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PROTEINS MASK GANGLIOSIDES IN MILK FAT GLOBULE AND ERYTHROCYTE MEMBRANES

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SUMMARY

Gangliosides in the membrane of erythrocytes and the fat globules of cow's milk were not degraded by neuraminidase treatment at pH 5.2 or 7.4. Removal of portions of the membrane protein by treatment with trypsin or by extraction with a solution of EDTA and 2-mercaptoethanol rendered these membrane-associated gangliosides accessible to neuraminidase attack. After trypsin treatment under conditions where the membranes are impermeant to this enzyme, gangliosides were exposed to neuraminidase. The results suggest that the carbohydrate groups of the gangliosides of bovine erythrocytes and milk fat globules are located primarily on the environmental face of the membrane and are shielded from neuraminidase attack by membrane proteins.

INTRODUCTION

During the course of studies on the accumulation of exogenously supplied gangliosides by cultured cells or isolated membrane fractions, it was observed that the endogenous membrane-bound gangliosides of 3T3 cells transformed by SV 40 virus [1], bovine milk fat globule membrane [1] and canine erythrocytes [2] were not degraded when the cells or membranes were treated with neuraminidase. Similarly, when L-cells [3], synaptosomes [4] or isolated plasma membrane vesicles [3, 5] were treated with neuraminidase, little or no loss of ganglioside sialic acid was observed. Carbohydrates of surface membrane glycoproteins are oriented along the outer or environmental face of the membrane [6, 7]. Since the carbohydrates of glycoproteins and glycosphingolipids are added by the same mechanism and in the same topological orientation (e.g. refs. 8 and 9), it is probable that the carbohydrate moieties of gangliosides are also oriented along the environmental face of surface membranes. It thus appeared probable that the resistance of ganglioside sialic acid to neuraminidase attack was due to masking by membrane constituents, most probably by proteins of the membrane. Recent observations on the labeling of membrane neutral glycosphingolipids by oxidation of terminal galactose residues with galactose oxidase and reduction with NaB^3H_4 also suggest masking of glycosphingolipids by membrane-

associated proteins [10, 11]. Since glycosphingolipid changes are general phenotypic expressions of tumorigenic transformation (e.g. refs 11 and 12), it appeared desirable to determine the orientation of ganglioside carbohydrate moieties in surface membranes. Results presented in this communication show that ganglioside sialic acids are oriented along the environmental face of the surface membrane of erythrocytes and the fat globule membrane of cow's milk but are masked or rendered structurally inaccessible to neuraminidase attack by membrane-associated proteins.

MATERIALS AND METHODS

Preparation of erythrocytes and erythrocyte ghosts

Blood was collected from steers at slaughter and diluted with phosphate-buffered saline (1 mM KH_2PO_4/10 mM Na_2HPO_4/154 mM NaCl/1 mM $MgCl_2$, pH 7.6) containing heparin as the anticoagulant. Erythrocytes were harvested by centrifugation at $4000 \times g$ for 15 min and were washed three times by suspension in phosphate-buffered saline and centrifugation, the supernatant and buffy coat being discarded after each centrifugation. After enzymatic treatment (see below), erythrocyte ghosts were prepared by lysis in phosphate buffer which was 30 mM in NaCl [13]. After lysis, ghosts were harvested by centrifugation at $100\,000 \times g$ for 30 min at 4 °C and the pellet was resuspended in lysis buffer and ghosts were harvested by centrifugation. This washing was repeated six to eight times until the supernatant was free of hemoglobin.

Preparation of washed fat globules and isolated milk fat globule membrane

Milk fat globules were isolated from milk obtained fresh from Holstein cows [14]. Milk fat globule membranes were released from washed globules and recovered as described previously [14]. Globules and globule membranes obtained in this manner are free of contaminating milk serum proteins.

Modification of fractions

(*a*) *Trypsin treatment.* Erythrocytes, milk fat globules or milk fat globule membranes were suspended in phosphate-buffered saline and treated with trypsin (EC 3.4.21.4) at a level of 25 μg/mg membrane protein. After incubation for the desired time (20 min unless specified otherwise) at 30 °C, a 3-fold by weight excess of trypsin inhibitor was added and incubation was continued for a further 30 min. Fractions were then recovered and washed by centrifugation prior to neuraminidase treatment. Trypsin-catalyzed hydrolysis of membrane proteins was monitored by electrophoresis in 10 % acrylamide gels containing sodium dodecyl sulfate, as described previously [14].

