Association of the Glycolipid Pattern with Antigenic Alterations in Mouse Fibroblasts Transformed by Murine Sarcoma Virus

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SUMMARY

The level of the neutral glycolipid, GalNAcβ1→4Galβ1→4Glc→Cer (asialo GM2), in BALB/c 3T3 mouse fibroblasts transformed by Kirsten murine sarcoma virus (3T3KiMSV) was greatly increased compared to the nontransformed parental cells (3T3). This elevated chemical quantity was found to be localized on the surface of intact cells and accessible to external reagents, as detected by immunofluorescence and labeling with galactose oxidase:NaB₃H₄. Furthermore, immunization of rabbits with 3T3KiMSV cells but not with 3T3 cells resulted in antibody production against asialo GM₂. These results demonstrate the potential usefulness of glycolipids as tumor-associated cell surface markers.

INTRODUCTION

Alterations of cellular glycolipid patterns have been described as being associated with the process of cancer. These changes have been observed as a common phenotypic marker of spontaneous tumors as well as tumor cells transformed by DNA viruses, RNA viruses, and chemical carcinogens (for reviews see Refs. 4 and 15). In many instances the blocked synthesis of more complex glycolipids in tumor cells is often accompanied by an accumulation of high levels of precursor glycolipid(s). Typical examples of this phenomenon include increased levels of lactosylceramide in a clone of BHK cells transformed by polyoma virus (16), lacto-N-neotetraosylceramide in hamster NIL cells transformed by polyoma virus (12, 33), and GM₃ and GD1α ganglioside in various hepatoma cells as compared with normal liver (6, 8, 29, 31, 34).

The biological implications of such alterations are not known. The primary objective of the present study was to determine whether such an accumulation of a precursor glycolipid would result in a change in the immunogenicity of the affected tumor cells. A previous study in this laboratory indicated that the accumulation of lacto-N-neotetraosylceramide (paragloboside) in polyoma-transformed hamster fibroblasts (NILpy cells) was accompanied by such a change of immunogenicity. Sera of hamsters bearing NILpy tumors contained detectable amounts of antibody-like material reactive with paragloboside as determined by complement fixation (33).

BALB/c 3T3 cells transformed by the nonproductive Kirsten strain of murine sarcoma virus (3T3KiMSV cells) (1) were reported previously to have a drastically altered glycolipid pattern due to the blocked synthesis of GM₂ ganglioside from GM₃ ganglioside (9, 10). We now report the striking accumulation of asialo GM₂ in these cells, in addition to the changes already described. Coupled with this chemical change are immunological alterations that indicate that asialo GM₂ could function as a tumor-associated antigen in 3T3KiMSV cells.

MATERIALS AND METHODS

Animals and Cells. New Zealand White male rabbits were purchased from Gunther Fur Products, Kent, Wash. BALB/c male mice were purchased from Simonsen Laboratories, Inc., Gilroy, Calif. BALB 3T3 (clone A31) and 3T3KiMSV (clone K-234) cells were obtained from Dr. M. Hatanaka, Frederick Cancer Research Center, Frederick, Md. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum in a 5% CO₂ atmosphere. Glycolipids were radioactively labeled by growing the cells for 48 hr with 0.3 μCi of [14C]galactose (uniformly labeled; specific activity, >200 mCi/mmol) per ml of medium. Cells were harvested by mechanical scraping, and the washed cell pellet was stored at -80°.

Glycolipid Analysis. Detailed procedures for the isolation, characterization, and structural analysis of glycolipids from various organs and cultured cells have been described recently (20). Briefly, frozen cell pellets and organs were extracted with chloroform:methanol (2:1, v/v). The ganglioside fraction was partitioned according to the method of
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 Folch et al. (11), followed by dialysis of the upper layer using dialysis tubing with a small pore size (Spectrapor 4000; Spectrum Medical Industries, Inc., Los Angeles, Calif.) to minimize loss of ganglioside. The neutral glycolipid fraction was purified by the acetylation procedure previously described (27). Purified neutral glycolipid and ganglioside fractions from radiolabeled cells were analyzed by separate TLC and autoradiography.

