# LACTO-N-NEOTETRAOSYLCERAMIDE ("PARAGLOBOSIDE") AS A POSSIBLE TUMOR)ASSOCIATED SURFACE ANTIGEN OF HAMSTER NILPY TUMOR

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Summary- A specific surface-labeled component of polyoma-transformed NIL cells (NILpy) was identified as "paragloboside" (βGal1→4βGlcNAc1→3βGal1→4 βGlc→ceramide) (C.G. Gahmberg and S. Hakomori, J. Biol. Chem. 250, 2438, 1975). The presence of sialylparagloboside in NIL cells, but not in NILpy, suggests that paragloboside accumulates in NILpy as a precursor, consequent to blocked synthesis of sialylparagloboside. Evidence is now furnished to support the possibility that this glycolipid is a tumor-associated surface antigen of NILpy tumor. Sera of NILpy tumor-bearing hamsters contain a specific antibody reactive with paragloboside. The amount of this antibody in sera was parallel to the size of tumors.

Tumor-associated antigens (TAA)[1] or tumor-associated transplantation antigens (TATA) at the tumor cell surface have been implicated in immunological rejection or enhancement of tumors (for a review, see 1). Although TAA have been characterized immunologically, their chemical nature has not been conclusively determined. Recent studies of Kahan (2), Appella (3), and others (4) have suggested TAA or TATA may be cell surface proteins. It has also been suggested that TATA may be modified histocompatibility antigens (5).

During the past several years, a number of studies have focused on chemical and enzymatic changes in glycolipids occurring as a result of malignant transformation (6). It has not been made clear whether there

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[1]Abbreviations: TAA: tumor-associated antigens, TATA: tumor-associated transplantation antigens.



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is a correlation between the observed chemical changes and the immunogenicity of the transformed cells.

Recently, polyoma virus-transformed NIL cells (NILpy) have been characterized by 1) a deletion or decrease of higher glycolipids (7-9), 2) the presence of a surface-exposed ceramide tetrasaccharide (as revealed by galactose-oxidase [$^3$H]-borohydride labeling technique) which is not present in NIL cells (10-11), and 3) a deletion of high molecular weight glycoprotein ("galactoprotein a") (10,12). The structure of the ceramide tetrasaccharide, mentioned above, was identified as βGal(1→4)βGlcNAc(1→3)βGal(1→4)Glc→ceramide. The same compound was previously isolated and characterized from human erythrocytes, and was termed "paragloboside" (13,19). The present report examines the immunological consequences of such biochemical alterations in the NILpy tumor cell surface.

## MATERIAL AND METHODS

*Cell Lines:* The NIL2K and polyoma transformed NILpy lines of hamster cells have been described previously (11). The NILpyT cell line used in the present studies was obtained from an in vivo NILpy tumor. This line possesses similar glycolipid and glycoprotein patterns as the NILpy cell line, but is significantly more tumorigenic in hamsters (11). NILpy cells, both in vivo and in vitro, were characterized by the presence of galactosyl residues in the ceramide tetrasaccharide area which could be surface-labeled by the galactose oxidase [$^3$H]-borohydride technique. This surface-labeled glycolipid was chemically characterized as lacto-N-neotetraosyl-ceramide ("paragloboside") on methylation analysis and enzymatic degradation (11). In contrast, ceramide tetrasaccharides on non-transformed NIL cells had no label in galactosyl residues but instead N-acetylgalactosaminyl residues were labeled and the labeled glycolipids were characterized as globoside (βGalNAcl→3αGall→4βGall→4βGlc→ceramide), and a new type of Forssman antigen with a structure αGalNAcl→3βGalNAcl→3αGall→4βGal→ceramide (11).

*Animals and Test Sera:* Syrian Golden Hamsters (Lakeview Hamster Colony) 2-3 months of age were each injected subcutaneously with $10^4$ NILpyT cells and 30-60 days later the animals were exsanguinated to obtain "tumor-bearing serum". Alternatively, hamsters were injected every 3 weeks with $10^6$ NILpyT cells, fixed with 0.25% glutaraldehyde (14); and "tumor-immune serum" was collected after 20 weeks. Stock hamsters were bled to collect "normal serum".

*Glycolipids and their Analysis:* Globoside (15,16) and Forssman hapten (αGalNAcl→3βGalNAcl→3αGall→4βGall→4Glc→cer) (17), were prepared from human and goat red blood cells respectively. Each was purified by column and thin-layer chromatography as described previously (18). Lacto-N-neotetraosylceramide (paragloboside: βGall→4βGlcNAcl→3βGall→4Glc→Cer) (13) was prepared by acid hydrolysis of sialylparagloboside (αNANA2→3βGall→4βGlcNAcl→3βGall→4Glc→cer), the major ganglioside of human red blood cells

(13,19,20). The product of acid hydrolysis was purified by column chromatography and sugar composition was verified by gas liquid chromatography. Glycolipids of cells were analyzed by thin-layer chromatography after extraction with chloroform-methanol as previously described (18).

