Tumor-associated Ganglio-N-triosylceramide

TARGET FOR ANTIBODY-DEPENDENT, AVIDIN-MEDIATED DRUG KILLING OF TUMOR CELLS*

(Received for publication, April 21, 1980)

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The tumor-associated glycolipid, ganglio-N-triosylceramide (GalNAcβ1 → 4Galβ1 → 4Glcβ1 → Cer; GgOsea2Cer) was used as a target for antibody-conjugated drug killing of tumor cells. Because of antibody inactivation by drug conjugation, targeting systems have been developed in which administration of biotinyl-anti-glycolipid antibodies was followed by successive addition of: (a) avidin and biotinyl drug (e.g. neocarzinostatin); (b) avidin and biotinyl phospholipid liposomes in which actinomycin D was encapsulated; and (c) drug encapsulated in liposomes covalently linked to avidin.

Successful targeting and killing of tumor cells expressing ganglio-N-triosylceramide was observed in System a above. Liposome targeting was successful in Systems b and c but liposome-targeted killing of the tumor cells was not efficient.

Anti-ganglio-N-triosylceramide antibodies were derivatized with biotin-N-hydroxysuccinimide with full retention of their antibody function including complement-dependent cytolysis. In both Systems a and b above, avidin was used to bridge biotin-substituted drugs or drug carriers to biotinyl antibody bound to cells. Targeting required the presence of biotin on both the antibody and the drug or drug liposomes. Absorption of biotinyl antibody with guinea pig red blood cells (which contain ganglio-N-triosylceramide) abrogated targeting. Furthermore, ganglio-N-triosylceramide, on the target cells was identified chemically as three glycolipid spots separated on thin layer chromatography. These were characterized as having the same carbohydrate but different ceramides. Biotinyl neocarzinostatin was synthesized with decreased biological activity, but it gained the capacity to be directed to cells with avidin. Biotinyl liposomes encapsulating actinomycin D were prepared from biotinylphosphatidylethanolamine, lecithin, and cholesterol.

In System c above, avidin was covalently linked to liposomes prepared with sialyllactosylceramide by reductive amination after the gentle periodate oxidation of the liposomes. This technique provides a useful basis for the covalent coupling of liposomes to biologically active proteins in general.

The idea of directing drugs specifically to tumor cells to avoid toxic effects on normal tissue is not new. More than 70 years ago, Paul Ehrlich (1) envisioned the use of "bodies which possess a particular affinity for a certain organ ... as a carrier by which to bring therapeutically active groups to the organ in question." Hormones (2), lectins (3,4), and antibodies have since been used in attempts to accomplish this goal. Efforts that have utilized antibody for this purpose (for review, see Refs. 5 and 6) have been hindered by various factors: (i) the lack of high titered tumor-specific antibody, (ii) the loss of antibody activity during conjugation with drug or drug carrier, (iii) the restricted specificity of antibodies directed to tumor-specific transplantation antigens (TSTA), that preclude their use as a common targeting reagent for various tumors, and (iv) the observation that many human cancers and spontaneous murine tumors are nonimmunogenic (7-10). Thus, major obstacles to this type of therapy have been the lack of chemically defined tumor-associated markers and well defined, high titer antibodies.

Glycolipids are attractive target candidates since they are chemically well defined cell surface antigens (11, 12), and affinity-purified polyclonal (12, 13) and monoclonal anti-glycolipid antibodies (14) have been prepared successfully. Two types of glycolipid changes have been observed associated with oncogenic transformation: (i) incomplete synthesis of a carbohydrate which frequently results in the accumulation of a precursor glycolipid (11, 13) and (ii) induction of synthesis of a glycolipid foreign to the host, such as Forssman antigen in human tumors and A-like antigen in blood group B or O hosts (11, 15, 16).

In either processes, (i) or (ii) above, a tumor-associated glycolipid can be found at the tumor cell surface which can serve as a well defined target. Recently, ganglio-N-triosylceramide (GalNAcβ1 → 4Galβ1 → 4Glc → ceramide) was established as a tumor-specific cell surface marker for the tumor in mice derived from Kirsten virus-transformed 3T3 cells (13). We have also found this glycolipid on L5178c127 lymphoma cells and use it as the basis for a targeting model.