(*b*) *EDTA extraction.* Proteins were removed from milk fat globule membrane by the extraction method used for the isolation of spectrin constituents from erythrocyte membranes [15]. Milk fat globule membrane preparations were suspended in 5 mM EDTA/5 mM 2-mercaptoethanol (pH 7.5) and dialyzed against several changes of this solution at 4 °C for 3.5 days. Membrane material was then recovered and washed by centrifugation prior to neuraminidase treatment.

(*c*) *Neuraminidase treatment.* Fractions were suspended in acetate-buffered saline (10 mM acetic acid/1 mM sodium acetate/154 mM NaCl/1 mM $MgCl_2$/1 mM

$CaCl_2$, pH 5.2) or in 50 mM phosphate buffer (pH 7.4) containing 0.02 I.U. neuraminidase (EC 3.2.1.18)/mg membrane protein. After incubation for 4 h at 37 °C, the reaction was stopped by adjusting the pH to 10.0 with 1 mM NaOH and fractions were recovered by centrifugation.

Chemical determinations

Lipids were extracted with chloroform/methanol and gangliosides were recovered by partitioning and dialysis as in previous studies [2, 16]. Ganglioside sialic acid was released by hydrolysis with 0.05 M H_2SO_4 for 1 h at 80 °C and was measured by the thiobarbituric acid method with *N*-acetylneuraminic acid as the standard [17]. The ganglioside composition of extracts was monitored qualitiatively by thin-layer chromatography [16].

Protein was measured with the Folin phenol reagent, using crystalline bovine serum albumin as the standard [18].

Enzymes

Trypsin, treated with diphenylcarbamyl chloride to inactivate chymotrypsin, was obtained from Sigma Chemical Company. Neuraminidase, extracted from *Clostridium perfringens*, was obtained from both Sigma Chemical Company and Worthington Biochemicals.

RESULTS

Milk fat globules are enveloped by apical plasma membrane during secretion from mammary epithelial cells, the secretory mechanism being such that the external surface of the membrane surrounding the fat globule is equivalent to the external surface of the plasma membrane of the secretory cell (reviewed in refs. 19 and 20). In composition, milk fat globule membrane and mammary plasma membrane fractions are virtually identical [19, 20]. As judged by lactoperoxidase-catalyzed iodination and brief digestion with trypsin, protein constituents of this membrane are asymmetrically disposed with respect to the plane of the lipid bilayer [14]. The membrane surrounding the fat globule remains impermeant to trypsin during incubation for periods of up to 30 min (ref. 14 and Fig. 1). When intact fat globules were treated with trypsin, only certain polypeptides were hydrolyzed (components 9, 12 and 15–17). In contrast, when isolated milk fat globule membrane was treated with trypsin (not shown) several of the polypeptides (components 1–8, 11, 14 and 21 in Fig. 1) were degraded in addition to those which were labile in intact fat globules [14].

When milk fat globule membrane was treated with neuraminidase there was little or no loss of sialic acid from the ganglioside fraction of the membrane (Fig. 2). Under these conditions, approximately 70 % of the total membrane-bound sialic acid is released from the membrane [14]. Identical results were obtained when the neuraminidase hydrolysis was performed at pH 5.2 or 7.4. However, a significant portion of the ganglioside sialic acid of the membrane was released after milk fat globule membrane was treated with trypsin for periods as brief as 2 min (Fig. 2). Treatment of milk fat globule membrane with trypsin for longer periods resulted in a progressive increase in the amount of the ganglioside sialic acid which became labile to neuraminidase. From 75 to 80 % of the ganglioside sialic acid was released from the mem-

