Inhibition of Tumor Cell Growth by Aggregation of a Tumor-associated Glycolipid Antigen: A Close Functional Association between Gangliotriaosylceramide and Transferrin Receptor in Mouse Lymphoma L-5178Y

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ABSTRACT

The growth of mouse lymphoma L-5178Y cells with a high degree of gangliotriaosylceramide expression (high-expressor clone AA12) was inhibited by the addition of a biotinylated mouse immunoglobulin M monoclonal antibody (2D4) directed to gangliotriaosylceramide followed by cross-linking with avidin or a second antibody (anti-mouse immunoglobulin M). This growth inhibition was observed in both serum-containing medium and a chemically defined medium containing transferrin as the only growth factor. Cell growth was not inhibited by the addition of biotinylated antibody 2D4 alone, avidin alone, or the biotinylated derivative of an immunoglobulin M monoclonal anti-N-acetyllactosamine antibody (1B2) cross-linked with avidin. The growth of lymphoma L-5178Y 27AV cells, which do not express gangliotriaosylceramide (non-expressor clone), was not inhibited by either of these monoclonal antibodies or their biotinylated derivatives plus avidin.

In the presence of biotinylated antibody 2D4 and avidin, cells of the high-expressor clone (L-5178Y AA12) displayed a capping of gangliotriaosyl antigen. In contrast, the transferrin receptor and the major glycoproteins (concanavalin A receptors) were not capped in the presence of biotinylated antibody 2D4 and avidin but were homogeneously distributed on the cell surface. Cells whose growth was inhibited by the addition of biotinylated antibody 2D4 and avidin showed an inhibition of $^{125}$I-transferrin internalization, although binding of $^{125}$I-transferrin to the cell surface was similar to that of control cells. These results indicate that the tumor antigen gangliotriaosylceramide is functionally associated with the transferrin receptor and may regulate the process of internalization of transferrin.

INTRODUCTION

The possibility that glycolipids in cell surface membranes may have a role in cell growth regulation has been suggested by changes in glycolipid composition and organization which are closely associated with oncogenic transformation, cell cycle, and cell growth inhibition (1–4). This possibility has been further reinforced by a few observations that the cell growth of mouse 3T3 and hamster NIH cells was inhibited by the addition of monovalent anti-GM1 antibody but not by anti-globoside antibody (5). On the other hand, the aggregation of gangliosides at the normal lymphocyte surface stimulated cell growth (6, 7). Some tumor cells accumulate large quantities of specific glycolipids that can be recognized by immunological methods as tumor-associated antigens (8). However, the functional role of these glycolipid antigens in tumor cells is largely unknown. A number of clones of lymphoma L-5178Y cells expressing different quantities of gangliotriaosylceramide have been isolated and characterized (9). The high expressors of this glycolipid antigen have been characterized as having 3 major molecular species of gangliotriaosylceramide which differ in their fatty acid composition (10) and susceptibility to NK cell killing (9). The growth of high-expresser clones as tumors in DBA/2 mice is inhibited by passive immunization with the monoclonal IgG3 antibody G10D11, but not by the monoclonal IgM antibody 2D4 (11). In contrast, nonexpressor L-5178Y 27AV cells, which do not contain gangliotriaosylceramide, are not susceptible to NK cell killing (9), and their tumor growth is not inhibited by either the G10D11 or 2D4 antibodies (10).

These lymphoma cells can be propagated in RPMI Medium 1640 containing a normal serum concentration or in RPMI Medium 1640 containing ethanolamine, selenium, and TF, a serum-free medium similar to that established for growing murine myeloma cells and hybridoma cells (12). It should be noted that L-5178Y clones do not require any growth factors except TF and that TF is a required basic growth factor for essentially all animal cells (13). TF receptors are expressed ubiquitously on actively proliferating cells, but not on nongrowing cells (14–17). A monoclonal antibody (4216) directed to human transferrin receptor blocks TF binding and inhibits cell growth of human tumors in vitro (16). In view of the remarkable growth-inhibitory effect of some antibodies directed to certain glycolipids present at the cell surface (18–21), we began to study the effect of monoclonal antibodies directed to the glycolipid tumor antigens of L-5178Y lymphoma cells, with particular attention to their effects on TF and the function of the TF receptor. We have observed that the cross-linking and capping of gangliotriaosylceramide in the membranes of an L-5178Y lymphoma clone, induced by biotinylated anti-gangliotriaosyl antibody (2D4) and avidin, result in the inhibition of cell growth in vitro. The capping of gangliotriaosylceramide is associated with an inhibition of TF internalization.