This paper describes novel, indirect targeting procedures that have been studied to establish glycolipids as effective tumor targets. Avidin was used to bridge biotin-substituted drugs or drug carriers to biotinyl antibody bound to cells. This method resulted in no loss of antibody activity. Furthermore, this system has the potential for amplification since every antibody can bind more than 1 molecule of avidin. Avidin, in turn, can bind more than 1 molecule of drug.

MATERIALS AND METHODS

Biotin Substitution of Anti-glycolipid Antibodies—The N-hydroxysuccinimide ester of biotin was coupled to 125I-labeled antibodies, IgG and IgM, at a molar ratio of 1:1000 by a modification of the method of Gamble et al. (15). The avidin-biotin conjugate was prepared by coupling avidin and biotin-N-hydroxysuccinimide ester in the presence of 1 M ethanolamine as described by Davis et al. (16). The extent of coupling was determined by the assay of the bound avidin by radioimmunoassay, or by the nitroblue tetrazolium reduction assay of the avidin-biotin conjugate.


1 Portions of this paper (including part of the "Materials and Methods," Figs. 10 to 12, Table III, and additional references) are...
ysecunimide ester of biotin (BNHS) was made according to an established procedure (17). One millimole (206 mg) of dicyclohexylcarbodiimide (Sigma Chemical Co., St. Louis, MO) was added to 4 ml of dimethylformamide containing 1 mmol (244 mg) of biotin (Sigma) and 1.3 mmol (150 mg) of N-hydroxysuccinimide (Sigma) and stirred under nitrogen at room temperature for 24 h. Insoluble dicyclohexylurea was removed by the addition of a solution of sodium metaperiodate for 10 min in ice. Glucose (final concentration 30 mM) was added to stop the reaction. The preparation was then dialyzed against NaCl/P, (pH 6.5) to remove non-encapsulated acylaminocytin D. The liposomes were then incubated with 8 nm (final concentration) sodium metaperiodate for 45 min in ice. Glycerol (final concentration 30 mM) was added to stop the reaction. The preparation was then dialyzed against NaCl/P, (pH 6.5, overnight. Avidin was added (1 mg/4 ml of lecithin) and incubated overnight at room temperature. Sodium cyanoborohydride (Aldrich Chemical Co., Milwaukee), 8 ml/100 ml of lecithin, was then added and incubation continued at 4°C with stirring for 48 h. Liposomes associated and free avidin were then separated on Sephar-yl S-200 (Pharmacia Chemicals, Piscataway, NJ).

Alternatively, in order to shorten the length of time for the procedure, liposomes were prepared as above and then directly oxidized. The oxidation was stopped with glycerol and 30 min later the prep-paration was passed over a Sephadex G-50 column. The void volume was collected, to which avidin (1 mg/4 ml of lecithin) and NaCNBH, (8 pmol/50 pmol of lecithin) were added together and incubated in the cold 48 h. Free avidin was separated from liposomes as previously described. Liposomes were washed in NaCl/P, by gel precipitation of ganglio-N- triosylceramide liposomes as previously described (14).

Biocytin Substitution of Neocarzinostatin and Phosphatidylethanol- anolamine—Neocarzinostatin (21) was provided by Dr. Kanamaru (Institute of Tuberculosis, Tokohu University, Sendai, Japan). It was supplied in 2-ml vials containing 2000 units of neocarzinostatin in sodium acetate/acetic acid buffer (0.15 M, pH 5.0); 1400 units were equivalent to 1 mg. A Sephadex G-25 column. Thirty minutes after the final addition of BNHS, 10 pmol of glycine was added to react with any remaining BNHS. Then biocytin neocarzinostatin was dialyzed in Spectropore tubing (Spectrum Chemical, Piscataway, NJ).

Biotinylation of Phosphatidylethanolamine (by the ability of biotin to compete with the binding of 2(4'-hydroxyazobenzene)benzoic acid to avidin (29)). Acalcycin D was quanti-tated by using H-labeled acylaminocytin D (4 Amersham, Arlington Heights, IL) as a tracer. Controls included in these experiments were liposomes made with GM3 but made without oxidation or liposomes that were made without GM3.