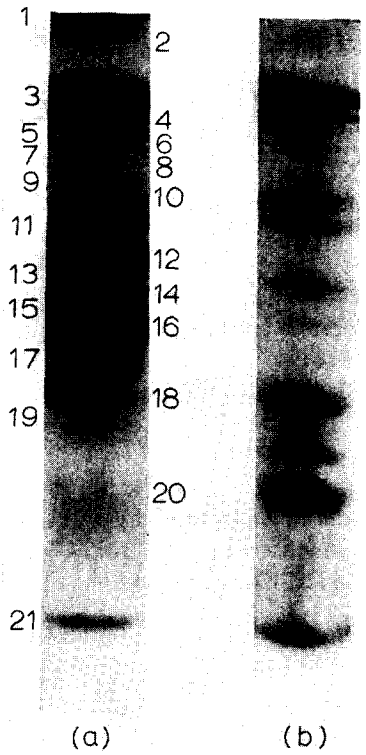

Fig. 1. Trypsin treatment of washed milk fat globules. Conditions for the trypsin treatment are described in ref. 14. Gel (a) washed cream (2.5 mg milk fat globule membrane protein/ml). Gel (b) washed cream (2.5 mg milk fat globule membrane protein/ml) treated with trypsin (50 μg/ml) for 30 min at 30 °C.

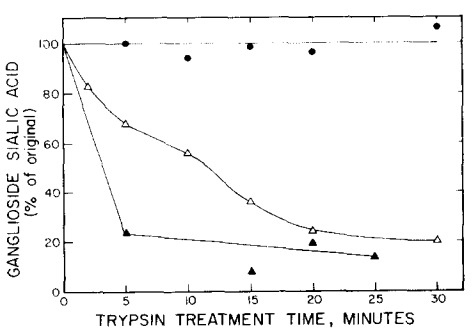

Fig. 2. The effect of trypsin digestion on the accessibility of ganglioside-sialic acid to neuraminidase in washed milk fat globules. Washed milk fat globules were treated with trypsin for the times indicated in the figure and then further treated with neuraminidase. Control with no trypsin treatment (●); samples treated with trypsin and then subjected to neuraminidase hydrolysis at pH 5.2 (△) and 7.4 (▲).

brane by neuraminidase digestion at pH 5.2 or 7.4 after trypsin treatment for 20–30 min. At least one of the molecules of sialic acid is structurally inaccessible to neuraminidase attack in certain ganglioside structures (cf. ref. 1). Approximately 80 % of the sialic acid of mixed milk fat globule membrane gangliosides is labile to neuraminidase in solution; since the predominant ganglioside in this membrane is disialolactosyl ceramide (GD_3) [16]. Thus, after trypsin treatment for 30 min, globule membrane gangliosides are nearly totally accessible to attack by neuraminidase. This observation strongly suggests that membrane proteins mask milk fat globule membrane gangliosides from attack by neuraminidase. Since isolated membranes are used, the localization of the carbohydrate moiety of gangliosides with respect to the plane of the lipid bilayer cannot be determined from these results.

Gangliosides of intact fat globules, which are contained nearly exclusively in the membrane surrounding the globules [16], were also resistant to neuraminidase hydrolysis (Table I). Treatment of the intact globules with trypsin for 20 min rendered about 75 % of the ganglioside sialic acid labile to attack by neuraminidase. Virtually the same results were obtained when globule membrane samples were treated in parallel with intact fat globules (Table I). Based on the original milk fat globule membrane protein content, trypsin treatment alone resulted in only minor loss of gangliosides from the membrane (Table I). Globules were observed to remain intact during neuraminidase treatment and, since they were shown to be impermeant to trypsin under the treatment conditions, the observations imply an external localization of the ganglioside sialic acid.

To clarify further the proteinaceous nature of the membrane constituents responsible for shielding gangliosides from neuraminidase attack, globules and milk fat globule membrane were extracted with EDTA/mercaptoethanol by a procedure

TABLE I

LABILITY OF GANGLIOSIDES IN BOVINE MILK FAT GLOBULE MEMBRANE TO DISGESTION BY NEURAMINIDASE

Intact milk fat globules or isolated milk fat globule membranes were treated with neuraminidase alone or were first treated with trypsin or extracted with EDTA/mercaptoethanol to remove protein before neuraminidase treatment. Gangliosides were then extracted and their amount measured by sialic acid assay. Details are given in the text. Essentially identical results were obtained when neuraminidase hydrolysis was performed at pH 5.2 or 7.4.