 Purified asialo GM₂ from 3T3KiMSV cells was obtained by preparative TLC. Maximum recovery of glycolipid was achieved by treating the silica gel suspension with Dowex 50 H⁺ as previously described (25). Structural characterization of asialo GM₂ was accomplished by: (a) carbohydrate analysis through gas chromatography, (b) oxidation with galactose oxidase followed by reduction with [³H]borohydride and examination of the ³H activity through radioactivity counting of sugar peaks obtained on gas chromatography, (c) methylation analysis (3, 21), (d) total mass spectrometry after permethylation and reduction (18), and (e) enzymatic degradation with β-N-acetylhexosaminidase and β-galactosidase of jack bean (22).

 The chemical quantity of asialo GM₂ in normal BALB/c organs was determined by TLC of free glycolipids in the solvent chloroform:methanol:water (60:35:8) and of acetylated glycolipids in the solvent 1,2-dichloroethane:methanol (91:9). The latter system made possible the identification of small amounts of asialo GM₂ in the presence of CTH and ceramide tetrasaccharide.

 Cell Surface Labeling. Cell surface labeling was carried out as previously described (12, 13). Washed cell monolayers were incubated for 2 hr at room temperature with 10 units of galactose oxidase (AB Kabi, Stockholm, Sweden) per 15-cm plate in 2 ml PBS, pH 7.0. The cells were harvested by mechanical scraping, washed by centrifugation, and suspended in 0.5 ml PBS, pH 7.4, containing 2.5 mCi of [³H]sodium borohydride. After 30 min incubation at room temperature, the cells were washed extensively and stored at −80°. Each frozen cell pellet was extracted with chloroform:methanol (2:1, v/v). The ganglioside fraction was partitioned according to the method of Folch et al. (11). The neutral glycolipid fraction was purified by the acetylation procedure (27). The ganglioside fraction was analyzed by TLC using solvent chloroform:methanol:water (60:35:1) and the neutral glycolipid fraction was analyzed with solvent chloroform:methanol:water (60:35:8). The locations of nonlabeled glycolipid standards were visualized on TLC between CTH and globoside, was isolated.

 Procedure for Immunization. Asialo GM₂ was prepared from guinea pig erythrocyte membranes by extraction with chloroform:methanol, followed by the acetylation procedure (27). The unknown glycolipid, which migrated a double spot was found in the transformed cells which was not readily detectable in the nontransformed counterpart. The unknown glycolipid, which migrated in between CTH and globoside, was isolated from the 3T3KiMSV cells by the acetylation procedure and preparative TLC. Methylation analysis indicated that the glycolipid contained equimolar proportions of 3,4,6-tri-O-methyl-(2-N-methylacetamido-2-deoxy)galactitol, 2,3,6-tri-O-methylgalactitol, and 2,3,6-α-O-methylneuraminic acid.
produced a spectrum with the identical properties of permethylated and reduced asialo GM$_2$ isolated from guinea pig erythrocytes. The glycolipid was degraded by β-N-acetylhexosaminidase of jack bean to give a CDH, and the degradation product, the CDH, was further degraded by β-galactosidase. Thus, the glycolipid was identified as asialo GM$_2$ (GalNACβ1→Galβ1→4Glc→Cer).

Asialo GM$_2$ was barely detectable in 3T3 cells when the cells were grown in [14C]galactose, followed by autoradiographic analysis of the extracted glycolipids as indicated in Figure 1. However, the relative intensities of glycolipid spots in the autoradiogram (Fig. 1) do not correlate with the chemical quantities of each glycolipid (Table 1), since the metabolic labeling from precursor sugars is effected by the rate of glycolipid turnover. In order to visualize asialo GM$_2$ in 3T3 cells on TLC, using orcinol spray, it was necessary to extract at least 1 ml of packed 3T3 cells. As shown in Table 1, the chemical amount of asialo GM$_2$ in 3T3KiMSV cells is about 20 times greater than the low amount found in 3T3 cells.

Analysis for the Presence of Asialo GM$_2$ in Various Organs and Tissues of BALB/c Mice. Table 1 indicates the difficulty of detecting asialo GM$_2$ in the organs of normal BALB/c mice. TLC was run on the neutral glycolipid fractions in both the free state and after acetylation, in an effort to visualize trace amounts of asialo GM$_2$. Among the organs assayed only spleen and erythrocytes yielded faint spots on TLC which migrated slightly more slowly than authentic acetylated asialo GM$_2$; all other organs were negative.