MATERIALS AND METHODS

Materials

**Antibodies.** Mouse IgM monoclonal antibodies 2D4 (21) and 1B2 (22), directed to gangliotriaosylceramide and N-acetyllactosamine structure, were used. This investigation has been supported by research grants from the NIH (CA20026 and GM23100). Present address: Department of Medicine, Okayama University School of Medicine, Okayama, Japan.

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respectively, were prepared as previously described. Five ml of hybri-
doma ascites were fractionated on a Sephadex G-25 column equilibrated
with 1.0 M NaCl and 0.1 M NaHCO₃, and the void fraction was collected
as IgM. The biotinylated derivative of the IgM antibody was prepared
according to the procedure of Bayer et al. (23) with some modification.
Five mg of biotin:N-hydroxysuccinimide in 0.25 ml of dimethyl formamide
were added slowly to 5 ml of the IgM antibody solution and incubated
at room temperature for 10 min. The biotinylated IgM antibody was
purified using an Ultrogel column (M, 200,000 to 350,000) in a cold room
(4°C; dimension of the column, 2.5 x 120 cm). Eluates were collected as
7-ml fractions on a fraction collector, and the titer of the anti-gangliotria-
syl ceramide antibody was determined by hemagglutination of guinea pig
erythrocytes. The titer of the anti-N-acetylfucosamine antibody in the
eulates was determined by hemagglutination of human erythrocytes. The
presence of biotin residue in the biotinylated antibody was determined by
the precipitation reaction with avidin in double-gel diffusion plates in 0.5%
avarage. The purified biotinylated IgM antibody had a hemagglutination
titer of 1:572 and a protein concentration of 0.8 mg/ml. These antibodies
were stored at −70°C.

The monoclonal IgG antibody (R17-217) directed to the mouse TF
receptor was the same preparation as previously described (24) and was
donated by Dr. Ian Towbridge (Salk Institute, La Jolla, CA).

Cells. Variant clones of mouse lymphoma L-5178Y, B10.D2 were prepared
as previously described (9). The high-expression AA12 clone was reisolated
from 1A1, which was previously described (9), and was kindly donated
by Dr. W. W. Young, Jr. (Department of Pathology, University of Virginia
Medical School, Charlottesville, VA). Gangliotriaosyl ceramide was ex-
erpressed on the surface of all cells (100% expression). The nonexpressor
clonel 27AV was isolated as previously described as an NK-insusceptible
cell line (25) and was kindly donated by Dr. David Urdal (Immunex Corp.,
Seattle, WA). The nonexpressor cells did not contain any detectable
chemical amount of gangliotriaosyl ceramide. These cells were all free
from Mycoplasma contamination.

Other Miscellaneous Materials. Biotin:N-hydroxysuccinimide was
purchased from Pierce Chemical Co. (Rockford, IL). Avidin, crystalline
bovine serum albumin, crystalline bovine insulin, and human TF were
obtained from Sigma Chemical Co. (St. Louis, MO). Pronase was ob-
tained from Worthington Biochemical Corp. (Freehold, NJ). Fluorescein
isothiocyanate-conjugated rabbit anti-mouse IgM and anti-mouse IgG
were obtained from Cappel Laboratories (Cochranville, PA), and fluores-
cein-conjugated concanavalin A was obtained from Calbiochem Behring
Co. (San Diego, CA). RPMI Medium 1640 and fetal calf serum were
purchased from Grand Island Biological Co. (Grand Island, NY). Ultrogel
was from LKB Co. (Rockville, MD), and 24-well and 96-well tissue culture
plastic plates were from Costar (Cambridge, MA).

Methods

Cell Culture. Cells were propagated in RPMI Medium 1640 supple-
mented with 10% heat-inactivated fetal calf serum. These cells were also
cultured in chemically defined medium, adopting the conditions used for
mouse myeloma or hybridoma cell culture (12). The medium consisted of
RPMI Medium 1640 supplemented with 1 mM pyruvate, 2 mM glutamate,
100 μM selenium, and 20 μM ethanolamine with various concentra-
tions of TF as indicated in "Results."

