Glycolipid-dependent agglutination of liposomes by *Croton tiglium* lectin

Kalyan K. Banerjee and A. Sen*

Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, Calcutta 700 009, India

Received 16 August 1983

Croton tiglium lectin, a protein with hemagglutinating and hemolytic activities and specific for complex carbohydrates only, agglutinates phospholipid–glycolipid vesicles in the presence of 1 mM $CaCl_2$. The agglutination of liposomes, which is not affected by phospholipid composition and ionic strength, is completely inhibited by trypsin-released glycopeptides from sheep erythrocyte surface indicating that the phenomenon is mediated by lectin–carbohydrate interactions. Since the lectin-reactive glycolipids all carry the sequence Gal–Gal or their *N*-acetylated derivatives as the common structural denominator, it appears that the disaccharide unit Gal–Gal or their *N*-acetylated derivatives constitute an essential part of the carbohydrate hapten of the lectin. Lack of evidence for non-carbohydrate-dependent hydrophobic interaction of CTL with phospholipids and glycolipids lends support to the view that hemolysis is also a carbohydrate-dependent function of the lectin.

Liposome agglutination Croton tiglium *lectin* *Glycolipid dependence*

1. INTRODUCTION

In [1], we reported the purification and properties of a non-toxic lectin from the seeds of *Croton tiglium* Linn., which agglutinated sheep and cow erythrocytes and lysed rabbit erythrocytes. While the lectin was not inhibited by simple sugars, trypsin-released glycopeptides (trypsin fragments) from sheep erythrocyte surface inhibited both hemagglutination and hemolysis, suggesting that the carbohydrate-binding site of the lectin, which might be too large to be complementary to a monosaccharide, was involved in inducing both the activities. However, since several D-galactose-specific lectins appeared to compete with CTL for the same receptor sites on rabbit erythrocyte surface, D-galactose or a related sugar was suggested [1] to be an essential part of the complex carbohydrate complementary to the lectin.

Our object was to study the agglutination induced by CTL of liposomes containing several glycosphingolipids in order to determine the essential structural features of the lectin-reactive complex carbohydrate. Like natural cells, the liposomes can be cross-linked and agglutinated by polyvalent lectins which bind non-covalently to specific carbohydrate structures of glycolipids [2–5]. Unlike natural cells, however, liposomes lack cytoskeleton and cellular energetic machinery which affect secondary membrane events in lectin-induced cellular agglutination [6]. Thus, although glycolipid-dependent agglutination of liposomes may be affected by phospholipid composition and ionic strength [5], the phenomenon is largely free from the complexities which characterize cellular agglutination. Like precipitin reactions of lectins with polysaccharides and glycoconjugates, such agglutination of liposomes can generally be used for the study of carbohydrate-specific lectin–glycolipid

Abbreviations: CTL, *Croton tiglium* lectin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; FA, Forssman antigen; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose

* To whom correspondence should be addressed.

Published by Elsevier Science Publishers B.V.

interactions. If a series of glycolipids with defined carbohydrate structures are available, such a study can give information regarding lectin–sugar complementarity. Moreover, studies of lectin-induced agglutination of liposomes can provide knowledge about the role of ionic and hydrophobic forces in lectin–cell interactions [5], which may have biological significance.

2. MATERIALS AND METHODS

CTL and trypsin fragments were prepared as in [1]. PC, PE and PS were obtained from Sigma (St. Louis MO). The purity of the phospholipids was checked by thin-layer chromatography. The glycolipids, viz. ceramide trihexoside from rabbit erythrocytes, globoside from pig erythrocytes, FA from sheep erythrocytes and GM1 ganglioside from human brain were generous gifts from Dr Subhas Basu, University of Notre Dame. The structures of the glycolipids are shown below [7]:

Ceramide Trihexoside; Gal $\alpha 1 \longrightarrow 4$ Gal $\beta 1 \longrightarrow 4$ Glc-ceramide *Globoside:* GalNAC $\beta 1 \longrightarrow 3$ Gal $\alpha 1 \longrightarrow 4$ Gal $\beta 1 \longrightarrow 4$ Glc-ceramide *FA:* Gal NAC $\alpha 1 \longrightarrow 3$ GalNAC $\beta 1 \longrightarrow 3$ Gal $\alpha 1 \longrightarrow 4$ Gal $\beta 1 \longrightarrow 4$ Glc-ceramide