*Glycoproteins:* Glycoproteins (GP) were solubilized by treating NILpyT or NIL2K cell sultures with papain-cellulose (Sigma), as previously described (21).

*Complement Fixation Test:* Dilutions of test sera were incubated overnight at 4° C in microtiter plates with 1.5 $CF_{50}$ units of complement; 25, 12, or 6 µg each of test glycolipid or glycoprotein antigen; and, in the case of glycolipid antigens 50, 25, or 12 µg each of cholesterol and lecithin. Sensitized sheep red cells were added and lysis was assessed visually after 30 minutes at 37° C. The lowest dilution of test serum producing 3+ complement fixation (see Footnote, Table I) with 25 µg test antigen is defined to be the titer for the serum. The general procedure followed the description by Casey (22).

## RESULTS

The results presented in Table I indicate that sera of tumor-bearing hamsters had significant levels of complement fixing antibody to paragloboside, but did not show reaction to globoside, or Forssman glycolipids,

TABLE I. Complement fixation reactions of hamster serum with glycolipid antigens from NIL2K and NILpyT cells.

| TEST ANTIGEN | TEST SERUM | | |
|---|---|---|---|
| | *TUMOR-BEARING* | *TUMOR-IMMUNE* | *NORMAL* |
| Paragloboside | | | |
| CF Titer: | 1:40* | 1:20 | none§ |
| | (5/5)† | (2/4) | (0/5) |
| Globoside | | | |
| CF Titer: | none | 1:10 | none |
| | (0/5) | (3/4) | (0/3) |
| Forssman | | | |
| CF Titer: | none | none | none |
| | (0/5) | (0/4) | (0/3) |
| Lecithin+Cholesterol | | | |
| CF Titer: | none | none | none |
| | (0/5) | (0/4) | (0/3) |

*Numbers indicate the complement fixation titer of sera (CF Titer) defined as the lowest dilution of test serum producing complete complement fixation (3+) with 25 µg of test antigen with 50 µg each of cholesterol and lecithin. The average of the titer is shown. "none" indicate no fixation was observed under the same condition. Incomplete complement fixation as indicated by the partial complement-dependent lysis of red blood cells was not regarded as positive test. †Numbers in parenthesis indicate number of serologically positive hamsters/total number of hamsters tested. §Some normal sera showed incomplete complement fixation at high serum concentration. This must be due to the presence of an ill-defined antibody-like material in normal hamster sera, cross-reacting with various glycolipid (27-29).

or to lecithin and cholesterol mixtures which were used as auxiliary lipids in the complement-fixation assay. The sera of hamsters immunized with NILpyT cells (glutaraldehyde treated, 14) showed a weaker reaction to paragloboside and an additional reaction to globoside. The sera of normal hamsters had no reaction with glycolipids and glycoproteins tested under the given condition. However, some sera of normal hamsters showed an incomplete fixation of complement at high serum concentration which could be a cross reaction due to the presence of unidentified antibody-like material directed towards unidentified structures (27-29).

The specific reaction of tumor-bearing sera with paragloboside was demonstrable by immune-precipitin reaction using Ouchterlony's double diffusion technique. No precipitin bands could be detected with globoside, Forssman, ceramide trihexoside ($\alpha$Gal1→4$\beta$Gal1→4$\beta$Glc→cer), or with glycoprotein fractions. The specific precipitin reaction with paragloboside was very weak but addition of auxiliary lipids, lecithin and cholesterol, intensified the reaction. The tumor-immune or normal sera did not show precipitin bands with either paragloboside or globoside.

In further studies, the level of paragloboside-reactive antibody was examined as a function of tumor size. The results presented in Fig. 1

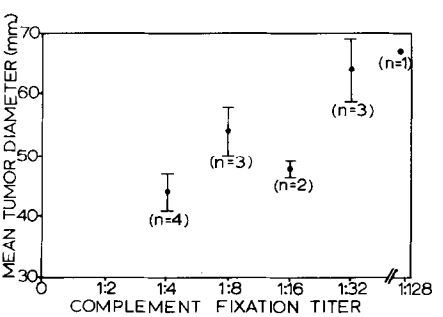


Figure 1. The correlation between tumor size and the titer of complement-fixing antibody to paragloboside in sera. The complement fixation titer was determined with 25 µg of paragloboside with cholesterol and lecithin as specified in the text. "n" denotes number of cases.

show that as the total tumor burden increased, the serum titer of antibody to paragloboside also increased.

The antigenicity of glycolipid and glycoprotein antigens from NIL2K and NILpyT cells was also evaluated by immunizing rabbits, rather than hamsters, with cells or cell surface materials released by papain treatment. The results presented in Table II indicate that Forssman antigen was the strongest glycolipid antigen in NILpyT and NIL2K cells, no antibody against paragloboside was detectable under these conditions. To determine whether cell surface glycoproteins contain antigens also found in glycolipids, monospecific rabbit anti-paragloboside and anti-globoside were reacted in Ouchterlony tests with glycoproteins from NILpy and NIL2K cells. Anti-paragloboside antiserum reacted only with NILpy glycoproteins while anti-globoside antiserum reacted with both NIL2K and NILpy glycoproteins.