Cell Growth Inhibition. Cell proliferation was determined in a 24-well
tissue culture plate. Three × 10⁶ cells in exponentially growing phase
were plated in each well and cultured in the presence or absence of
biotinylated antibody and avidin for 48 h. The cell number was determined
using a Model 281 Coulter Counter (Coulter Electronics, Inc., Hialeah,
FL). The degree of cell proliferation was expressed as the percentage of
increase in cell number, specifically, N/N₀ × 100%, where N₀ and N are
the numbers of cells at 0 and 48 h, respectively. The percentage of
increase in cell number in the absence of biotinylated antibody and avidin
was taken as 100% and considered to be no growth inhibition.

Lodination of TF. Iron-free human TF was saturated with iron, using
ferric ammonium citrate according to the procedure of Larrick and
Cresswell (26). Four hundred μg of iron-saturated TF [TF(Fe)₄] were
lodinated with 2 ml of carrier-free Na¹²⁵I by a modified chloramine T
method (27). Briefly, TF was dissolved in 0.3 ml of 0.3 M phosphate
buffer, pH 7.4, and reacted sequentially with 20 μl of Na¹²⁵I (2 mCi) and
4 μg of chloramine T dissolved in 15 μl of PBS, and the mixture was
incubated at room temperature for 60 s. After further addition of 15 μg
of sodium metabisulfitde and 2 mg of potassium iodide (KI), iodinated TF
was purified on a 10-ml Sephadex G-25 column and stored in 0.2% bovine
serum albumin album in PBS at −70°C. The specific activity of the
product was about 1.7 μCi/mg. It was used within 4 weeks after
preparation.

Binding of TF on the Cell Surface. Exponentially growing lymphoma
cells were divided into 2 groups. One group was cultured in the presence
of biotinylated antibody 2D4 (20 μg/ml) and avidin (50 μg/ml) in RPMI
Medium 1640 supplemented with 10% heat-inactivated fetal calf serum
for 24 h (thus growth was inhibited) and consequently washed 3 times
with the binding buffer (RPMI Medium 1640 supplemented with 15 μm
N-2-hydroxyethylpiperazone-N'-2-ethanesulfonic acid and 0.1% bovine
serum albumin, pH 7.3) at 4°C. Another group of cells was cultured under
the same conditions but in the absence of avidin (cells of this group
were continuously growing). Each group of 5 × 10⁶ cells was incubated with
34 ng of ¹²⁵I-TF(Fe)₂ and various amounts of native TF(Fe)₂ in a final
volume of 0.3 ml at 0°C for 45 min. At the end of the incubation
period, TF bound to the cells was separated from free TF by passage of
the cells through a cushion of 30% sucrose in a Beckman Model 12
Microfuge at Speed 10 for 5 min. After the buffer and the sucrose layers
were aspirated, the tips of tubes containing the cell pellet were excised,
and radioactivity was counted in a Beckman Model 7000 gamma counter.
Nonspecific binding of ¹²⁵I-TF(Fe)₂, determined as bound radioactivity in
the presence of 3.3 mg of native TF(Fe)₂, was less than 3% of total
binding and was subtracted from the total binding to get specific binding.

Internalization of Cell-bound TF. To examine internalization of sur-
face-bound TF, cells were first incubated with ¹²⁵I-TF(Fe)₂ at 0°C for 45
min as above, the unbound TF was washed off with the binding buffer,
and the cells were incubated at 37°C in a water bath. At indicated times,
the incubation mixture was cooled to 0°C in an ice bath. One aliquot
was analyzed to determine the total cell-associated TF, after separation
of cells through a sucrose layer using a Beckman Microfuge as described
above. Another aliquot was digested with 10 μg of Pronase per 100 μl
of cell suspension at 0°C. After 60 min, the cell-associated and free TF
were separated. The cell-associated TF was considered to be internal-
ized TF, and the Pronase-releasable TF was considered to be a sum of
cell surface-bound, exocytosed, and degraded TF.

