studies on the temperature dependence of *Ricinus communis* 120 agglutinating activity [12,13]. In these studies, the extent of agglutination of phosphatidylcholine vesicles containing the glycosphingolipid, lactosyl ceramide, was found to decrease as the temperature increased above 25°C.

The anchoring of asialo-G_{M_1} into the phosphatidylcholine bilayer must depend on the physical state of the two lipid components of the bilayer. Thus, the glycolipid, if molecularly dispersed in the bilayer, would be expected to be more firmly anchored into a gel state than into a liquid-crystalline state phospholipid bilayer. Alternatively, if the glycosphingolipid is organized into gel-like domains in a liquid-crystalline phospholipid bilayer, then extraction of the glycolipid would also be very difficult. There is considerable evidence that a simpler neutral glycosphingolipid, glucosylceramide, is in fact present in microdisperse gel state domains in liquid-crystalline phosphatidylcholine bilayers [14,15]. The firm anchoring of asialo-G_{M_1} into the phosphatidylcholine bilayer at a temperature at which the phospholipid is in the liquid-crystalline state suggests that asialo-G_{M_1} may also be organized into microdisperse gel state domains in this bilayer. Preliminary support for this interpretation has been obtained from measurements of the rate of interbilayer transfer of asialo-G_{M_1} (16).

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