GM1 Ganglioside: Gal $\beta 1 \longrightarrow 3$ GalNAc $\beta 1 \longrightarrow 4$ Gal $\beta 1 \longrightarrow 4$ Glc-Ceramide
↓
2*N*A-NA

(*N*ANA = *N*-Acetyl neuraminic acid)

All other reagents were of analytical grade and used as received. Glycolipid-containing liposomes were prepared as in [2]. Phospholipid and glycolipid, dissolved in chloroform–methanol (2:1, v/v) were mixed in appropriate proportions in a glass tube. The solvent was evaporated under reduced pressure at 25°C. The lipids were then suspended in 10 mM Tris-HCl buffer containing 150 mM NaCl (pH 7.2) to yield 2 mg phospholipid/ml. The suspension was sonicated for 15 min in a bath sonicator and finally centrifuged at $8000 \times g$ for 10 min at 4°C to remove multilamellar liposomes.

CTL solution in the above buffer was added to the liposome suspension, mixed rapidly and transferred immediately to a spectrophotometer cuvette. Agglutination of liposomes was monitored by observing the increase in A_{500} of a suspension of liposome incubated with the lectin against a blank without lectin.

The concentration of the lectin was determined spectrophotometrically using $\epsilon_{1\ cm}^{1\%} = 15.6$ at 280 nm [1].

3. RESULTS

3.1. *Effect of phospholipid composition and Ca^{2+} ions on the glycolipid-dependent agglutination of liposomes*

Fig.1 shows the results of incubation of CTL at 250 μg/ml with liposomes devoid of glycolipids, as well as with FA-containing liposomes. It is seen that the lectin does not agglutinate PC, PC-PS and PC-PE vesicles devoid of glycolipid in the presence or in the absence of 1 mM $CaCl_2$. However, PC, PC-PE and PC-PS vesicles in which FA has been incorporated, are agglutinated by CTL only in the presence of Ca^{2+} as indicated by the increase in turbidity of the liposome suspension. This agglutination is completely reversed in the presence of 1 mM EDTA indicating that the agglutination of liposomes by CTL has an absolute requirement for the simultaneous presence of a

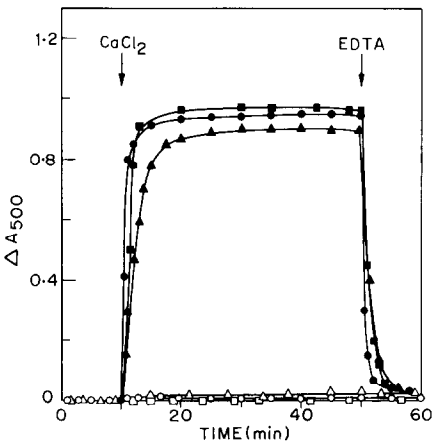

Fig.1. Effect of phospholipid composition on CTL-induced agglutination of liposomes (by vol.): (○) PC; (△) 1:1 PC-PS; (□) 1:1 PC-PE; (●) 2:1 PC-FA; (▲) 1:1:1 PC-PS-FA; (■) 1:1:1 PC-PE-FA liposomes were incubated with CTL at a concentration of 250 μg/ml. Addition of $CaCl_2$ and EDTA to final concentrations of 1 mM are shown by arrows.

carbohydrate-bearing structure in liposome and Ca^{2+} in the medium. Furthermore, an increase to 300 mM NaCl (not shown) did not affect agglutination, showing that the ionic forces were not important in glycolipid-dependent agglutination of liposomes by CTL. Since CTL agglutinates FA-containing PC, PC-PE and PC-PS vesicles almost equally (fig. 1), it appears that the process is not affected by the charge of the phospholipid.