Immunofluorescence Assays. Cells were cultured in the presence of
20 μg of biotinylated antibody 2D4 per ml and 50 μg of avidin per ml.
After various time periods, cells were washed 3 times with cold PBS at
4°C. Cells were either stained directly or after incubation with antibody
2D4 for 60 min at 0°C or with fluorescein isothiocyanate-conjugated
rabbit anti-mouse IgM and anti-mouse IgG antibodies which specifically
react with mouse IgM but not with mouse IgG.
RESULTS

Cell Growth Inhibition. The growth of high-expressor AA12 cells was inhibited in the presence of biotinylated IgM anti-gangliotriaosylceramide antibody (2D4) and avidin, but not by avidin or biotinylated antibody 2D4 alone; the growth of non-expressor 27AV cells was not inhibited by the biotinylated antibody 2D4 and avidin (see Chart 1 and Table 1). This growth inhibition was not due to the effect of avidin or the avidin:biotinylated antibody complex, since the addition of biotinylated IgM anti-N-acetyllactosamine antibody (1B2) and avidin had no effect on cell growth (Chart 2). The cell growth of AA12 cells was increasingly inhibited by increasing concentration of avidin in the presence of biotinylated antibody 2D4 (Chart 3). These results suggest that the cell growth inhibition induced by the biotinylated antibody 2D4 and avidin is due to cross-linking of the antibody at the cell surface. This conclusion is supported by the fact that cell growth was also inhibited by the antibody 2D4 cross-linked with a second anti-mouse IgM antibody, as shown in Table 2. The growth of inhibited cells returned to normal after they were washed and placed in culture medium without the biotinylated antibody 2D4 and avidin; the increase in cell number was almost the same as that of control cells (data not shown). This indicates that the growth inhibition induced by the biotinylated antibody 2D4 and avidin is a reversible cell surface process and that the continuous presence of the cross-linking reagent is necessary to effect the growth inhibition.

Inhibition of TF-dependent Cell Growth in Chemically Defined Medium by the Biotinylated Antibody 2D4 and Avidin. The cell growth of the high-expressor clone can be maintained in chemically defined medium, as described in "Materials and Methods," with TF as the only required growth factor. Even a trace quantity (0.02 µg/ml) of TF could stimulate cell growth 300% compared with cells grown without addition of TF. TF addition in the presence of biotinylated antibody 2D4 and avidin gave only slightly over 100% stimulation of cell growth. Essentially no further stimulation of cell growth was observed by increasing the concentration of TF (see Chart 4). Addition of insulin stimulated cell growth, but cells grew without insulin. Neither the conditioned medium of AA12 cells nor the medium dialyzed and concentrated promoted cell growth when added to

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Table 1

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<th>Conditions</th>
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<td>Biotinylated antibody 2D4</td>
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* Mean ± SD of the percentage of inhibition of cell growth of 3 determinations.

Table 2

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<th>Anti-mouse IgM antibody</th>
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<td>0 ± 1.2*</td>
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<td>-3.2 ± 3.3</td>
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<td>+</td>
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<td>65.4 ± 2.0</td>
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* Mean ± SD for 3 determinations.
TF-dependent cell growth stimulation was almost completely inhibited by the addition of biotinylated antibody 2D4 and avidin but was not inhibited by the addition of antibody alone, as shown in Chart 4. Growth-inhibited cells became refractory to TF stimulation even at high concentrations of TF (10 to 30 μg/ml). The binding of 125I-labeled TF to AA12 cells in the presence or absence of biotinylated antibody 2D4 and avidin is shown in Chart 5. Cells whose growth was inhibited by brief pretreatment with biotinylated antibody and avidin exhibited slightly fewer TF binding sites as compared to noninhibited cells grown in the presence of the biotinylated antibody 2D4 alone (without avidin). However, the difference between the growth-inhibited and control cells was much less than the difference demonstrated by the rate of cell proliferation (cell number increase).