Fluorescent Liposomes—Liposomes consisting of biotinyl-PE (0.1 pmol), lecithin (1 pmol), cholesterol (0.75 pmol), and GM3 (0.1 pmol) were prepared by injecting the lipid mixture (40 ml of ethanol) into 1 mol of 0.05 M carbonate-buffered isocin, pH 9.0. Forty micrograms fluorescein isothiocyanate (Sigma) was then added in the cold and the solution stirred overnight at 4°C. The fluorescent liposomes were then collected from the void volume of a Sephadex G-20 column (Pharmacia Chemicals).

Cell Culture—Kirsten virus-transformed 3T3 cells were cultured as previously described (13). L5178x127 cells were obtained from Dr. Christopher Henney (Basic Immunology, Fred Hutchinson Cancer Research Center, Seattle, WA). They were cultured in RPMI 1640 medium (NHCO, Grace Bio-Chemistry, Rockville, MD) + 200 nm to 0.1% dialyzed fetal calf serum, penicillin, streptomycin, and 2 mM pyruvate. Cell counts were made on a Coulter model ZBI cell counter. The proliferation assay for determining drug toxicity was performed with cells from log phase cultures (less than 900,000 cells/ml). Cells were washed in NaCl/P, pH 7, and suspended at 2000 cells/ml in growth medium. One-half milliliter of this suspension was added to 0.5 ml of growth medium containing increasing concentrations of neocarzinostatin or biotinyl neocarzinostatin (three wells per drug dilution). 0.5 ml aliquots of cells were counted at this time to deter-mine the cell number at time zero. Forty-eight hours later each well was counted. Cell proliferation was determined as the per cent in-crease in cell number (the number of cells at 48 h + the number of cells at time zero) divided by the number of cells at time zero x 100%. The growth seen in cultures without drug was normalized to 100% (0% inhibition) and the proliferation in experimental cultures ex-pressed above growth seen in control cultures. Proliferation in all cases without drug was at least 60%.

The assay for drug targeting was done on ice. 4.2 million cells were washed in NaCl/P,. The pellet was resuspended in 100 ml of antibody (1 mg/ml, H.A. titer 1:15) and incubated on ice for 45 min. Six milliliter of NaCl/P, containing 0.5% dialyzed fetal calf serum were then added to wash the cells. The pellet from this wash was suspended in 1 mmol of avidin (1 mg/ml) and incubated for 45 min and then washed as before. This pellet was suspended in 4 ml of NaCl/P,. One-half milliliter of this suspension was added to 0.5 ml of increasing concentra-tions of drug in NaCl/P, and incubated on ice for 15 min. Then, 6 ml of growth medium was added to stop the reaction. The final cell pellets were suspended in 5 ml of growth medium and 1 ml aliquots (100,000 cells) were added to wells (three per drug dilution) on a 24-
well microtiter plate (Falcon Plastics) and incubated at 37°C for 48 h. One milliliter of the cell suspension was counted to determine the number of cells at time zero for each drug dilution. Viable cells were examined by trypan blue exclusion.

RESULTS

Biotin Substitution of Antibody—Antiserum derivatized with biotin (Fig. 1, Well B) could precipitate avidin (Fig. 1, Well I) in gel diffusion. It retained its antigenic character as recognized by anti-rabbit immunoglobulin (Fig. 1, Well 3) and anti-rabbit whole serum (Fig. 1, Well 2) and it could still precipitate liposomes containing ganglio-N-triosylceramide (Fig. 1, Well 4). Substitution of up to 70% of the amino groups with biotin had little effect on the ability of antibody to agglutinate guinea pig red blood cells (Table I). Furthermore, biotin-substituted antibody could still fix complement (Table I).

![Fig. 1. Gel diffusion of antibodies to ganglio-N-triosylceramide (GgOse3Cer) before (Well A) and after (Well B) substitution with biotin. Well 1, avidin 1 mg/ml; Well 2, sheep anti-whole rabbit serum; Well 3, goat anti-rabbit IgG; Well 4, GgOse3Cer liposomes.]