Cell Surface Labeling. Using sequential treatment of intact cells with galactose oxidase followed by tritiated borohydride, the labeling of asialo GM$_2$ in 3T3KiMSV cells was more than 30 times greater than that found in 3T3 cells (Table 2). Clearly, asialo GM$_2$ was not only present in higher chemical levels in the transformed cells, but it was also accessible to interaction with these cell surface labeling reagents. The only other glycolipid of the transformed cells that was labeled was a ceramide tetrasaccharide showing the identical migration rate on TLC as rat kidney globoside (or cytolipin A) (21, 30). In 3T3 cells the ganglioside GM$_1$ was the only glycolipid that was heavily labeled.

Reactivity of 3T3 and 3T3KiMSV Cell Monolayers with...
The background count of neutral glycolipids was 6 to 10 cpm after purification of the neutral glycolipid fraction by the acetylation procedure. Gangliosides GM₂ and GM₃ were analyzed directly following Folch partitions (11) and showed a background count of 100 to 200 cpm.

### Table 2

**Extent of glycolipid exposure on the cell surface of 3T3 and 3T3KiMSV cells**

<table>
<thead>
<tr>
<th></th>
<th>3T3</th>
<th>3T3KiMSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMH*</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>CDH</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>CTH</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Asialo GM₃</td>
<td>23</td>
<td>816</td>
</tr>
<tr>
<td>CTT</td>
<td>77</td>
<td>292</td>
</tr>
<tr>
<td>CP</td>
<td>318</td>
<td>536</td>
</tr>
<tr>
<td>GM₁</td>
<td>4262</td>
<td>315</td>
</tr>
</tbody>
</table>

* The abbreviations used are: CMH, ceramide monohexoside; CTT, ceramide tetrasaccharide; CP, ceramide pentasaccharide.

### Anti-Asialo GM₂ Serum

Rabbit anti-asialo GM₂ antibodies reacted specifically by indirect immunofluorescence with 3T3KiMSV cells in monolayers (Fig. 2A) but not with 3T3 cells. This rabbit serum, which had been purified to remove anti-albumin antibodies (see "Materials and Methods"), was found to be specific for asialo GM₂ by immunodiffusion and complement fixation and did not react with other glycolipids including GM₂ ganglioside. The strong cell-membrane-associated fluorescence of the transformed cells could be abolished completely by absorption of the rabbit antibodies with asialo GM₂ liposomes. The diffuse nonspecific fluorescence that remained after such treatments was not associated with the plasma membrane and was similar to that found by treating 3T3KiMSV monolayer cells with rabbit anti-GM₁ antibodies (Fig. 2C). This serum was specific for GM₁, ganglioside, which is present in high levels in 3T3 cells but only in trace amounts in 3T3KiMSV cells.

Trypsinization of the cells followed by treatment of the cell suspension by indirect immunofluorescence intensified the difference in reactivity of the 2 cell types with anti-asialo GM₂ (photographs not shown). These results relate directly to the question of whether the antibodies are reacting with asialo GM₂-like structures in glycoproteins. These data suggest that the same structures as found in gangliosides may not be present in large quantities in trypsin-sensitive membrane glycoproteins of these cells. Fig. 2D demonstrates that intense, membrane-associated fluorescence can be obtained when 3T3 cells are reacted with anti-GM₁ ganglioside serum.

