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De-*N*-acetyl-gangliosides in Humans: Unusual Subcellular Distribution of a Novel Tumor Antigen[1]

Roger Chammas,[2] Justin L. Sonnenburg, Nicki E. Watson, Tadashi Tai, Marilyn G. Farquhar, Nissi M. Varki, and Ajit Varki[3]

Glycobiology Program and Cancer Center, Divisions of Hematology-Oncology and Cellular and Molecular Medicine, University of California San Diego, La Jolla, California 92093-0687 [R.C., J.L.S., N.E.W., M.G.F., N.M.V., A.V.], and Tokyo Metropolitan Institute, Tokyo 113, Japan [T. T.]

ABSTRACT

The disialoganglioside G_{D3} is a major antigen in human melanomas that can undergo 9-*O*-acetylation of the outer sialic acid (giving 9-OAc-G_{D3}). Monoclonal antibody SGR37 detects a different modification of the G_{D3}, de-*N*-acetylation of the 5-*N*-acetyl group (giving de-*N*-Ac-G_{D3}). We found that conventional immunohistochemistry of the SGR37 antigen is limited by a reduction in reactivity upon fixation with aldehydes (which presumably react with the free amino group) or with organic reagents (which can extract glycolipids). We optimized conditions for detection of this antigen in unfixed frozen tissue sections and studied its distribution in human tissues and tumors. It is expressed at low levels in a few blood vessels, infiltrating mononuclear cells in the skin and colon, and at moderate levels in skin melanocytes. In contrast, the antigen accumulates at high levels in many melanomas and in some lymphomas but not in carcinomas. In positive melanomas, expression is sometimes more intense and widespread than that of G_{D3}.

Both 9-*O*-acetylation and de-*N*-acetylation of G_{D3} seem to occur after its initial biosynthesis. Isotype-matched antibodies against G_{D3}, 9-*O*-acetyl-G_{D3} and de-*N*-acetyl-G_{D3} were used to compare their subcellular localization and trafficking. 9-*O*-acetyl-G_{D3} colocalizes with G_{D3} predominantly on the cell surface and partly in lysosomal compartments. In contrast, de-*N*-acetyl-G_{D3} has a diffuse intracellular location. Adsorptive endocytosis of antibodies indicates that whereas G_{D3} remains predominantly on the cell surface, de-*N*-acetyl-G_{D3} is efficiently internalized into a compartment that is distinct from lysosomes. Rounding up of melanoma cells occurring during growth in culture is associated with relocation of the internal pool of de-*N*-acetyl-G_{D3} to the cell surface. Thus, a minor modification of the polar head group of a tumor-associated glycosphingolipid can markedly affect the subcellular localization and trafficking of the whole molecule. The high levels of the SGR37 antigen in melanomas and lymphomas, its selective endocytosis from the cell surface, and its relocation to the cell surface of rounded up cells suggest potential uses in diagnostic or therapeutic approaches to these diseases.

INTRODUCTION

Gangliosides are Sia[4]-containing glycosphingolipids found on the outer plasma membrane leaflet of most vertebrate cells (1, 2). They contribute to physical properties of the membrane (3), serve as receptors for bacterial and viral adhesins (4), and interact with/modulate the functions of growth factor and extracellular matrix receptors (5, 6). Gangliosides are also potential ligands for endogenously active animal lectins, such as selectins (7, 8) and I-type lectins (9, 10). Most common gangliosides derive from LacCer (Galβ1–4Glcβ1–1′ceramide), which is further modified by different glycosyltransferases. The monosialoganglioside G_{M3} (Siaα2–3LacCer) can be modified by the action of a specific sialyltransferase (ST8Sia I; Refs. 11 and 12), generating the disialoganglioside G_{D3} (Siaα2–8Siaα2–3LacCer). The latter is a well-known marker for cellular activation and malignant transformation (13–16) and is among the ganglioside antigens targeted for the immunotherapy of cancer (17–24).

Various modifications of the common sialic acid *N*-acetyl-neuraminic acid increase ganglioside diversity further (25, 26). For example, *O*-acetylation of the terminal *N*-acetyl-neuraminic acid in ganglioside G_{D3} generates 9-*O*-acetyl-G_{D3} (an epitope also detected by CD60 antibodies; Refs. 27 and 28). MAbs have also been used to detect the loss of the *N*-acetyl group from C5 of *N*-acetyl-neuraminic acid, *i.e.,* de-*N*-acetylated gangliosides containing free amino groups (29–31). Such structural modifications have potential functional implications. For example, *O*-acetylation at C9 of sialic acids masks binding of proteins that recognize the exocyclic chain of Sias *e.g.,* influenza A and B hemagglutinins (32), I-type lectins (33, 34), and complement factor H (35–37). When added exogenously, synthetic de-*N*-acetyl-G_{M3} was found to antagonize the effects of G_{M3} upon epidermal growth factor receptor kinase activity (38).

MAbs against several glycolipid tumor antigens are presently under active study as diagnostic and therapeutic agents. Using the MAbs SGR37 and SMR36 that were raised against chemically synthesized de-*N*-acetyl-G_{D3}, we previously reported reactivity in the human melanoma cell lines Melur and M21 (31). Here, we have compared the distribution of de-*N*-acetyl-G_{D3} (SGR37 antigen) with its parental molecule G_{D3} in human tissues and malignant tumors. We have also evaluated the subcellular distribution of G_{D3} in human melanoma cells in comparison with that of two structurally related derivatives, 9-*O*-acetyl-G_{D3}, and de-*N*-acetyl-$G_{D3.}$ Despite the subtle structural differences among these molecules, we found marked differences in their tissue distribution, subcellular localization, and trafficking.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all reagents used were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and Antibodies. Hybridoma R24 (anti-G_{D3}, purchased from American Tissue Culture Collection; 13) and hybridoma 27A (anti-9-O-acetyl-G_{D3}; Ref. 39) were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS. Hybridoma SGR37 (raised against chemically synthesized de-*N*-acetyl-G_{D3}; Ref. 31) was grown as above in the presence of recombinant interleukin 6 (1 ng/ml). R24, 27A, and SGR37 are all mouse IgG3 monoclonal antibodies. The FLOPC-21 immunoglobulin was used as an additional isotype control for all immunochemical assays. The immunoglobulin concentration in hybridoma supernatants was typically 8–12 μg/ml. Purification of immunoglobulins, when necessary, was done batchwise using protein A-Sepharose (Pharmacia), according to the manufacturer's specifications, and the final concentration was adjusted to 1 mg/ml in PBS. Human melanoma M21 and Melur cell lines (provided by R. Reisfeld, The Scripps Research Institute, La Jolla, CA) were grown in DMEM (regular glucose), supplemented with 10% heat-inactivated FCS. A monospecific rabbit polyclonal antibody against human lamp-1 was a kind gift from Dr. Minoru Fukuda (The Burnham Institute, La Jolla, CA).