<table>
<thead>
<tr>
<th>Table I</th>
<th>Biologic activity of antisera before and after substitution with biotin</th>
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<tr>
<td>Serum</td>
<td>Biotin substituted NH2 groups</td>
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<tr>
<td>1. Nonimmune</td>
<td>0</td>
</tr>
<tr>
<td>2. Anti-GgOse3Cer</td>
<td>68%</td>
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<tr>
<td>3. Anti-GgOse3Cer</td>
<td>64%</td>
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* Agglutination of guinea pig red blood cells.
** Complement fixation with purified GgOse3Cer from guinea pig red blood cells.

- , no reaction; n.d., not done.

Synthesis of Biotin Neocarzinostatin— Ninety-six per cent of the amino groups on neocarzinostatin were substituted with biotin following our procedure. Biotin neocarzinostatin, in contrast to native neocarzinostatin, could precipitate avidin in double gel diffusion (Fig. 2) and the line of identity between biotin antibody (Fig. 2, Well I) and biotin neocarzinostatin (Fig. 2, Well 2) established the feasibility of the proposed indirect targeting mechanism.

Biological Activity of Biotin Neocarzinostatin—The toxicity of biotin neocarzinostatin was measured by the inhibition of cell proliferation. The activity of biotinyl neocarzinostatin was lower than the inhibitory activity of neocarzinostatin (Fig. 3). The ID50 of neocarzinostatin was extrapolated to 1.8 nM whereas the ID50 of biotin neocarzinostatin was 12 nM. Cells were not lysed immediately when treated with a dose of biotinyl neocarzinostatin or neocarzinostatin that completely inhibited cell proliferation. The cells would swell up but they continued to exclude trypan blue for 48 to 72 h before they began to deteriorate.

Targeting of Biotin Neocarzinostatin to Cells—Under the conditions used for targeting only biotinyl neocarzinostatin inhibited cell proliferation (Fig. 4). The controls presented in Fig. 4 emphasize that the presence of the biotin moiety on both the antibody and the drug was required for effective targeting.

The major glycolipid on guinea pig red blood cells is GgOse3Cer. Absorption of biotinyl antibody on to guinea pig red blood cells abrogated targeting (Fig. 5). In contrast, sheep red blood cells, which do not express ganglio-N-triosylceramide, could not remove the target specificity from the antibody preparation.

Liposome Encapsulated Actinomycin D Targeting to the Cells—In order to avoid the direct chemical modification of drugs, we investigated the targeting of liposome-encapsulated actinomycin D to cells. We synthesized biotinylphosphadiethanolamine. Single compartment vesicles prepared with this compound could precipitate avidin in gel diffusion (Fig. 6) which indicated that the biotin group was exposed on the surface of the liposome and that it could bind to avidin. Actinomycin D was encapsulated into single compartment liposomes (12.5 ± 2 ng/µg of cholesterol (mean ± S.D. of four experiments)) and examined for specific inhibition of cells. L5178x127 cells treated with biotinyl-anti-GgOse3Cer or unsubstituted anti-GgOse3Cer and avidin were compared at liposome-encapsulated actinomycin D concentrations of 400 nM. Cells treated with the biotin antibody and avidin were inhibited 95% and 30%, respectively (ID50 = 175 nM), while cells treated with unsubstituted antibody and avidin were inhibited 85% and 15% (ID50 = 220 nM) at the same drug concentrations. Clearly, the difference between targeted and nontargeted cells was minimum. We looked at Kirsten virus-transformed 3T3 cells which also contained GgOse3Cer and found that the ID50 under targeting conditions (biotinyl-anti-GgOse3Cer versus unsubstituted anti-GgOse3Cer) was 25 nM and 45 nM, respectively. Thus, these cells were more sensitive to actinomycin D and the specific effects of targeting were more suggestive. The inhibition of [3H]uridine uptake into acid-precipitable material was used as a more specific assay for actinomycin D activity. As illustrated in Table II, no specific inhibition of [3H]uridine uptake was observed under targeting conditions. Biotinyl antibody and biotinyl liposomes precipitate with avidin in gel diffusion, yet we could not demonstrate dramatic killing of cells under conditions that should guarantee the specific association of liposomes with cells. In order to convince ourselves that targeting was taking place, we prepared fluorescein isothiocyanate-labeled liposomes. Fig. 7 illustrates that only cells...
Targeting Drugs to Glycolipid