Inhibition of TF Internalization by the Biotinylated Antibody 2D4 and Avidin. Based on the results described above, we measured the rate of TF internalization by the procedure described in "Materials and Methods." Cells whose growth was inhibited by pretreatment with the biotinylated antibody 2D4 and avidin showed a much lower amount of cell-associated, Pronase-resistant 125I-TF, as compared to the value for control cells pretreated with the biotinylated antibody 2D4 alone (Chart 6). These results suggest that cells whose growth was inhibited by the biotinylated antibody 2D4 and avidin had a much lower rate of TF internalization than control cells. This conclusion is supported by the larger quantity of Pronase-releasable and Pronase-sensitive TF at the surface of growth-inhibited cells as compared to the control cells, as shown in Chart 7. A rapid initial decrease and the subsequent slow increase of Pronase-releasable TF, which occurs when cells are brought to 37°C, indicate the process of internalization and release of the radioactive degradation product of TF, respectively.
Morphological Changes in Growth-inhibited Cells. The high-expressor cells whose growth was inhibited in the presence of biotinylated antibody 2D4 and avidin were of variable size and became slightly larger in diameter (poikilocytosis) (Fig. 1a) than cells grown in the absence of avidin or the biotinylated antibody 2D4, whose growth was not inhibited. The diameter of the growth-inhibited cells returned to normal after they were washed and cultured in the absence of the biotinylated antibody and avidin (Fig. 1b). Cells grown in the presence of the biotinylated antibody 2D4 alone (Fig. 1c), avidin alone (Fig. 1d), or the antibody 2D4 (without biotinyl substitution) and avidin (Fig. 1e) showed normal cell size and exhibited no poikilocytosis.

The distribution of gangliotriaosylceramide was studied by fluorescein-conjugated antibody 2D4 alone (Fig. 2). A capping of gangliotriaosylceramide was observed in the presence of the biotinylated antibody 2D4 and avidin, in striking contrast to the homogeneous distribution of the antigen in the presence of the biotinylated antibody 2D4 alone. The degree of capping was obvious at 6 h after the addition of the cross-linking reagent and had increased somewhat at 24 h. No capping was induced even after 24 h in the absence of avidin.

The distribution of concanavalin A binding sites was studied by staining with fluorescein-conjugated concanavalin A, as shown in Fig. 3. Concanavalin A binding sites were homogeneously distributed around the cell surface, and no capping was induced by the biotinylated antibody 2D4 and avidin.

The distribution of the TF receptor was studied using a monoclonal antibody directed to the TF receptor. As shown in Fig. 4, the TF receptors were distributed around the cell surface and were not capped, in contrast to gangliotriaosylceramide which was capped in the presence of the biotinylated antibody 2D4 and avidin. The receptor distribution was patchy, but not capped, when cells were cultured in the presence of the biotinylated antibody 2D4 without avidin. The distribution of the TF receptor was identical in the absence of biotinylated antibody 2D4 and avidin.

**DISCUSSION**

A variety of experimental approaches have been used to evaluate whether membrane glycolipids participate in cell growth regulation. Exogenous addition of glycolipids in cell culture, which incorporate into plasma membranes and enrich specific types of glycolipids in membranes, has been one approach that has been utilized to observe modifications of the cell cycle, cell proliferation, and growth factor-mediated receptor function (28–33). A second approach has been the alteration of glycolipid metabolism induced by specific reagents such as a sphingosine analogue5 and tunicamycin (34, 35). These reagents modify glycolipid composition through direct inhibition of their synthesis (for sphingosine analogues) or through a yet-unidentified mechanism (for tunicamycin). A third approach which has been used is the application of antibodies directed to specific types of glycolipids. Either whole antibodies or their monovalent derivatives (Fab) included in culture medium modify the metabolism and organization of glycolipids, resulting in either enhancement or inhibition of glycolipid synthesis (18–20, 36). Monovalent affinity-purified antibodies to G\(\alpha\)\(\delta\), but not to globoside, induce enhanced synthesis of G\(\alpha\)\(\delta\), inhibit cell growth of nontransformed BHK or 3T3 cells, and reduce cell saturation density (18). Significant growth inhibition of astrocytoma cells is induced by anti-G\(\alpha\)\(\delta\) and anti-G\(\alpha\)\(\delta\) antibodies and is accompanied by increased adenylyl cyclase and decreased guanylate cyclase activities (20). Antibodies to G\(\alpha\)\(\delta\) or G\(\alpha\)\(\gamma\) preincubated with rat kidney NRK cells or 3T3 cells inhibit phenotypic changes associated with oncogenic transformation caused by tumor viruses (19), although the mechanism of inhibition has not been elucidated. Cell growth of human melanoma was inhibited by IgG3 monoclonal antibody (524) to

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Fig. 3. Distribution of concanavalin A binding sites on AA12 cells. Cells were pretreated with biotinylated antibody 2D4 and avidin (a) or biotinylated antibody 2D4 alone (b) for 24 h. Cells were then stained with fluorescein isothiocyanate-conjugated concanavalin A at 0°C, and pictures were taken under a fluorescent microscope.