Tissues and Immunohistochemistry. Cryosections (5-μm-thick) of human tissues (both normal and tumor samples) were obtained through the

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[2] Present address: Ludwig Institute for Cancer Research and Unidade de Oncologia Experimental, Universidade Federal de Sao Paulo, Sao Paulo, Brazil 04023-062.

[3] To whom requests for reprints should be addressed, at Glycobiology Program, University of California San Diego Cancer Center, Room 1065, 9500 Gilman Drive, La Jolla, CA 92093-0687.

[4] The abbreviations used are: Sia, sialic acid; LacCer, lactosyl-ceramide; MAb, monoclonal antibody.

University of California San Diego Cancer Center Histology Core facility. Different fixation procedures were tested to ensure minimum losses of the antigen. After cryosectioning, sections were thaw-mounted onto Superfrost Plus slides (Surgipath), air-dried at room temperature for 1 h, and then subjected to chemical fixation with various agents including phosphate-buffered paraformaldehyde (2, 4, and 8%), for 30 min at room temperature; cold acetone, for 10 min at −20°C; or 2% glutaraldehyde in PBS for 30 min at room temperature. Because these treatments altered SGR37 reactivity (see "Results"), some sections were fixed only after initial probing with the antibodies and secondary reagents. Organic solvents such as methanol (which also act as fixatives) were also used to ascertain the lipid nature of the antigens for R24 and SGR37 MAbs. Periodate oxidation was done as described elsewhere (27, 31) using either 1 mM sodium periodate in PBS (15 min at 4°C, mild oxidation, specific for the exocyclic chain of Sias; Ref. 40) or with 46 mM sodium periodate in PBS (15 min at room temperature, strong oxidation, reacting with all vicinal hydroxyl groups present in carbohydrates; Ref. 41). Oxidation reactions were usually stopped by addition of 1 or 46 mM $NaBH_4$ solution in PBS for 15 min (similar results were obtained after simply washing away the periodate). Tissues were then washed and processed for immunohistochemistry. All sections were blocked with 10% goat serum in PBS for 30 min, incubated with primary antibody (5 μg/ml of R24, up to 25 μg/ml of SGR37, and 25 μg/ml of FLOPC21, all mouse IgG3). Incubations were either carried for 1 h at room temperature or overnight at 4°C. Incubations with a biotinylated secondary antibody and appropriate streptavidin conjugates and substrate color development followed routine procedures.

Indirect Immunofluorescence Studies on Whole Cells. Melanoma cells were grown on coverslips (Fisher Scientific) or tissue culture slide chambers (LabTek) up to 70–80% confluency. To avoid inactivation of the SGR37 antigen, cells were lightly fixed with 2% phosphate-buffered paraformaldehyde for 30 min at room temperature and promptly washed with PBS containing 50 mM lysine to quench the excess of paraformaldehyde. Permeabilization was achieved by treating cells with saponin (0.03% in PBS for 15 min and then present throughout subsequent incubations). Optimal primary antibody concentrations for R24 and 27A were 5 μg/ml, and for SGR37, 20 μg/ml (the isotype control FLOPC21 was also used at 20 μg/ml). Secondary antibodies were either fluorescein- or rhodamine-conjugated (Jackson ImmunoResearch, West Grove, PA) and used at a final concentration of 10 μg/ml. Cells were observed in an epifluorescence microscope (Zeiss, Germany), equipped with appropriate filters (Omega Optical, Brattleboro, VT).

Indirect Immunofluorescence Studies on Semithin Sections. Melur cells were lightly fixed on tissue culture plates for 1 h at room temperature with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), scraped off, and pelleted. After washing (1×) with PBS, the cells were infiltrated with 2.3 M sucrose in 0.1 M phosphate containing 20% polyvinylpyrroline (M_r 10,000). The pellet was trimmed, mounted on aluminum cryopins, and frozen in liquid N_2. Semithin cryosections (0.5–1.0 μm) were cut with a Reichert Ultracut E ultramicrotome equipped with an FC-4 cryoattachment and transferred to gelatin-coated slides. Sections were double labeled with the primary antibodies for 3 h at room temperature, followed by secondary antibodies $F(ab)_2'$ donkey anti mouse FITC and $F(ab)_2'$ donkey anti-rabbit Texas Red. Micrographs were taken with a Zeiss Axiophot equipped for fluorescence.

Immunogold Labeling. Ultrathin sections were cut from the same pellets as above and transferred to Formvar-coated nickel grids. Antibodies were incubated overnight, followed by a rabbit anti-mouse bridge. The sections were then labeled with 5 nm, gold-conjugated goat anti-rabbit IgG for 1 h. The sections were stained with 2.0% neutral uranyl acetate (30 min), followed by adsorption staining with 0.2 uranyl acetate, 0.2% methyl cellulose, and 3.2% polyvinyl alcohol and observed in a Philips CM-10 electron microscope. In some experiments, the cells were treated with Streptolysin-O before fixing, pelleting, and ultrathin sectioning.

Flow Cytometric Analysis. Melur cells were typically harvested at 70–80% confluency. Nonadherent cells were collected by aspiration, whereas adherent cells were harvested using ATV solution (0.5 g/l trypsin and 0.2 g/l EDTA). Nonadherent cells were treated with ATV in parallel to adherent cells. After trypsin inactivation with serum-containing medium, cells were washed with PBS containing 1% BSA. When indicated, cells were permeabilized with 0.03% saponin in PBS for 15 min at room temperature. Primary antibodies were used at the same concentrations as indicated above in incubations of 1–2 h at 4°C. After washing, cells were incubated with 10 μg/ml FITC-labeled anti-mouse IgG for 1 h at 4°C. After fixation with 1% formaldehyde, cells were analyzed in a FACScan (Beckton & Dickinson, San Jose, CA). Extensive trypsin digestion (1 mg/ml in PBS for 1 h at 37°C) prior to antibody incubation was done to study the possibility of masking of gangliosides by cell surface glycoproteins (42). Simultaneous analysis of cell cycle status and ganglioside expression was done by modifications of methods described previously (43). Briefly, cell permeabilization after antibody binding was done with 0.03% saponin in PBS for 15 min at room temperature. Permeabilized cells were then incubated with propidium iodide (Life Technologies, Inc.; 20 μg/ml) in RNase A (Boehringer Mannheim; 40 μg/ml) containing PBS for 30 min. After washing, cells were analyzed in a FACScan.

Subcellular Fractionation. Melur cells were lysed by nitrogen cavitation, as described elsewhere (31). The cell lysate was centrifuged at 600 × *g* for 10 min at 4°C. One ml of the supernatant (postnuclear supernatant) was applied on top of 17 ml of Percoll (20% in Tris-buffered saline, pH 8.0). A Percoll gradient was formed by ultracentrifugation at 20,000 × *g* for 50 min using a fixed angle rotor. Fractions of 1 ml were collected using a fraction delivery system (Beckman) and analyzed for β-hexosaminidase and 5′-nucleotidase activity, as described elsewhere (44). Lipids from each fraction were extracted and analyzed for the presence of R24 and SGR37 antigens by ELISA, as described previously (30, 31).