Concurrent with the experiments described for biotinyl-phosphatidylethanolamine, we covalently linked avidin directly to liposomes. Single compartment liposomes made up of lecithin, cholesterol, and N-glycolyl hematoside (NGlyNeuα2 → 3Galβ1 → 4Glc → Cer) were oxidized with sodium metaperiodate. Avidin was then coupled to the resulting aldehyde at C7 of the sialic acid by reductive amination in the presence of NaCNBH3. Fig. 8A illustrates that protein co-elutes with liposomes and actinomycin D after this procedure. Liposomes that were not oxidized (Fig. 8B) or liposomes that were oxidized but that did not contain hematoside (Fig. 8C) did not have any protein associated with them. The covalent nature of the protein-liposome association was demonstrated by NaDodSO4 polyacrylamide gel electrophoresis.

that had been treated with biotinyl-anti-GgOse₃Cer and avidin were fluorescent. Cells treated with unsubstituted anti-GgOse₃Cer and avidin (Fig. 7B) or avidin alone were negative. These results suggested that targeting of liposomes was occurring (evidenced by the specific labeling of cells with fluorescent lipid label) but that delivery of the liposome-encapsulated drug was not taking place (suggested by poor growth inhibition of cells and no specific inhibition of [³H]uridine uptake).
To the sharp avidin subunits seen with free avidin (Fig. 1, Lane 1), treated with unsubstituted anti-GgOse:Cer + avidin + fluorescent liposomes; B, cells treated with unsubstituted anti-GgOse:Cer + avidin + fluorescent liposomes. Cells treated with fluorescent liposomes alone were also negative. All incubations were done on ice.

**FIG. 7.** Targeting of fluorescent, biotinylphosphatidyl-ethanolamine liposomes to Kirsten cells. A, cells treated with biotinyl anti-GgOse:Cer + avidin + fluorescent liposomes; B, cells treated with unsubstituted anti-GgOse:Cer + avidin + fluorescent liposomes. Cells treated with fluorescent liposomes alone were also negative. All incubations were done on ice.

**FIG. 8.** Sephacryl S-200 separation of free avidin (Peak II) from liposome-associated avidin (Peak I). A, hematoside liposomes oxidized and coupled with NaCNBH3 to avidin; B, hematoside liposomes not oxidized and treated with NaCNBH3 and avidin; C, liposomes without hematoside oxidized and coupled with NaCNBH3 to avidin. Abscissa, elution volume (milliliters) from column (column volume was 170 ml, void volume was 70 ml); , micrograms per ml of cholesterol in fractions (left ordinate); , micrograms per ml of avidin (left ordinate), micrograms per ml of actinomycin D in fractions (right ordinate).

(Fig. 9) Liposome-associated avidin (Fig. 8A, Peak I) demonstrated marked smearing on gels (Fig. 9, Lane 3) in contrast to the sharp avidin subunits seen with free avidin (Fig. 9, Lanes 2 and 4). The coupling of avidin to liposomes resulted in no loss of biotin-binding activity, both Peaks I and II had activities of 12.5 units/mg of protein. Actinomycin D incorporated into these liposomes (2.7 ± 0.3 ng/µg of cholesterol) was less than that encapsulated into biotinylphosphatidyl-ethanolamine liposomes, most likely due to the extended preparation time required for making the avidin-associated liposomes. In spite of this effective method of covalently binding avidin to liposomes, specific killing of avidin-associated liposomes. In spite of this effective method of covalently binding avidin to liposomes, specific killing of avidin-associated liposomes. In spite of this effective method of covalently binding avidin to liposomes, specific killing of avidin-associated liposomes. In spite of this effective method of covalently binding avidin to liposomes, specific killing of avidin-associated liposomes. In spite of this effective method of covalently binding avidin to liposomes, specific killing of avidin-associated liposomes.