Fraction	Treatment	Ganglioside sialic acid (nmol/mg membrane protein)
Globules	None	5.5
Globules	Neuraminidase	5.2
Globules	Trypsin, neuraminidase	1.3
Globule membrane	None	5.2
Globule membrane	Trypsin	5.0
Globule membrane	Neuraminidase	4.5
Globule membrane	Trypsin, neuraminidase	0.7
Globules	EDTA	3.5
Globules	EDTA, neuraminidase	0.5
Globule membrane	EDTA	3.2
Globule membrane	EDTA, neuraminidase	0.6

that removes spectrin constituents from erythrocyte membranes [15]. This extraction results in solubilization of about 20 % of the milk fat globule membrane protein. While several membrane proteins are present in this soluble fraction, the major constituent of the fraction is a glycoprotein (Weber, K. and Keenan, T. W., manuscript in preparation). About 30–40 % of the membrane ganglioside sialic acid was also lost during this extraction and could be quantitatively recovered in lipid fractions extracted from the supernatant. Of the ganglioside remaining in the membrane after EDTA/mercaptoethanol extraction, 80 % or more was labile to degradation by neuraminidase (Table I).

To extend the findings to another membrane type, bovine erythrocytes were also treated with trypsin and neuraminidase. Gangliosides of bovine erythrocytes have apparently not been characterized. Thin-layer chromatographic analysis of erythrocyte ghost ganglioside fractions revealed the presence of only one substantial sialic acid-containing constituent. This ganglioside had a thin-layer chromatographic mobility identical to that of the disialoganglioside GD_3 (nomenclature of Svennerholm [21]; disialolactosylceramide); the structure of which has been confirmed by constituent analysis (in preparation). Thirteen separate analyses revealed the ganglioside sialic acid content of bovine erythrocyte ghosts to be 9.03 ± 1.46 nmol/mg protein. Neuraminidase treatment of intact erythrocytes caused only minor release of ganglioside sialic acid (Table II). Similarly, treatment of intact erythrocytes with trypsin alone also caused but minor loss of gangliosides from the membrane. However, neuraminidase treatment following trypsin hydrolysis resulted in the release of nearly 50 % of the ganglioside sialic acid of the membrane (Table II). Since we have not established optimum conditions for trypsin hydrolysis of erythrocyte membrane proteins, it is possible that an even larger amount of the ganglioside would be rendered labile to neuraminidase with more extensive protein hydrolysis.

TABLE II

LABILITY OF GANGLIOSIDES IN BOVINE ERYTHROCYTE MEMBRANE TO DIGESTION BY NEURAMINIDASE

Erythrocytes were treated with neuraminidase either alone or after trypsin treatment. After stopping the enzymatic reaction, ghosts were prepared, the ganglioside fraction was recovered and measured by sialic acid assay. Details are given in the text.

Treatment	Ganglioside sialic acid (nmol/mg ghost protein)
None	10.7
Neuraminidase	10.0
Trypsin	9.8
Trypsin, neuraminidase	5.6

DISCUSSION

Our results reveal that the carbohydrate moieties of gangliosides are oriented along the environmental face of both bovine erythrocytes and milk fat globule membranes, which are derived primarily from the apical plasma membrane of mammary epithelial cells. Membrane proteins mask these gangliosides from neuraminidase

attack. Such masking by membrane proteins is probably also the case in L-cell plasma membranes [3], synaptosomes [4], canine erythrocytes [2] and intestinal epithelia [22], where the gangliosides are also cryptic to neuraminidase. Since a low pH can have a variety of effects on cell membranes (e.g. refs. 23–30), it was important to confirm our results with neuraminidase hydrolysis at a physiological pH. Without trypsin hydrolysis, milk fat globule membrane gangliosides were resistant to neuraminidase hydrolysis at both pH 5.2 and pH 7.4. These results are in contrast to those of Yogeeswaren et al. [31] with transformed 3T3 cells and Wherrett [32] with the major ganglioside (*N*-acetylneuraminyl-galactosyl-*N*-acetylglucosaminyl-galactosyl-glucosylceramide) of human erythrocytes. The gangliosides of these cells were labile to neuraminidase, suggesting possible difference in topography of membranes in this regard among cell types.

Glycosphingolipids are antigenically active [33] and, in the surface membrane, are believed to function as recognition molecules [34, 35]. Nevertheless, it appears that at least in certain cell types the membrane gangliosides are shielded from the approach of external proteins. It is possible that the degree of exposure of glycosphingolipids of the surface membrane changes during certain stages of the cell cycle [11] or in certain pathological states. For example, Gahmberg and Hakomori [10] have observed glycosphingolipids containing a terminal galactose residue to be more accessible to attack by galactose oxidase in transformed hamster fibroblasts (NILpy cells) than in the untransformed counterparts. Our observations suggest that neuraminidase can be used to explore such changes occuring with at least certain gangliosides of the surface membrane.