Adsorptive Endocytosis Assay. Melur cells were grown on coverslips or tissue culture slide chambers (LabTek) up to 70–80% confluency. The adsorptive endocytosis assay (45–47) consists of incubating cells in the presence of antibodies (20 μg/ml) under culture conditions and determining the subcellular distribution of the antibodies after a chase. The uptake of dextran-FITC (0.1 mg/ml) was used as a marker for lysosomes. The mixture containing the antibodies and organelle probe was pulsed for 30 min and chased for 8 h. Cells were then fixed with 2% phosphate-buffered paraformaldehyde, and the subcellular distribution of the endocytosed antibody was analyzed using anti-mouse IgG antibodies.

RESULTS

Optimization of Conditions for Detection of SGR37 and R24 Antigens in Human Tissue Sections. The original SGR37 reagent (31) is an IgG3 antibody that was raised by immunizing mice with chemically synthesized de-*N*-acetyl-G_{D3}. It was shown to react in a highly specific manner with this ganglioside structure, requiring the presence of a nonacetylated amino group of a neuraminic acid residue, as well as the intact side chain of the outer Sia residue. The antibody also reacts with human melanoma cells in culture and with an appropriately migrating TLC band in lipid extracts from these cells (it is still unclear which of the two Sia residues must be de-*N*-acetylated to allow recognition; Ref. 31). We now wished to use this antibody to explore the expression and distribution of the antigen in normal human tissues, in comparison with that of the G_{D3} antigen (detected by antibody R24, also an isotype-matched mouse IgG3). Conditions for optimal recognition of the antigens in tissue sections were first worked out as described in "Materials and Methods." As expected for glycolipid antigens, tissue section reactivity with both antibodies was markedly diminished by fixation in organic solvents (*e.g.*, methanol) or by the paraffin-embedding process typically used in routine histological processing (48). Even with mild acetone fixation, which has been previously used successfully with R24 (49), there was some loss of the SGR37 antigen. With other conventional glycolipid antigens, the way to circumvent these extraction problems is to avoid organic solvents and to fix frozen sections with aldehydes instead (see "Materials and Methods"). Although this works well with MAb R24 (anti-G_{D3}), SGR37 reactivity (anti-de-*N*-acetyl-G_{D3}) was progressively lost with increasing paraformaldehyde fixation (data not shown, reactivity was obviously diminished at concentrations of paraformaldehyde that were >2%). This is likely because the free amino groups that are critical determinants of the SGR37 antigen are also covalently modified by the paraformaldehyde. Similar problems were noted with glutaraldehyde fixation (data not shown). We therefore turned to the

use of fresh, unfixed, frozen tissue sections (50), which gave good reactivity with both antibodies.

The SGR37 Antigen Is a Rare but Naturally Occurring Structure in Normal Human Tissues. Using unfixed frozen sections, the distribution of both R24 and SGR37 antigens were determined in normal human tissues. Some examples of the results are shown in Fig. 1, and the overall findings are summarized in Table 1. R24 antigen positivity was seen in skin melanocytes, blood vessels, pancreatic islets, adrenal medullary cells, marginal zone lymphocytes in the spleen, interfollicular zone lymphocytes in the tonsil, Leydig cells of the testis, and smooth muscle cells in many tissues. This correlates reasonably well with prior reports, except that our use of unfixed sections may have somewhat limited the extent of R24 reactivity (16, 20, 49–53). Reactivity to SGR37 was present only in melanocytes and blood vessels in some tissues, such as in the ovary and in the pia mater and white matter of the brain. The only cells that were negative for the R24 antigen but positive for the SGR37 antigen were a few infiltrating mononuclear cells in the skin and colon.

From these data, we conclude that MAb SGR37, which was originally raised against a synthetic ganglioside, recognizes a naturally occurring antigen in a few normal human cell types (melanocytes, some blood vessels, and a few mononuclear cells). It is of interest that many R24-positive areas did not also stain for SGR37, indicating that G_{D3} expression is not always accompanied by de-*N*-acetylation. The only cell types positive for SGR37 but not R24 were infiltrating mononuclear cells in certain tissues. This implies either that de-*N*-acetylation of G_{D3} is essentially complete in this particular cell type, or that there are other structurally related gangliosides that do not react with R24 in the *N*-acetylated form but cross-react with SGR37 when present as a de-*N*-acetyl form. Another theoretical possibility is the existence of 9-*O*-acetyl/de-*N*-acetyl-G_{D3}.

How certain can we be that the antigens detected are indeed gangliosides? The antibodies used are known to be highly specific for specific structural details of their ganglioside targets. In the case of SGR37, the specificity for the free amino group and the side chain of the Sia residue have been demonstrated previously (31). The extractability of the antigens by organic solvents or paraffin embedding (see above) indicates that the natural epitope is on a lipid, and the sensitivity of the SGR37 antigen to aldehydes (see above) fits with the requirement of the antibody for a free amino group. Strong periodate oxidation (46 mM, neutral pH) abolished both SGR37 and R24 reactivity, indicating that the epitopes in the tissues for both antibodies are indeed carbohydrate dependent (data not shown). We also tried mild periodate oxidation, which should give controlled oxidation of the Sia side chain and destroy reactivity of both antibodies when performed against purified gangliosides *in vitro* (31). This treatment did not affect binding of either antibody in cells or tissue sections. However, using cultured melanoma cells as models, we noted that, unlike the case with Sia residues on glycoproteins, mild periodate oxidation is not able to access the side chain of Sias attached to gangliosides on intact cell surfaces (data not shown). We speculate that molecules such as monoglycerides on cell membranes compete with the oxidation by low concentrations of periodate. Regardless, the sensitivity to organic solvents and aldehydes as well as the abolition of reactivity by strong periodate reassures us that the molecule being detected in the tissues is either de-*N*-acetyl-G_{D3}, or some closely related structural analogue.

The SGR37 Antigen Accumulates in Most Melanomas and Some Lymphomas but not in Carcinomas. Compared with the small amounts of reactivity seen with SGR37 in a few normal tissues, many clinical tissue samples of human melanomas (which are known to be G_{D3} positive) were found to immunostain strongly for the SGR37 antigen (see examples in Fig. 2 and summary in Table 1). Indeed, in some samples, reactivity with the R24 antibody is more focal, compared to that with SGR37. This implies that naturally occurring tumors *in situ* may have larger levels of de-*N*-acetylation than that of the corresponding cultured cells. Immunoreactivity with SGR37 was also seen in some of the malignant-appearing cells present in lymphoma samples (particularly of the T-cell type). In contrast, R24 immunoreactivity in lymphomas occurred mainly on stromal elements. Unlike the case with melanomas and lymphomas, there was no SGR37 immunoreactivity detected in a number of carcinomas studied (Fig. 2 and Table 1; note that R24 immunostains stromal elements in these tumors).