**FIG. 9.** NaDodSO4 polyacrylamide gel electrophoresis of liposome-associated avidin. 1 and 8, molecular weight standards (BDH Chemical Ltd., Poole, England); 2, avidin, 10 µg; 3 and 6, liposome-associated avidin, 10 µg (Fig. 8A, Peak I); 4, 10 µg of Peak II (Fig. 8A); 5, avidin (10 µg) with liposomal lipids (hematoside, lectin, cholesterol); 7, 10 µg of Peak II with liposomal lipids. Samples were run on 12.5% polyacrylamide gels according to the basic stacking procedure of Laemmli (31) after boiling 5 min in sample buffer containing 2% NaDodSO4 and 5% 2-mercaptoethanol. Gels were stained with Coomassie Blue R-250.

**The Chemistry of the Target**—The presence of GgOse:Cer on Kirsten-transformed 3T3 cells has been described (13). The glycolipid profile of L5178y127 cells is available from the JBC data repository as Document No. 80M-780B, in the form of one microfiche or photocopy. Orders for supplementary material should specify the title, author(s), and reference to this paper and the JBC Document number, the form desired (microfiche or photocopy) and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by remittance to the order of the Journal in the amount of $2.50 per microfiche or $1.00 per photocopy.

The glycolipid profile of L5178y127 cells is shown in Fig. 10 (Lane 1). Bands a, b, and c, that migrated in the region of guinea pig ganglio-N-triosylceramide and human erythrocyte ganglio-N-tetraosylceramide (GbOse:Cer) were purified to homogeneity. Twenty-five micrograms of each glycolipid was included in liposomes and examined for reactivity with various anti-glycolipid antibodies. Surprisingly, all three bands reacted with antibodies to guinea pig ganglio-N-triosylceramide by gel diffusion (Fig. 11, Wells 4, 5, and 6), hemagglutination inhibition, and complement fixation. None of them reacted with anti-globo-N-tetraosylceramide. The three bands had the same sugar composition with a molar ratio GalNAGal:Glc of 1:1:1 (Table III). Digestion of Bands a, b, and c with a mixture of hexosaminidase and β-galactosidase from Jack bean yielded ceramide monohexoside (Glc-ceramide) in each case and established β-anomeric linkages between the sugars (Fig. 12, A, B, and C). Bands a and b were permethylated and subjected to direct probe mass spectrometry. The presence of a terminal hexosamine was indicated by peaks at m/e 250.
Hydrolysis of the permethylated glycolipid, followed by reduction, acetylation, and examination by GC-MS for permethylated hexose aldol acetates revealed only 2,3,6-trimethyl-1,5-diacyt galactose and 2,3,6-trimethyl-1,5-diacyt galactose which confirmed the carbohydrate structure of Bands a and b as GalNAcβ1→4Galβ1→4Glc→ceramide.

The difference observed in the mobility of the glycolipids on TLC was due to difference in the carbohydrate portion of the molecules. C18 sphingosine was the only long chain base identified by GC-MS for Bands a and b. Band a was composed of fatty acids C24:0 (39%), C24:1 (53%), C23:0 (2%), and C22:0 (4%). Band b was composed of C16:0 (78%), C18:0 (15.6%), and C18:1 (6%) fatty acids. Ganglio-N-triosylceramide from guinea pig erythrocytes had C24:0 (53%), 24:1 (16%), 23:0 (3.1%), 22:0 (26.2%). This difference in fatty acids between glycolipid a and b was also obvious from the direct probe mass spectrometry analysis. Band a had prominent mass peaks at m/e 661, 659, 863, and 831 (863 → 464 + 2) (C24:0; and 833 (C22:0). Band b, on the other hand, had major peaks at m/e 549, 759, and 721 (753 → 32) which are representative of ceramide fragments containing C16:0 fatty acid. Quantities of Band c have been insufficient to permit detailed chemical analysis.

Glycolipids d and e reacted with antibodies to ganglio-N-tetraosylceramide and were digested with β-galactosidase from Jack bean to Bands a and b (Fig. 9, D and E). These bands were thus identified as ganglio-N-tetraosylceramide.