### Immunogenicity of 3T3 and 3T3KiMSV Cells in Rabbits

#### A. Hemagglutination of guinea pig erythrocytes

<table>
<thead>
<tr>
<th>Serum</th>
<th>Titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-3T3 1</td>
<td>0</td>
</tr>
<tr>
<td>Anti-3T3 2</td>
<td>1:2</td>
</tr>
<tr>
<td>Anti-3T3KiMSV 1</td>
<td>1:64</td>
</tr>
<tr>
<td>Anti-3T3KiMSV 3</td>
<td>1:64</td>
</tr>
<tr>
<td>Anti-3T3KiMSV 4</td>
<td>1:16</td>
</tr>
</tbody>
</table>

#### B. Inhibition by glycolipid of guinea pig erythrocyte hemagglutination caused by anti-3T3KiMSV rabbit serum

<table>
<thead>
<tr>
<th>Glycolipid concentration</th>
<th>CTH</th>
<th>Asialo GM₂</th>
<th>Globoside</th>
<th>Forssman</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

#### C. Complement fixation

<table>
<thead>
<tr>
<th>Rabbit serum</th>
<th>Control</th>
<th>GM₂</th>
<th>Asialo GM₂</th>
<th>GM₁</th>
<th>Asialo GM₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-3T3 1</td>
<td>AC</td>
<td>1:32</td>
<td>AC</td>
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<td>1:64</td>
</tr>
<tr>
<td>Anti-3T3 2</td>
<td>AC</td>
<td>1:32</td>
<td>AC</td>
<td>1:64</td>
<td>1:64</td>
</tr>
<tr>
<td>Anti-3T3KiMSV 1</td>
<td>0</td>
<td>1:256</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti-3T3KiMSV 3</td>
<td>AC</td>
<td>1:32</td>
<td>1:256</td>
<td>AC</td>
<td>AC</td>
</tr>
<tr>
<td>Anti-3T3KiMSV 4</td>
<td>0</td>
<td>1:16</td>
<td>1:64</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Titer represents the highest serum dilution that caused obvious hemagglutination (2+) of intact guinea pig erythrocytes. Pre-immune sera from all rabbits tested had no detectable titer.

* Minimum dose of glycolipid in μg/0.1 ml which inhibits 3 hemagglutination doses of serum from rabbit Anti-3T3KiMSV 3 as in A above; values determined with auxiliary lipids.

* Titer represents the highest serum dilution that caused complete fixation of 1.5 units of guinea pig complement.

* AC indicates that sera were anticomplementary at the dilution 1:16 for Anti-3T3 Sera 1 and 2 and at 1:8 for Anti-3T3KiMSV Serum 3. All sera were diluted 1:4 before testing.

### DISCUSSION

The present study clearly indicates the close relationship between an anomaly of glycolipid metabolism and antigenic alterations of transformed cells. Accumulation of asialo GM₂ in 3T3KiMSV cells results in changes of antigenicity as well as immunogenicity of these cells. In agreement with the results of Fishman et al. (9, 10) we have found a deletion of GM₃ ganglioside and an associated increase in GM₂ ganglioside in 3T3KiMSV cells (Fig. 1). However, in their studies, the pattern of neutral glycolipids in 3T3 cells was described as being identical to that of the transformed cells. In striking contrast we found a marked accumulation of asialo GM₂ in the 3T3KiMSV cells. The reason for this discrepancy...
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is not known. Although clonal variation between cells studied in 2 laboratories is possible, preliminary studies of various nonproductive clones of 3T3KiMSV showed a similar immunofluorescence staining with anti-asialo GM\textsubscript{2} (M. Hatanaka, W. Young, and S. Hakomori, unpublished observation). The increased level of asialo GM\textsubscript{2} could be caused by blockage of degradation of asialo GM\textsubscript{2} to CDH. However, studies comparing the catabolism of GM\textsubscript{1}, GM\textsubscript{3}, and asialo GM\textsubscript{2} in 3T3 and 3T3KiMSV cells found no essential differences in the activities of various glycosyl hydrolases when radiolabeled glycolipids were used as substrates (G. Rosenfelder and Y. Tonegawa, unpublished observation). Thus, the inhibition of GM\textsubscript{3} synthesis accounts not only for an elevated level of GM\textsubscript{3} in the transformed cells as previously described (9, 10), but also for increased amounts of asialo GM\textsubscript{2}.