SGR37 Antigen Is Difficult to Detect on Human Melanoma Cell Surfaces but Accumulates in Intracellular Compartments. We used the IgG3 antibody SGR37 against de-*N*-acetyl-G_{D3} and isotype-matched antibodies against G_{D3} (R24) and 9-*O*-acetyl-G_{D3} (27A) to study the distribution of the corresponding antigens in cultured Melur and M21 human melanoma cells, which are known to contain all three molecules. As demonstrated in Fig. 3, flow cytometric analysis indicates that although G_{D3} (and 9-*O*-acetyl-G_{D3}, data not shown) were expressed at high concentrations on the cell surface, SGR37 reactivity was very low. Glycolipids may not be readily accessible for interact-

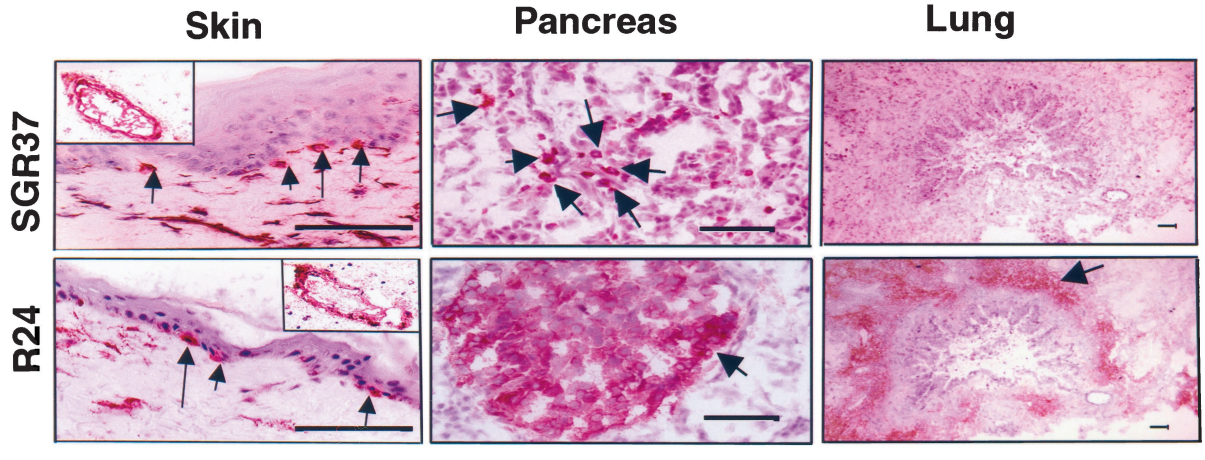

Fig. 1. Expression of SGR37 antigen in normal human tissues in comparison with the R24 antigen shown are representative examples of immunostaining of unfixed human tissue sections with MAbs R24 and SGR37 (red-brown peroxidase reaction product). In normal skin, both MAbs immunostain melanocytes (*arrows*) and some dermal blood vessels (*insets*, examples of blood vessels at lower magnification). In the pancreas, R24 strongly stains islet cells, whereas SGR37 stains rare cells (*arrows*); *bars*, 50 μm. In the lung, R24 immunostains smooth muscle (*arrow*), whereas SGR37 does not.

Table 1 *Distribution of R24 and SGR37 antigens in normal and malignant human tissues*

Unfixed sections of human tissues and tumors were stained with the R24 or SGR37 antibodies as described in "Materials and Methods."

	R24	SGR37
Normal tissues		
Skin	Melanocytes and blood vessels	Melanocytes, few infiltrating mononuclear cells, and blood vessels
Brain	Blood vessels	Blood vessels in pia matter and white matter
Pancreas	All islet cells	Rare islet cells
Adrenal	Medulla	–[a]
Colon	Smooth muscle	Few infiltrating mononuclear cells
Spleen	Marginal zone lymphocytes	–
Tonsil	Interfollicular zone lymphocytes	Blood vessels
Thymus	–	–
Testis	Leydig cells	–
Ovary	Stromal cells	Blood vessels
Heart	Stromal cells	–
Lung	Bronchial smooth muscle	–
Liver	Some blood vessels	–
Kidney	Some blood vessels	–
Placenta	–	–
Tumors		
Melanoma (14)[b]	Focal areas (14)	Diffuse reactivity (12)
Lymphoma (9)	Stromal cells	Scattered malignant cells (5)
Colon adenocarcinoma (2)	Stromal cells	–
Breast adenocarcinoma (2)	Stromal cells	–
Lung adenocarcinoma (2)	Stromal cells	–
Ovarian adenocarcinoma (2)	Stromal cells	–

[a] –, no staining seen.
[b] Numbers in parentheses indicate numbers of tumors studied.

ing with antibodies on the cell surface (54, 55). Epitopes could also be cryptic because of shielding by cell surface glycoproteins (42) or possibly because of interactions with other lipids (54). Alternatively, some gangliosides are thought to be in an intracellular compartment (56–60). To evaluate whether the SGR37 antigen was cryptic, Melur cells were treated with either trypsin or saponin. We used a concentration of trypsin shown previously to cleave cell surface glycoproteins exposing underlying glycolipids to macromolecular probes (42). This trypsin treatment did not increase SGR37 reactivity on the cell surface (data not shown). However, saponin, a reagent typically used to permeabilize intact cells, clearly increased cellular reactivity to SGR37 (Fig. 3). In contrast to this increase, R24 reactivity of human melanoma cells was actually somewhat decreased after the saponin treatment.

Indirect immunofluorescence microscopy of the saponin-permeabilized cells showed that the cultured melanoma cells displayed large amounts of G_{D3} and 9-*O*-acetyl-G_{D3} on the cell surface (appearing as clustered spots) and in some internal vesicle-like elements (Fig. 4, *A* and *B*). In contrast, the SGR37 antigen (Fig. 4*C*) was diffusely cytoplasmic, concentrated in the perinuclear area of some cells, and was seen only at very low levels on the cell surface. Saponin permeabilizes cells by specifically intercalating with cholesterol in cell membranes. Thus, we conclude that SGR37 antigen is either completely intracellular and/or is partly cryptic on the cell surface, concentrated in a cholesterol-rich domain that can be disrupted by saponin. Permeabilization of cells with streptolysin O (which makes holes only in the plasma membrane, without affecting cholesterol content) revealed only a small amount of the SGR37 antigen (data not shown). This suggests that the intracellular pool cannot be accessed easily without a membrane-perturbing reagent like saponin. Regardless, it is evident that the intracellular distribution of the SGR37 antigen is different from those of the other two closely related structures. As an independent check, we also used the IgM mouse monoclonal antibodies SMR36 (against de-*N*-acetyl-G_{D3}), GMR14 (against G_{D3}), and Jones (against 9-*O*-acetyl-G_{D3}). Again, we observed the same differential distribution of these gangliosides (data not shown).