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REFERENCES

1 Keenan, T. W., Schmid, E., Franke, W. W. and Wiegandt, H. (1975) Exp. Cell Res. 92, 259–270
2 Keenan, T. W., Franke, W. W. and Wiegandt, H. (1974) Hoppe Seyler's Z. Physiol. Chem. 355, 1543–1548
3 Weinstein, D. B., Marsh, J. B., Glick, M. C. and Warren, L. (1970) J. Biol. Chem. 245, 3928–3937
4 Dicesare, J. L. and Rapport, M. M. (1973) J. Neurochem. 20, 1781–1783
5 Barton, N. W. and Rosenberg, A. (1973) J. Biol. Chem. 248, 7353–7358
6 Hirano, H., Parkhouse, B., Nicholson, G. L., Lennox, E. S. and Singer, S. J. (1972) Proc. Natl. Acad. Sci. U.S. 69, 2945–2949
7 Nicholson, G. L. and Singer, S. J. (1974) J. Cell Biol. 60, 236–248
8 Schachter, H., Jabbal, I., Hudgin, R. L., Pinteric L., McGuire, E. J. and Roseman, S. (1970) J. Biol. Chem. 245, 1090–1100
9 Keenan, T. W., Morré, D. J. and Basu, S. (1974) J. Biol. Chem. 249, 310–315
10 Gahmberg, C. G. and Hakomori, S. (1975) J. Biol. Chem. 250, 2447–2451
11 Hakomori, S. (1975) Biochem. Biophys. Acta 417, 55–89
12 Brady, R. O. and Fishman, P. H. (1974) Biochem. Biophys. Acta 355, 121–148
13 Hanahan, D. J. and Ekholm, J. E. (1974) Methods Enzymol. 31A, 168–172
14 Mather, I. H. and Keenan, T. W. (1975) J. Membrane Biol. 21, 65–85

15 Marchesi, S. L., Steers, E., Marchesi, V. T. and Tillack, T. W. (1970) Biochemistry 9, 50–57
16 Keenan, T. W. (1974) Biochim. Biophys. Acta 337, 255–270
17 Warren, L. (1959) J. Biol. Chem. 234, 1971–1975
18 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19 Keenan, T. W., Morré, D. J. and Huang, C. M. (1974) in Lactation: A Comprehensive Treatise (Larson, B. L. and Smith, V. R., eds.), Vol. 2, pp. 191–233, Academic Press, New York
20 Patton, S. and Keenan, T. W. (1975) Biochim. Biophys. Acta 415, 273–309
21 Svennerholm, L. (1963) J. Neurochem. 10, 613–619
22 Holmgren, J., Lonnroth, I., Mansson, J. E. and Svennerholm, L. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2520–2524
23 Pinto da Silva, P. (1972) J. Cell Biol. 53, 777–787
24 Nicolson, G. L. (1973) J. Cell Biol. 57, 373–387
25 Eagle, H. (1973) J. Cell. Physiol. 82, 1–8
26 Mackenzie, G. G., Mackenzie, J. B. and Beck, P. (1961) J. Biophys. Biochem. Cytol. 9, 141–156
27 Nigra, T. P., Martin, G. R. and Eagle, H. (1973) Biochem. Biophys. Res. Commun. 53, 272–281
28 Calothy, G., Croce, C. M., Defendi, V., Koprowski, H. and Eagle, H. (1973) Proc. Natl. Acad. Sci. U.S. 70, 366–368
29 Croce, C. M., Koprowski, H. and Eagle, H. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1953–1956
31 Yahara, I. and Edelman, G. M. (1973) Nature 246, 152–155
31 Yogeeswaran, G., Sheinin, R., Wherrett, J. R. and Murray, R. K. (1972) J. Biol. Chem. 247, 5146–5158
32 Wherrett, J. R. (1973) Biochim. Biophys. Acta 326, 63–73
33 Rothfield, L. and Romeo, D. (1971) in Structure and Function of Biological Membranes (Rothfield, L., ed.), pp. 251–284, Academic Press, New York
34 Roseman, S. (1970) Chem. Phys. Lipids 5, 270–297
35 Cuatrecasas, P. (1974) Annu. Rev. Biochem. 43, 169–214