The accumulation of glycolipids in many transformed cells has been shown to be indicative of incomplete carbohydrate chain synthesis (6). It was reasoned that paragloboside might be a precursor for sialylpara-

TABLE II. Ouchterlony reactions of rabbit anti-sera with glycolipid and glycoprotein antigens from hamster NIL2K and NILpyT cells.

| | PRECIPITIN LINE PRESENT WITH TEST ANTIGEN* | | | | |
|---|---|---|---|---|---|
| TEST ANTI-SERUM | Paragloboside | Globoside | Forssman | GP-NILpyT | GP-NIL2K |
| Anti-NIL2K cells† | -(0/2) | -(0/2) | +(2/2) | -(0/2) | -(0/2) |
| Anti-NILpyT cells† | -(0/2) | -(0/2) | +(1/2) | -(0/2) | -(0/2) |
| Anti-GPNIL2K§ | -(0/2) | -(0/2) | +(2/2) | +(2/2) | +(2/2) |
| Anti-GP-NILpyT§ | -(0/2) | -(0/2) | +(2/2) | -(0/2) | -(0/2) |
| Anti-paragloboside¶ | +(1/1) | -(0/1) | -(0/1) | +(1/1) | -(0/1) |
| Anti-globoside¶ | -(1/1) | +(1/1) | -(0/1) | +(1/1) | +(1/1) |

* + or - indicate presence or absence of a precipitin line; numbers in parenthesis indicate the number of positive test/total number of rabbits tested.
† Immunization of rabbits with gluteraldehyde fixed cells (14), see text.
§ GP: glycoprotein. Glycoprotein was liberated by papain-cellulose (21), dialyzed and lyophilized. Two mg of lyophilized material was injected into rabbits with complete Freund adjuvant, subsequent injections were made during the 4th and 5th week, and antisera was collected after 7 weeks.
¶ Prepared according to the method described by Siddiqui and Hakomori (17).

globoside, which has been shown to be the major ganglioside in human extraneural tissues (13,19,20). Therefore, gangliosides of NIL and NILpy cells were analyzed, sialyl-lacto-N-neotetraosylceramide (sialylparaglobоside) was found in NIL cells. The chemical concentration of this glycolipid was found to be greatly reduced in NILpy cells as seen in Table III.

## DISCUSSION

Glycosphingolipids are important cell surface markers constituting essential blood group isoantigens (23,24); and, heterogenetic Forssman antigen (17). Immunization of heterologous animals with tissue homogenates often elicits antibodies which are reactive with glycolipids extracted from the same tissue (25). Purified glycolipids are also, in general, antigenic in heterologous animals if complexed with heterologous protein (26). However, it has not been unequivocally demonstrated that glycolipids on cell surfaces are immunogenic in homologous animals.

The results presented in this communication provide the first clear indication that a structurally well-defined glycolipid on cell surface could be a tumor-associated immunogen in homologous animals. The results appear to indicate that paragloboside, a specific surface component of

TABLE III. Gangliosides of NIL and NILpy cells*

| | *NIL sparse* | *NIL confluent* | *NILpy sparse* | *NILpy confluent* |
|---|---|---|---|---|
| Hematoside | ++++ | +++ | ± | ± |
| Sialylparagloboside | ++ | + | ± | - |
| Paragloboside† | nd | - | nd | + |

*Packed NIL and NILpy cells (approximately 1 ml of packed volume) was extracted with chloroform-methanol 2:1, the "Upper layer glycolipid" was separated by repeated partition and finally analyzed on thin-layer chromatography with resorcinol reagent (18). The comparison was made on the same cell residue basis.
++++ (200-300 μg), +++ (150-200 μg), ++ (50-100 μg), + (10-50 μg), ± (0-5 μg) per 100 mg of protein residue.
†Data from Gahmberg and Hakomori (11) nd: not determined.

NILpyT cells, is recognized by the host-immune system and elicits an antibody response. The observation clearly supports the chemical data that paragloboside could be a tumor-associated antigen of NILpy tumors (11).

A possibility that paragloboside could be a polyoma virus-associated antigen can be excluded since mouse 3T3 fibroblasts transformed with polyoma virus did not contain paragloboside (30).

The present results indicate that paragloboside in normal hamster cells is masked by sialyl residues. Sialyl-paragloboside has also been isolated from other sources (17,19,20). Apparently, NILpy cells accumulate paragloboside as a consequence of blocked synthesis of sialylparagloboside. It is also possible that paragloboside in normal cells could be masked by other glycosyl residues such as β-galactosyl (31), and/or α-galactosyl (32).

The present results indicate that the amount of serum antibody reactive to paragloboside increases with increasing tumor size, which could indicate increased synthesis of antibody or decrease binding of antibody by tumor tissue. Antibody reactive with paragloboside could function as "blocking antibody" (33) to stimulate tumor growth.

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