Given that both saponin and streptolysin O perturb the plasma membrane in different ways, it was important to obtain independent

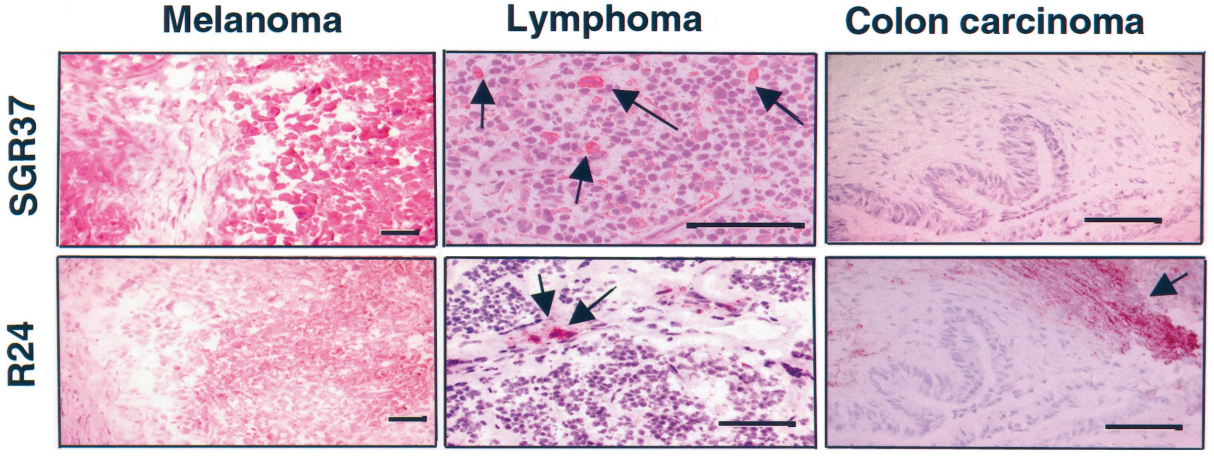

Fig. 2. Expression of SGR37 antigen in human tumors in comparison to the R24 antigen shown are representative examples of immunostaining of unfixed human tumor sections with MAbs R24 and SGR37. Most melanomas were positive for both antibodies (red-brown peroxidase reaction product). Note that R24 reactivity tends to be focal, whereas a larger number of cells accumulate the SGR37 antigen. In lymphomas, scattered malignant-appearing cells are positive for SGR37, but while stromal elements are positive for R24 (*arrows*). An example of a negative colon carcinoma is shown, with some R24 reactivity of the stroma (*arrow*). Bars, 50 μm.

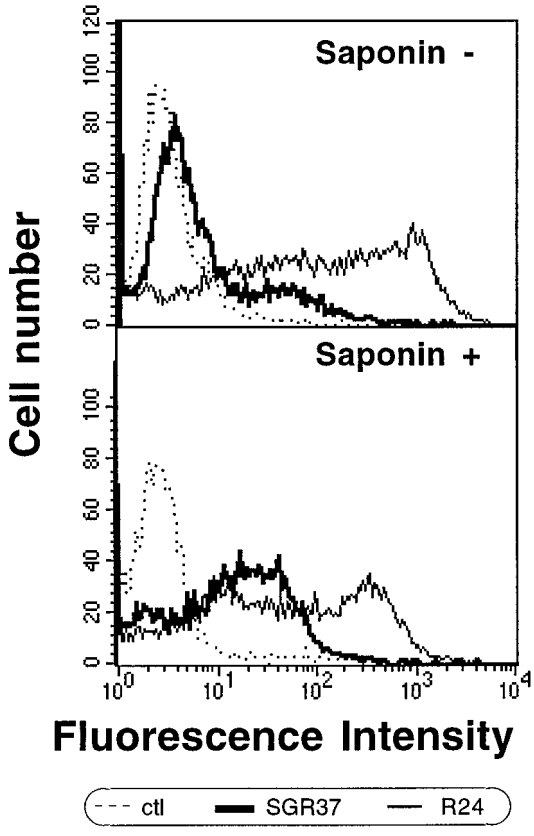

Fig. 3. Flow cytometry of human melanoma cells and effects of saponin treatment on detection of G_{D3} and de-*N*-acetyl-$G_{D3.}$ Human melanoma cells were incubated with MAbs R24 (*continuous line*), SGR37 (*bold line*), or an isotype control, FLOPC 21 (*dashed line*), either in the absence (*upper panel*) or presence (*lower panel*) of saponin. Whereas R24 antigen is readily available on the cell surface, the SGR37 antigen becomes much more accessible upon saponin permeabilization.

verification of the subcellular distribution of these gangliosides. Accordingly, we carried out immunofluorescence studies on semithin sections of the cultured cells harvested without permeabilization. This approach (which sections the cells directly without disrupting membrane structures) strongly corroborated the above results, *i.e.*, G_{D3} and 9-*O*-acetyl-G_{D3} are predominantly on the cell surface, whereas de-*N*-acetyl-G_{D3} is detected in a diffuse cytoplasmic internal distribution (see *left panels* of Fig. 5 for examples).

Unlike G_{D3}, the de-*N*-acetyl-G_{D3} Antigen Is Not Enriched in Plasma Membranes or Lysosomes. To further address the differential compartmentalization of these related antigens, subcellular fractionation and double indirect immunofluorescence experiments were also performed. Subcellular fractionation of cells disrupted by nitrogen cavitation showed that R24 and SGR37 antigens were enriched in two distinct fractions. The denser fraction was enriched in β-hexosaminidase activity, whereas the lighter one was enriched in 5′-nucleotidase activity. These fractions are compatible with lysosomes and plasma membrane, respectively (data not shown). However, both fractions could be potentially contaminated with other subcellular organelles on such a gradient. Double immunofluorescence studies were therefore performed on the semithin sections of cultured cells (Fig. 5). R24 and 27A stained the cell surface and the intracytoplasmic vesicles, which were identified as lysosomes, by double labeling with a polyclonal antibody against the lysosomal-associated membrane protein lamp-1. In contrast, the diffuse cytoplasmic internal distribution of the SGR37 antigen did not overlap with that for the lamp-1 lysosomal marker (see Fig. 5) nor with that for calnexin (an ER marker, data not shown). The signal also does not have a perinuclear Golgi-like pattern. Taken together, these results indicate that at the steady state of cultured adherent cells, the SGR37 antigen is mainly intracellular and absent from lamp-1 positive lysosomes. On the other hand, R24 and 27A antigens are mainly found on the cell surface or in lamp-1-positive lysosomes.