3.2. *Effect of incorporation of different glycosphingolipids on the agglutination of liposomes*

Fig.2 shows that CTL induces agglutination of liposomes prepared from a 1:1:1 (by. vol.) mixture of PC, PE and one of the following glycolipids, viz. ceramide trihexoside, globoside, FA and GM1 ganglioside. It will be seen that agglutination, which occurred only in the presence of 1 mM Ca^{2+} in all cases, was completely reversed by trypsin fragments. Addition of Gal, GalNAc or lactose did not affect CTL-induced agglutination of liposomes (not shown). The complete inhibition of CTL-induced agglutination of liposomes by trypsin-released glycopeptides appears to indicate that the process is mediated by binding of the lectin to specific carbohydrate moieties of the glycolipids and not by non-specific hydrophobic interaction between lectin and phospholipids. It may be observed that there is no significant difference in the extent of agglutination of liposomes containing different glycosphingolipids. All the glycolipids carry oligosaccharide chains containing the sequences GalNAc-GalNAc, GalNAc-Gal, Gal-GalNAc and Gal-Gal, as well as Gal-Glc. These disaccharide units, except Gal-Glc, however, differ in the type of glycosidic linkage between the two monosaccharide units. Inhibition of glycolipid-dependent, CTL-induced agglutination of liposomes by trypsin fragmets and not by GalNAc, Gal or lactose (Gal-Glc) suggest that a disaccharide unit consisting of either two Gal or two GalNAc residues or one unit with Gal and GalNAc, constitutes an essential part of the carbohydrate hapten of the lectin. However, it is not possible to say whether the presence of a non-reducing terminal Gal or GalNAc residue is essential for a complex carbohydrate to bind to the lectin.

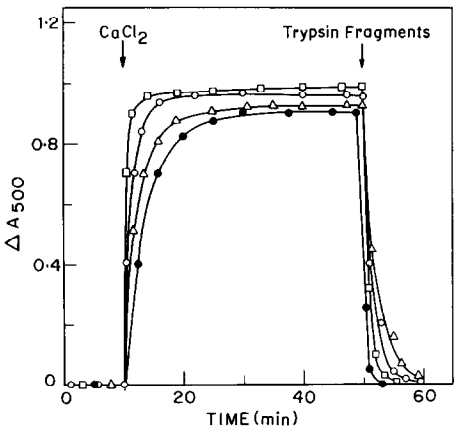

Fig.2. Effect of incorporation of different glycolipids on the agglutination of liposomes (by vol.): (□) 1:1:1 PC-PE-FA; (○) 1:1:1 PC-PE-GM1 ganglioside; (△) 1:1:1 PC-PE-globoside; and (●) 1:1:1 PC-PE-ceramide trihexoside liposomes were incubated with CTL at 250 μg/ml. Addition of $CaCl_2$ and trypsin fragments to final concentrations of 1 mM and 1 mg/ml, respectively, are shown by arrows. Concentration of trypsin fragments was determined as a glucose equivalent.

3.3. *Effect of CTL concentration on agglutination of liposomes*

Fig.3 shows the effect of CTL concentration on the agglutination of PC-PE-FA (1:1:1, by vol.) liposomes in presence of Ca^{2+}. As the lectin concentration is increased from 25-500 μg/ml, there

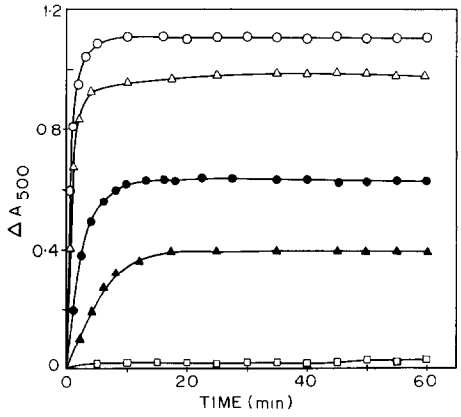

Fig.3. Increase of turbidity of 1:1:1 PC-PE-FA liposomes as a function of CTL concentration: (○) 500 μg/ml; (△) 250 μg/ml; (●) 150 μg/ml; (▲) 100 μg/ml; (□) 25 μg/ml.

is, as expected, an increase in the extent of agglutination. However, there is hardly any increase in turbidity at 25 μg lectin/ml, suggesting that there is a threshold number of receptor sites on liposomes, which must be occupied before agglutination occurs.