Glycolipids were also analyzed from L5178Yc127 tumors grown in mice (Fig. 10, Lane 4). Bands a and b from the tumor were identical with Bands a and b from in vitro grown cells by direct probe mass spectrometry. Tumor Bands a and b reacted with anti-ganglio-N-triosylceramide, and tumor Bands d and e reacted with antibodies to ganglio-N-tetraosylceramide. To our surprise, tumor glycolipids from the Band c area reacted with antibodies to globo-N-tetraosylceramide (GalNAcβ1→3Galα1→4Galβ1→4Glc→ceramide) as well as to anti-ganglio-N-triosylceramide. Thin layer chromatography of permethylated Band c revealed two spots. The upper spot on permethylated analysis yielded equimolar amounts of 2,3,6-trimethyl-1,5-diacyt galactose, 2,3,6-trimethyl-1,5-diacyt galactose, and 2,4,6-trimethyl-1,5-diacyt galactose which confirmed the presence of globo-N-tetraosylceramide. In addition, we found a prominent band that migrated with sheep erythrocyte Fossmann acetylated or deacetylated. Purified, it reacted with anti-Forssman antibodies. Thus, in addition to the glycolipids with a gangliosyl backbone found in cultured cells, tumors contained glycolipids with the globosyl oligosaccharide chain.

DISCUSSION

Our results clearly indicate the successful targeting of drugs to tumor cells that express a specific glycolipid marker, ganglio-N-triosylceramide, as the target. Of the three procedures employed, i.e. treatment of cells with biotinyl anti-ganglio-N-triosylceramide followed by the successive addition of avidin and biotinyl neocarzinostatin successfully killed target cells. Killing was specific for the glycolipid determinant and required the presence of a biotin group on both neocarzinostatin and antibody. Two of the three procedures employed, i.e. the treatment of cells with biotinyl antibodies followed by the addition of avidin and biotinyl liposomes, or with biotinyl antibodies followed by the addition of avidin covalently bound to liposomes resulted in successful targeting of the liposomes but ineffective killing of cells. Targeting was taking place but the subsequent delivery of drug into the cell was unsuccessful. Weinstein et al. (32) and Lessman et al. (33) have reached a similar conclusion from studies of the incorporation of liposome-trapped carboxyfluorescein into lymphocytes after targeting. Clearly more sophisticated liposomes have to be constructed to facilitate the penetration of drugs to the inside of the cell. Steps in this direction have been taken by the synthesis of liposomes containing fusogenic compounds such as the Sendai virus F protein that promotes the fusion of liposomes to cells (34, 35). Other workers (36) are combining liposome encapsulation with localized hyperthermia to promote the release of liposome-entrapped materials at in vivo target sites. Thus, the potential of liposomes for drug delivery remains to be realized. Targeting is effective, but the ultimate delivery is not.

Neocarzinostatin was the most successful drug used in this study. It was chosen because of reports that its pharmacologic activity can be mediated at the surface of the cell (37). This property eliminated the problem of drug penetration after targeting. Moreover, the amino acid sequence of neocarzinostatin is known (38), and the two free amino groups present on the molecule and used for derivatization are not essential for drug activity (39).

There has been a great deal of interest in the use of liposomes as vehicles to deliver materials to cells (40–44). Specific targeting of liposomes has been studied with lectins (45), aggregated immunoglobulin (46, 47), asialo glycoprotein (48), and antibody nonspecifically attached (48, 49) to vesicles. The noncovalent association of protein with liposomes has been examined extensively more recently (50) but few methods have been described for the covalent binding of protein to liposome (51, 52). We found that reductive amination was an effective means of covalently coupling protein to liposomes. Restriction of the aldehyde group to the liposome and the amino groups to the protein molecule prevented the formation of protein–protein or liposome–liposome hybrids that can be a problem when cross-linking reagents (glutaraldehyde) are used to associate protein to liposome (51). Unlike other studies that used a synthetic lipid to couple immunoglobulin to liposomes (52), our procedure used naturally occurring lipids that are native to the host. Avidin was efficiently bound to liposomes in a covalent stable bond with no loss of biotin-binding activity. The technology for the covalent linking of biologically active proteins to liposome surfaces is important for any potential studies on liposome applications in cell biology and immunology. The method described here will be of potential importance for future studies.