Immunoelectron microscopy with gold bead-tagged antibodies was carried out on the same preparations. The R24 antibody labeled large and small vesicular structures, as well as plasma membrane, when used with a rabbit anti-mouse bridge (data not shown). The SGR37 gave a weaker signal; the few gold beads that were seen appeared to be simply scattered in the cytoplasm with no labeling of specific membranous structures. Indeed, large internal vesicular structures (lysosomes) that labeled with R24 were completely devoid of label with SGR37. Upon treatment with streptolysin O prior to semithin sectioning and fixing with 2% paraformaldehyde, membranes continued to be heavily labeled with R24, but the small amount of SGR37 labeling was lost. The Melur cells were also prepared using freeze substitution and Lowicryl embedding described previously for glycolipids (59, 61). However, neither antibody worked in this preparation. Overall, the EM studies confirmed, but did not further extend, the conclusions from the immunofluorescence evaluation. Taken together, all of the data indicate that the R24 and 27A antigens behave as expected for typical gangliosides, being predominantly located on

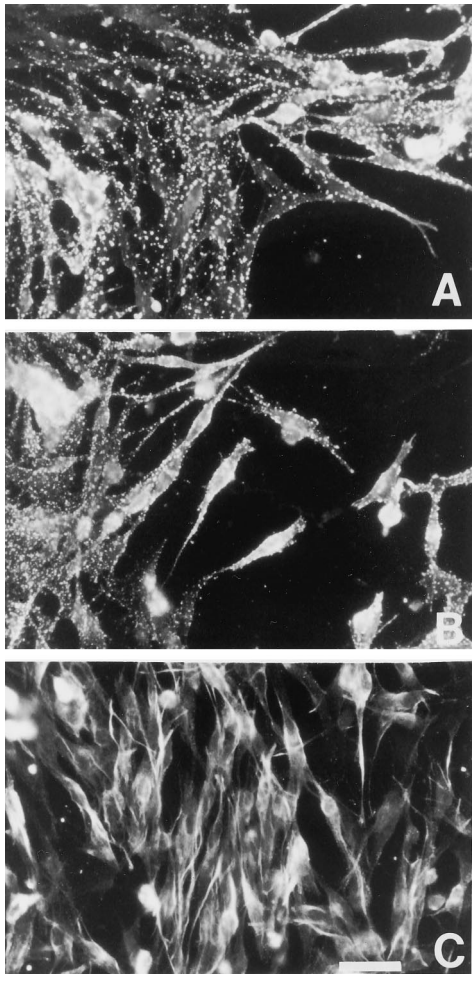

Fig. 4. Indirect immunofluorescence microscopy of saponin-permeabilized melanoma cells. Melur cells were cultured on coverslips, fixed lightly, permeabilized with saponin, and stained with MAbs R24 for G_{D3} (*A*), 27A, for 9-*O*-acetyl-G_{D3} (*B*), and SGR37, for de-*N*-acetyl-G_{D3} (*C*). Both R24 and 27A stained plasma membrane and large intracellular vesicles. In contrast, MAb SGR37 diffusely stained intracellular structures, with cell surface staining observed only in a few cells.

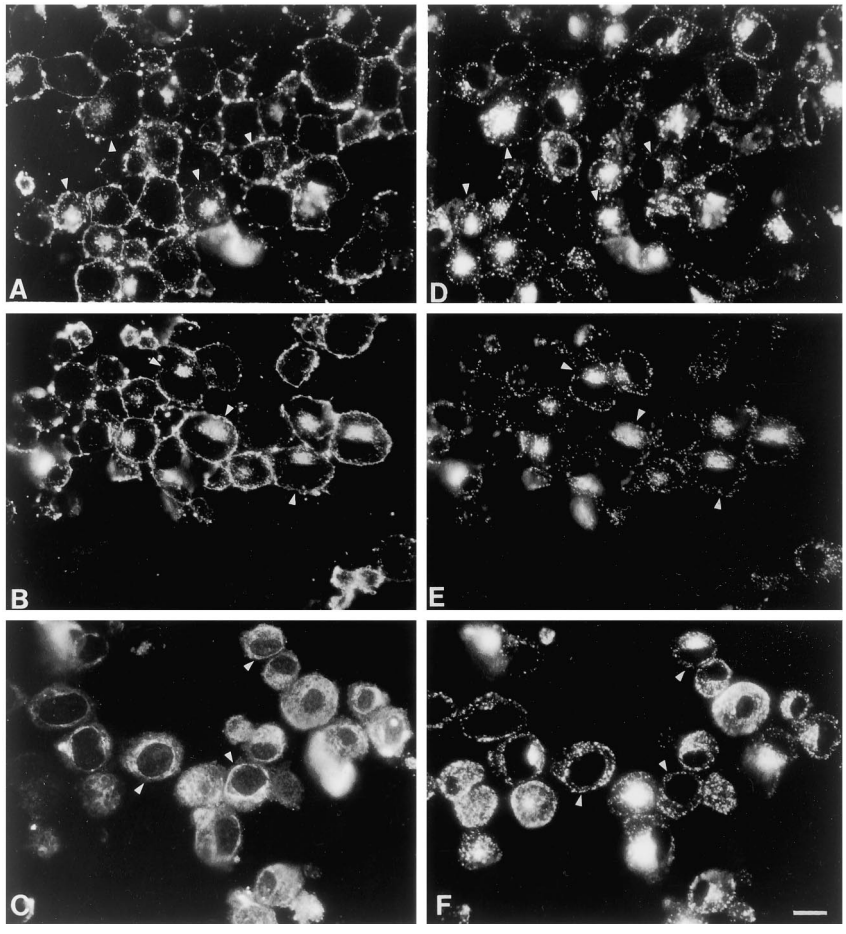

Fig. 5. Subcellular distribution of ganglioside antigens detected on semithin sections. Each pair of panels depicts the same field of semithin sections of Melur cells double-labeled with anti-ganglioside antibodies (*left panels: A*, 27A; *B*, R24; *C*, SGR37, detected with FITC-conjugated donkey antimouse IgG) and with polyclonal rabbit anti-lamp-1, a marker for lysosomes (*right panels*, detected by Texas Red-conjugated donkey anti-rabbit antibody). R24 and 27A stain cell surface and intracellular vesicles, and the latter colocalize with lamp-1-containing structures. SGR37 diffusely stains the cytoplasm, and there is no clear segregation with lamp-1-positive compartments. *Arrowheads*, identical cells in each pair of exposures. *Bar*, 10 μm.

the plasma membrane and in internal endosomal and lysosomal organelles. In contrast, the SGR37 antigen appears to be predominantly in the cytoplasm, without clear association with any membrane-bound organelle.

SGR37 Antigen Is Transiently Expressed on the Cell Surface and Then Delivered to an Internal Compartment. Biochemical considerations indicate that de-*N*-acetyl-G_{D3} is very likely to be derived from G_{D3} by de-*N*-acetylation after its initial synthesis in the Golgi (30, 31). Thus, the question arises whether the de-*N*-acetyl variant is targeted directly to its internal compartment from the Golgi or gets to that location after passing through the plasma membrane. The small amounts of cell surface SGR37 reactivity seen in flow cytometry suggests the latter possibility. To study this, we exploited the concept of adsorptive endocytosis using antibodies. When antibodies or lectins to potential cell surface antigens are added to the medium of live cultured cells, adsorptive endocytosis is antigen dependent, and the final intracellular distribution of the probe follows the antigen distribution (45–47, 62). Cells were incubated with the different monoclonal antibodies under standard culture conditions. Uptake of antibodies was then monitored by indirect immunofluorescence (Fig. 6). Upon incubation with MAb R24, Melur cells tended to extend neurite-like processes. Although it was possible to observe intracellular staining (*i.e.*, some antibody internalization), a considerable amount of MAb R24 was retained on the cell surface. On the other hand, SGR37 did not have the neurite promoting effect and was internalized and concentrated in cytoplasmic vesicular structures. Controls using the isotype-matched mouse IgG (FLOPC21) showed that it was also not significantly internalized in the course of these experiments (data not shown).