Fig. 4. Independent distribution of TF receptor and gangliotriaosylceramide on AA12 cells. AA12 cells were precultured in the presence of biotinylated antibody 2D4 and avidin for 24 h. Cells were then stained with the monoclonal IgG antibody (R17-217) directed to the mouse TF receptor and fluorescein isothiocyanate-conjugated rabbit anti-mouse γ-chain antibodies (a). Another aliquot of cells was stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgM (μ-chain specific) antibodies (b). Control cells (not treated with biotinylated antibody 2D4 and avidin) stained with anti-mouse TF receptor and fluorescein isothiocyanate-conjugated rabbit anti-mouse γ-chain antibodies (c) and untreated control cells stained with antibody 2D4 and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgM antibodies (d) are also shown.

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GLYCOLIPID ANTIGEN AND TF RECEPTOR

G_{3} ganglioside (36), but not by IgM monoclonal antibody (4.2) directed to the same ganglioside antigen. The present study shows that the growth of AA12 and 27AV lymphoma cells is not inhibited by either the 2D4 or 1B2 monoclonal antibodies, in general agreement with the notion that tumor cells are less susceptible than normal cells to surface-mediated growth inhibition by antibodies (18).

In this study, however, new procedures using biotinylated antibodies and avidin have been used to effect growth inhibition. A similar, effective growth inhibition was observed with the anti-glycolipid antibody and the secondary antibody. Thus, a cross-linking of antibodies bound to glycolipid tumor antigens is essential to induce growth inhibition, because no growth inhibition was observed in nonexpressor cells, and no effect was observed with the biotinylated anti-lactosaminyl antibody 1B2 and avidin. The lactosaminyl antigen defined by antibody 1B2 was absent from these cells. The cross-linking of gangliotriaosylceramide at the surface of high-expressor cells has been assessed by capping of the antigen in normal medium as well as in chemically defined medium. The capping was specifically induced by the biotinylated antibody 2D4 and avidin, but not by the biotinylated antibody 1B2 and avidin.

The mechanism of cell growth inhibition induced by cross-linking of glycolipid antigens has not been elucidated in depth; however, a close correlation between glycolipid capping and dysfunction of the TF receptor has been indicated by the fact that the internalization of TF is greatly inhibited when cell growth is inhibited by biotinylated 2D4 and avidin, although the TF receptor is not capped together with gangliotriaosylceramide and is distributed independently from gangliotriaosylceramide. A few possibilities can be considered to explain the mechanism for the inhibition of TF internalization induced by cross-linking of gangliotriaosylceramide. (a) A capping of gangliotriaosylceramide may cause a qualitative defect in the function of the lipid bilayer as well as its associated cytoskeletal system, thus causing the TF receptor present at the remaining plasma membrane to become dysfunctional. (b) A more attractive mechanism is based on the assumption of the presence of an as yet unidentified membrane component X which may be closely associated with glycolipids as well as with the TF receptor. The component X could be essential in maintenance of TF receptor function and its internalization. The component X could be moved with the glycolipid antigen when the glycolipid antigen is capped by cross-linking, thus inhibiting clustering of the TF receptor. Such a mechanism may not be unrealistic in view of recent observations that some membrane proteins are associated with glycolipids. An association of specific membrane proteins with a specific glycolipid has been postulated. Globoside, the major glycolipid of human erythrocytes, may be associated with unidentified proteins having molecular weights of 50,000 and 76,000. This association has been detected by cross-linking with bifunctional, photosensitive, cross-linking reagents coupled to anti-globoside antibody (37). In addition, the association of a low-molecular-weight polypeptide antigen (Paul-Bunnell antigen) with the ganglioside G_{4} and the specific affinity of this antigen with ganglioside G_{4} have been demonstrated (38).