The higher level of asialo GM\textsubscript{2} in 3T3KiMSV cells greatly enhances the antigenicity of this glycolipid. Clearly, there is much more asialo GM\textsubscript{2} on the surface of intact transformed cells as compared to nontransformed cells that is accessible to interaction with external reagents as revealed by indirect immunofluorescence (Fig. 2) and surface labeling with galactose oxidase:NaB\textsubscript{3}H\textsubscript{4} (Table 2). The trace amount of asialo GM\textsubscript{2} in normal 3T3 cells was neither antigenic nor immunogenic. This situation is similar to that of the P\textsuperscript{a} antigen present in P individuals (23). Only P\textsuperscript{a} erythrocytes showed reactivity toward anti-P\textsuperscript{a} serum, even though P erythrocytes do contain a significant amount of P\textsuperscript{a} antigen which was identified as CTH. In another case, human fetal erythrocytes were found to be much more reactive to antigloboside as compared to adult erythrocytes, even though the chemical level of globoside in fetal and adult erythrocytes was nearly identical (14). In this latter case the extent of crypticity and glycolipid organization within the membrane must determine the reactivity of cells with antiglycolipid sera. However, the enhanced surface reactivity of 3T3KiMSV cells can be attributed mainly to the elevated chemical quantity of asialo GM\textsubscript{2} rather than to a change of crypticity of asialo GM\textsubscript{2} in the tumor cell membranes.

Accumulation of asialo GM\textsubscript{2} in 3T3KiMSV cells also resulted in enhanced immunogenicity in a heterologous host. Rabbit antisera prepared by repeated i.v. injection of 3T3 and 3T3KiMSV cells showed a sharp distinction in reactivity against glycolipids (Table 3). Only those rabbits given injections of the transformed cells produced sera that reacted strongly and specifically with asialo GM\textsubscript{2} as indicated by complement fixation as well as hemagglutination and hemagglutination inhibition of guinea pig erythrocytes. Sera raised against 3T3 cells displayed moderate titers against gangliosides GM\textsubscript{2} and GM\textsubscript{3}, but essentially no reactivity toward asialo GM\textsubscript{2}. These results correlate well with the chemical levels of the glycolipids in these cells. Witebsky (35) described the presence of tumor-distinctive lipid haptons in some human tumors which reacted in complement fixation specifically with rabbit antisera that were directed against whole human tumor tissue homogenates. Rapport and Graf (26) observed that such lipid haptons of some tumors are glycolipids; typical examples are "cytolipin H" (lactosylceramide; CDH) of human epidermoid carcinoma and "cytolipin R" of rat lymphosarcoma. The biochemical basis for these immunological phenomena has not been clarified since both CDH and cytolipin R have been found as normal components of the respective host's tissue (30). However, it is conceivable that the "cytolipin phenomenon" could be related to a possible accumulation of the particular glycolipid in the tumors, whereas the same glycolipid may be cryptic in normal cell membranes.

In contrast to the results obtained using rabbits as hosts, it has not been possible to obtain a detectable immune response against asialo GM\textsubscript{1} in BALB/c mice (W. Young, unpublished observation). Attempts to date have included immunization with intact 3T3KiMSV cells, guinea pig erythrocytes, and glycolipid-protein complexes by a variety of routes and schedules. The great difficulty of obtaining any antibody production against 3T3KiMSV cells in syngenic animals has been reported previously (2, 32). However, the failure of a glycolipid-heterologous protein mixture to elicit an antiglycolipid response in BALB/c mice is in striking contrast to the strong responses to such immunogens that generally occur in rabbits (19, 24). It is possible that asialo GM\textsubscript{2}, being a trace constituent of normal mouse tissues (Table 1), may not be immunogenic due to a self-tolerance phenomenon. However, such a situation does not exist in rabbits where antibodies can be produced against glycolipids such as CDH and hematoside which are present in normal rabbit tissues. Further systematic study is in progress to determine whether the glycolipid antigen bound covalently to a suitable carrier molecule will result in a detectable antiglycolipid response in BALB/c mice.

REFERENCES

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Fig. 2. Indirect immunofluorescence of 3T3 and 3T6KMSV cell monolayers. Cells were incubated with specific rabbit antiserum to 3T6KMSV cell membranes, followed by fluorescein-labeled goat anti-rabbit immunoglobulin serum. A, 3T6KMSV cells, anti-serum; B, 3T3 cells, anti-serum; C, 3T3KMSV cells, anti-GM, serum; D, 3T3 cells, anti-GM, serum. × 100.
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