Previous studies have indicated that internalized gangliosides tend to be delivered to lysosomes (1, 63). To test whether the SGR37-positive vesicles detected by adsorptive endocytosis were functional lysosomes, cells were simultaneously pulsed with MAb SGR37 and with dextran-FITC, which is known to be concentrated in lysosomes. As shown in Fig. 7, SGR37 and dextran-FITC were concentrated into different compartments. Even in experiments using shorter times (30-min pulse, followed by 30-min chase), SGR37 accumulated in intracytoplasmic vesicles, while R24 was present mainly on the cell surface. These results indicate that SGR37 antigen is at least transiently expressed on the cell surface but then becomes predominantly an intracellular antigen, concentrated initially in a location other than in functional lysosomes.

SGR37 Antigen Accumulates on the Cell Surface of Rounded Nonadherent Cells, but This Is Not Related to Cell Cycle Status. The next question we addressed is whether the internal pool of de-*N*-acetyl-G_{D3} can be mobilized to the cell surface under any circumstances. In immunocytochemical studies of cultured Melur melanoma cells, we observed that SGR37 reactivity was strikingly higher in the small number of rounded-up cells, as compared with the bulk population of flattened, spread-out cells (Fig. 8*A*). To study this further, the rounded-up cells were shaken loose, harvested and compared with the adherent cells by flow cytometry (Fig. 8*B*). This confirmed that the rounded-up cells had an increased cell surface reactivity to SGR37 but not to R24. This fits with results we reported previously, where the microtubule-disrupting agent nocodazole increased cell surface density of SGR37 antigen (31). In cultured cells, mitosis is usually associated with temporary detachment from the substratum. The proportions of such rounded-up cells in different cell

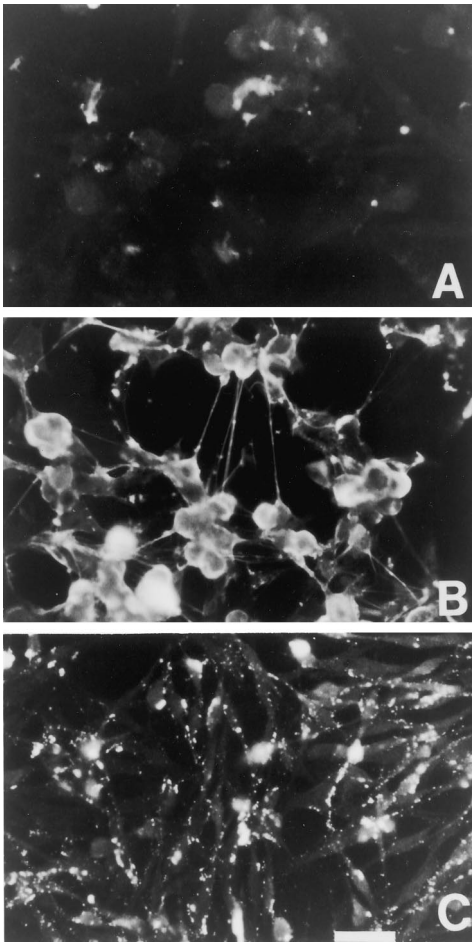

Fig. 6. SGR37 antigen is transiently expressed on the cell surface and then endocytosed. Melur cells were grown on coverslips, incubated with mouse MAbs FLOPC 21 (*A*), R24 (*B*), and SGR37 (*C*) for 8 h, under standard culture conditions, washed, and incubated with plain medium for 30 min before fixation with 2% paraformaldehyde and processing for immunofluorescence studies. Most of MAb R24 is retained on the cell surface, whereas antibody SGR37 was internalized and concentrated in vesicular compartments.

cycle phases was therefore determined using a method for simultaneous analysis of DNA content and ganglioside expression, which we developed (see "Materials and Methods"). In fact, most of these cells are actually in interphase (Fig. 9). Furthermore, no significant variation in either cell surface or global expression of SGR37 antigen was observed in different phases of the cell cycle (G_0-G_1 *versus* S-G_2-M). Thus, the SGR37 antigen accumulates on the cell surface of weakly adherent/rounded-up cells, regardless of their cell cycle phase. Taken together, these results suggest that accumulation of SGR37 antigen on the cell surface is associated with cell shape/adhesion state and not with distribution of cells in different phases of the cell cycle.

DISCUSSION

The disialoganglioside G_{D3} is a well known tumor marker for melanomas and neuroblastomas, as well as an activation marker for lymphocytes. Several other gangliosides have previously been suggested as tumor-specific markers for human melanoma cells, including G_{D2}, G_{M2}, and 9-*O*-acetyl-G_{D3}. However, on closer inspection, the specificity of these molecules for malignant cells has turned out to be relatively poor, *e.g.*, 9-*O*-acetyl-G_{D3}, is also present in leukocytes, as the CD60 antigen. This is not surprising, given the widespread distribution and regulated expression of the enzymes involved in producing such structures. Here we have pursued evidence for a rare and unusual variation on the conventional gangliosides of melanoma and lymphoma cells, the removal of the *N*-acetyl group on Sias, which generates a free amino group, giving so-called de-*N*-acetyl-gangliosides. The existence of these molecules in cultured cell lines has been suggested previously by us and by others (29–31, 38).

We have also identified and solved a technical problem that may have prevented previous attempts to detect these antigens in tissue sections. It is well known that organic solvent fixation (including some steps in the paraffin-embedding process) result in extraction of glycolipid antigens. With this particular antigen, even the use of mild acetone fixation, which actually enhances detection of some other gangliosides (49), is not suitable. The conventional alternative of aldehyde fixation is also detrimental, because exposure to this reagent destroys the antigen when used at the concentrations typically required for fixing sectioned tissues. We circumvented these problems by using completely unfixed sections (50), which were directly attached to the slides by drying. Fixation was done only after the primary antibody and secondary reagent binding was completed, thus preserving the ability to detect the antigen in tissue sections. We recognize that this approach may not optimally expose the G_{D3} antigen (49) but settled for this, because both antigens are being studied under similar conditions. It should also be noted that very light aldehyde fixation can be used for cultured cells without major loss of the antigen, as demonstrated by the positive reaction of the rounded up cells seen in Fig. 8*A*.