4. DISCUSSION

Results presented above show that addition of CTL to a suspension of several glycosphingolipid-containing liposomes leads to increase in turbidity indicating cross-linking or agglutination of the vesicles. The process, like CTL-induced hemagglutination or hemolysis of erythrocytes, was inhibited by trypsin fragments. Since glycolipid-containing liposomes and trypsin fragments share carbohydrate as the only common constituent, the above result indicates that the agglutination of liposomes is mediated by specific lectin–carbohydrate interactions. Specific role of carbohydrate is also suggested by the inability of the lectin to agglutinate liposomes when glycolipid is absent. Recently there has been increasing evidence that lectins, in addition to their carbohydrate binding function, can also interact hydrophobically with glycoproteins and phospholipids [8,9]. It is not known whether these non-carbohydrate-dependent interactions of lectins with cell membrane constituents are of any significance in lectin-induced cellular events such as agglutination or mitogenesis. However, it is well known that proteins can induce hemolysis or erythrocytes by interacting hydrophobically with cell surface [10]. Our results suggest that hydrophobic interaction of CTL with cell surface is unlikely, and thus lend further evidence to the observation [1] that hemolysis of rabbit erythrocytes is induced by binding of the lectin to cell surface carbohydrate receptors.

Since the hemagglutinating and hemolytic activities of CTL are not affected by Ca^{2+} (unpublished), it appears that the presence of Ca^{2+} ions is not required for binding of the lectin to glycolipid. However, Ca^{2+} ions are known to affect the mobility of the glycolipid receptors in liposomes by interacting with phospholipids [5,11], and may, thereby, play a crucial role in CTL-induced agglutination of liposomes.

The saccharide-binding specificity of the lectin is yet to be defined. However, the results suggest that the presence of a disaccharide structure consisting of two Gal or GalNAc residues is of paramount importance in determining the reactivity of complex carbohydrates with CTL. Apparently the carbohydrate-specificity of the lectin bears some similarity to that of peanut lectin (primary specificity, Gal β1 $\longrightarrow$ 3 GalNAc) which, however, is weakly inhibited by the monosaccharides Gal and GalNAc [12]. It may be noted that the peanut lectin competes with CTL for the same receptor sites on rabbit erythrocyte surface [1].

ACKNOWLEDGEMENT

We thank Dr S. Basu of the University of Notre Dame, USA for helpful discussions.

REFERENCES

[1] Banerjee, K.K. and Sen, A. (1981) Arch. Biochem. Biophys. 212, 740–753.
[2] Surolia, A., Bachhawat, B.K. and Podder, S.K. (1975) Nature 257, 802–804.
[3] Curatolo, W., Yau, A.O., Small, D.M. and Sears, B. (1978) Biochemistry 17, 5740–5744.
[4] Redwood, W.R. and Polefka, T.G. (1976) Biochim. Biophys. Acta 455, 631–643.
[5] Hampton, R.Y., Holz, R.W. and Goldstein, I.J. (1980) J. Biol. Chem. 255, 6766–6771.
[6] Nicolson, G.L. (1974) in: International Review of Cytology (Bourne, G.H. and Danielli, J.F. eds) vol.39, pp.89–190, Academic Press, New York.
[7] Esselman, W.J., Laine, R.A. and Sweeby, C.C. (1972) in: Methods in Enzymology (Guinsburg, V. ed) vol.28, pp.140–156, Academic Press, New York.
[8] Sparman, M., Ochoa, J.L. and Kristiansen, T. (1979) Protides of Biological Fluids, Proceedings of the 27th Colloquium, 431–434.
[9] Boldt, D.H., Speckart, S.F., Richards, R.L. and Alving, C.R. (1977) Biochem. Biophys. Res. Commun. 74, 208–214.
[10] Alouf, J.E. (1977) in: The Specificity and Action of Animal, Bacterial and Plant Toxins (Cuatrecasas, P. ed) series B, vol.1, pp.219–270, Chapman and Hall, London.
[11] Trauble, H. and Eible, H. (1974) Proc. Natl. Acad. Sci. USA 71, 214–219.
[12] Lotan, R., Skutelsky, E., Danon, D. and Sharon, N. (1975) J. Biol. Chem. 250, 8518–8523.