In conclusion, glycolipids were shown to be effective targets for antibody-mediated drug therapy. Moreover, the targeting system that we described can be readily adapted to any molecule having tumor specificity (antibody, lectin, or hormone). Simple substitution with biotin grafted a new function on to the antibody molecule with little effect on its ability to fix complement or to bind to antigen. In a similar fashion, neocarzinostatin gained the capacity to be directed specifically to tumor cells. Thus, although the physiologic function of glycolipids is still obscure, they can be exploited effectively as tumor cell targets.

REFERENCES

Targeting Drugs to Glycolipid

Glycolipid isolation.

Glycolipids were isolated from tissue and cultured cells by extraction with chloroform/methanol (2:1) and 1:2 followed by the Folch partition with water (3:1) according to the procedure published previously (25). The upper (v) phase was dried under argon and subsequently the neutral lipid was saponified with NaOH and Na2CO3 into free fatty acids and long chain glycolipids. The lower (w) phase was acetylated and purified as folllows (25). The acetylated glycolipids were then separated by preparative TLC in 25:40:40:40 ethyl acetate:hexane:methanol:formic acid (v:v:v:v) on a 0.5 mm silica gel coated plate (Merck 5554). Fractions obtained from this procedure were deacylated and purified by preparative TLC in ethyl acetate:methanol:water at 8:4:1 (v:v:v) to get a semi-purified plate with water or by spotting the plate with 0.5% fluorescent at 0.4:1:water:methanol (v:v:v). Individual compounds were visualized with UV light (365 nm) and scraped from the plates and eluted with 25% MeOH-water (v:v).

Enzymatic activity.

The carbohydrate composition was determined by one step chromatography (39 0.0255 on 8.0-100 mesh Bio-Gel P-2) of water soluble extracts obtained from the hydrolysis of glycolipid with 1 N acetic acid containing 0.5% sulfuric acid under nitrogen at 90°C for 8 hours followed by reduction and acetylation as previously described (25).

Purified glycolipids were obtained after hydrolysis in methanol-water: HCl 0.1 M 1:1.5 for 18 hours at 90°C as previously described (26). Water fatty acids and neutral fatty acids were separated on silica gel by the method of Bouboure and Kambouchner (27) and then analyzed by gas chromatography and gas chromatography-mass spectrometry (GC-MS) on a 0.25 mm 10% Supelcoport column (28). The long chain bases were analyzed by GC as 10% on 1% SE-30 at 250°C as previously described (28).

Information on the carbohydrate sequence and acetylenic structure of the glycolipids was obtained from the analysis of permethylated glycolipids (24). Permethylation was achieved with diazomethane. The permethylated glycolipids was isolated by GLC chromatography on a 2 m column at average solvent. If necessary, the permethylated products were further purified by TLC on 0.25 mm silica gel 60 (Merck) with hexane:ethylacetate at 1:1. The permethylated glycolipids were then hydrolyzed with 1 N HCl in methanol-water 1:1 on a 0.25 mm Supelcoport column (50). Final concentrations were 10° or glycolipids in 45° butanol phase. After extraction, the 10% solution was treated at 90°C for 30 minutes, samples were dried under nitrogen and extracted with TFA.

Hydrolysis standards were obtained from the following sources and purified as previously described (25): Galactosyl-trisaccharide from plasma glycoconjugates, glycolipid from sheep erythrocytes, glycolipid from human erythrocytes, glycolipid from sheep erythrocytes; Glc, and Glc, from potato extract. Glycolipid tetrocarboxylate was prepared with mild and extensive hydrolysis of Glyc.

Differential activity.

Purified glycolipids were mixed with eq. (30 mg ml-1) and cholesterol (150 mg ml-1) and dried under nitrogen (molar ratio 0.11-1.17-0.75). The dried residue was reconstituted in 4 ml of ethanol and dispersed into a stability solution of the 1 ml 11.2. These preparations were used with various anti-glycolipid antibodies in Dot blotting, hemagglutination inhibition or complement fixation as described (14).

References for supplemental MATERIALS AND METHODS