A dysfunction of the TF receptor associated with cross-linking of the glycolipid antigen is clearly indicated by the inhibition of TF internalization. A decrease of cell-associated, Pronase-resistant TF was induced by the biotinylated anti-glycolipid antibody and avidin. The results show clearly that internalization is inhibited in a time-dependent manner. TF is a ubiquitous growth factor for essentially all animal cells, although the requirement for TF varies significantly (13, 39). The TF receptor is expressed on the surface of actively proliferating cells such as stem cells, virus-infected cells, and malignant cells, but not on nonproliferating cells (14–18). Both the high-expressor AA12 cells and the nonexpressor 27AV cells can be grown in chemically defined medium in which TF is the only growth factor. These cells do not require insulin, hydrocortisone, epidermal growth factor, or platelet-derived growth factor, in striking contrast to many animal cells which require these growth factors in addition to TF (39). Furthermore, these cells do not seem to depend on an autocrine effect. Since the growth of high-expressor AA12 cells was specifically inhibited by the biotinylated antibody 2D4 and avidin, but the AV27 cells were not, it is logical to assume that the growth inhibition depends on capping of the TF receptor. Cells whose growth was inhibited became refractory to TF stimulation and did not internalize surface-bound TF, although the binding capacity to TF was close to that of noninhibited cells.

High-expressor cells are susceptible to NK cells, in contrast to nonexpressor cells which are insensitive to NK cells (9). Although antibody 2D4 does not inhibit NK killing (9), other monoclonal antibodies directed to gangliotriaosylceramide can inhibit NK killing (40). A possible correlation between NK susceptibility and the expression of gangliotriaosylceramide has been postulated (9, 41); however, various sublines showing a great deal of variation in gangliotriaosylceramide expression all show a similar NK susceptibility (9). More recent studies have found evidence that the TF receptor could be a target structure for NK cells (42). An interesting possibility is that a complex consisting of gangliotriaosylceramide or other glycolipids and the TF receptor could be the essential target for NK cells. If either of the components of this complex were absent, NK susceptibility would be lost. For example, the nonexpressor AV27 cells do not possess gangliotriaosylceramide, but they have the TF receptor and therefore are not susceptible to NK killing. In agreement with previous results (11), nonexpressor clones of gangliotriaosyl antigen showed a significantly higher ability for host killing than high-expressor clones in vivo. The average life span of DBA/2 mice inoculated with 10^6 high-expressor clone AA12 was 40 days, while that of mice inoculated with 10^6 nonexpressor clone AV27 was 22 days. These high- and nonexpressors showed identical growth curves in vitro in either serum-containing medium or chemically defined medium. In addition to providing NK susceptibility, it is possible that the presence of gangliotriaosylceramide greatly modifies growth factor-mediated cell growth by ligands which could bind to gangliotriaosylceramide. The ligands could be antibodies, lectins, glycosyltransferase, or glycosylhydrolases. The glycolipid antigens that characterize the lineages and genetic origin of tumor cells may also greatly affect growth regulation at the cell surfaces and may define the degree of cancer.

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Fig. 1. AA12 cells were cultured in the presence of biotinylated antibody 2D4 and avidin (a), biotinylated antibody 2D4 (c), avidin (d), unsubstituted biotinylated antibody 2D4 and avidin (e), and biotinylated antibody 1B2 (f) for 48 h, and pictures were taken under a phase-contrast microscope. AA12 cells pretreated with biotinylated antibody 2D4 and avidin for 48 h were washed free of antibody and avidin and cultured for an additional 24 h in the absence of biotinylated antibody and avidin (b).
Fig. 2. Biotinylated antibody 2D4 in AA12 cells. AA12 cells were precultured in the presence of biotinylated antibody 2D4 and avidin for 6 h (A, B) or 24 h (E, F), or in the presence of the biotinylated antibody 2D4 alone for 6 h (C, D) or 24 h (G, H). Pretreated cells were stained with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgM, and pictures were taken under a fluorescent microscope (A, C, E, G) and under a phase-contrast microscope (B, D, F, H).
Inhibition of Tumor Cell Growth by Aggregation of a Tumor-associated Glycolipid Antigen: A Close Functional Association between Gangliotriaosylceramide and Transferrin Receptor in Mouse Lymphoma L-5178Y

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