Accumulating evidence indicates that glycosphingolipids are not

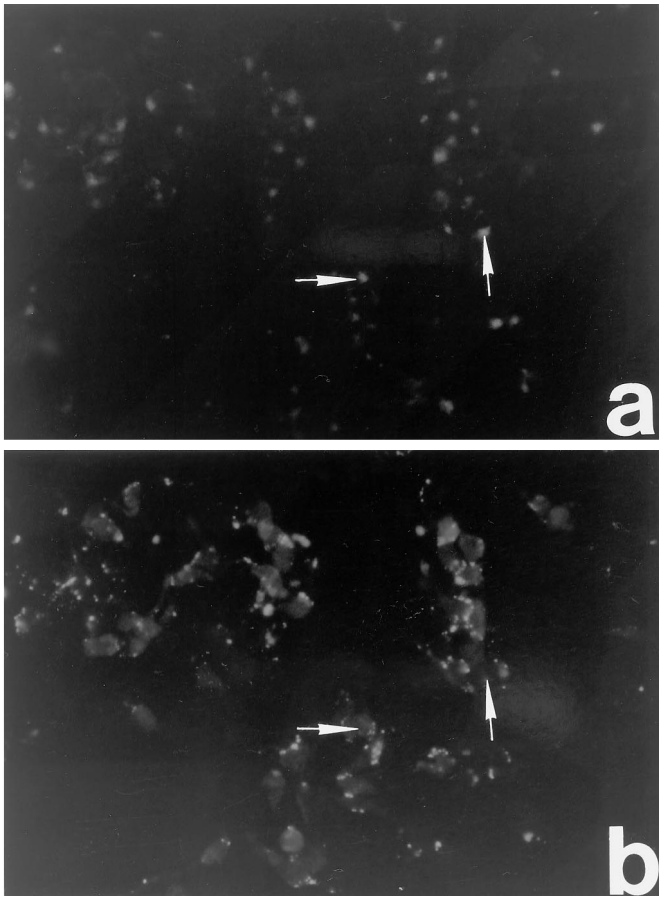

Fig. 7. Endocytosed SGR37 antigen is not delivered to functional lysosomes. The internal location of endocytosed MAb SGR37 (*b*) was followed in comparison with that of coincubated dextran-FITC (0.1 mg/ml), a fluid-phase marker for uptake into functional lysosomes (*a*). SGR37-containing vesicles are clearly segregated from dextran-FITC-positive vesicles. *Arrows*, identical cells.

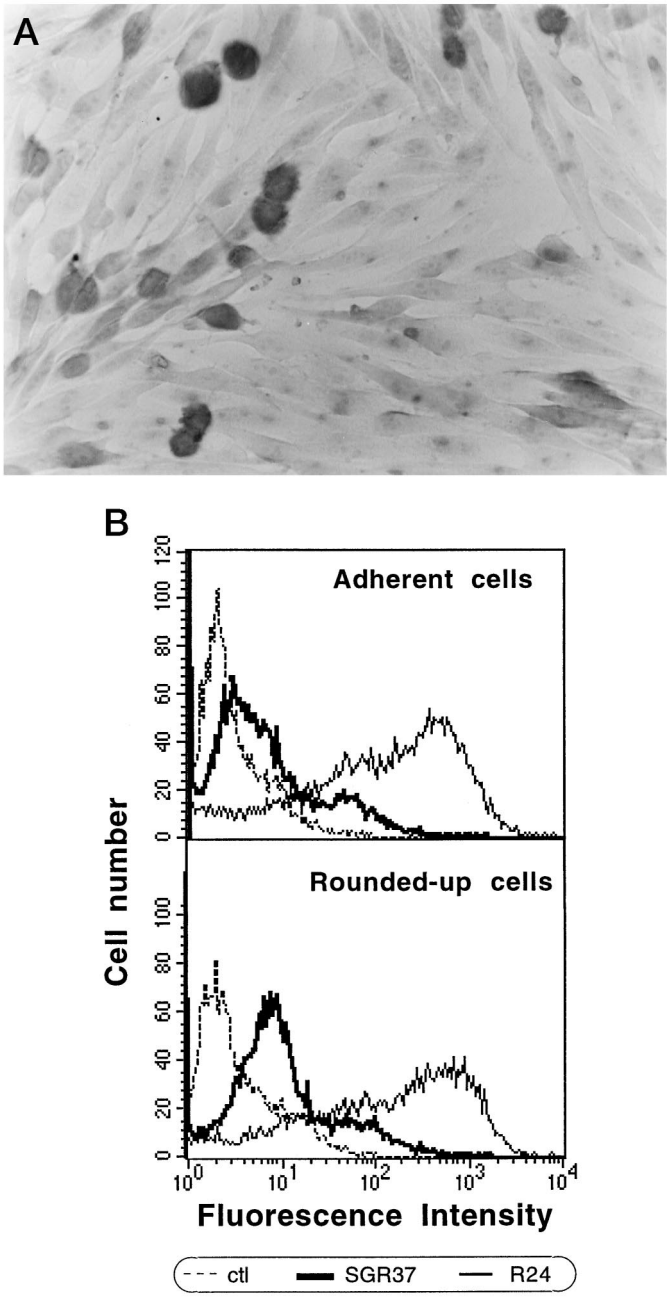

Fig. 8. Nonadherent cells accumulate the SGR37 antigen on the cell surface (*A*). Immunocytochemistry of Melur cells showing that rounded up cells stained intensely with SGR37, whereas adherent cells were positive only in discrete areas in the perinuclear zones of the cytoplasm. In *B*, adherent and nonadherent Melur cells were separated mechanically and analyzed by flow cytometry for both antigangliosides and isotypic control. This confirmed that nonadherent cells accumulated higher amounts of SGR37 antigen on the cell surface as compared with adherent cells. No differences were observed for ganglioside G_{D3} expression on the cell surface, as evaluated with MAb R24.

randomly distributed on the cell surface but rather are present in plasma membrane microdomains (64–66). These microdomains or lipid clusters are thought to be formed through interactions of lipid hydrophobic tails among each other and with cholesterol and possibly through weak interactions between the polar groups of (glyco)sphingolipids (66). Such microdomains are also enriched in glycosylphosphatidyl inositol-linked glycoproteins (66) and may provide a phase for partitioning and/or anchoring of cytosolic proteins with hydrophobic acyl chains. In polarized epithelial cells, sphingolipid (glycosphingolipids and sphingomyelin) microdomains are enriched in the apical membrane, whereas phosphoglycerolipids tend to be segregated into the basolateral membranes (67). In some cell types, these gangliosides associate with glycosylphosphatidyl inositol-linked glycoproteins and are found not only on plasma membranes (plasmalemma propria) but also in plasmalemmal invaginations (caveolae) and related vesicles (66, 68–70). Although gangliosides and glycosphingolipids at the steady state are preferentially enriched on the cell surface, they can also be present in both intra- and extracellular compartments. Sakakibara *et al.* (71) presented evidence for distribution of galactocerebrosides in association with microtubule-associated intracellular compartments. Gillard *et al.* (72) showed that although glycosphingolipid biosynthesis can involve many complex intracellular pathways, some are associated with the intermediate filament network, and that their biosynthesis is reduced in vimentin-deficient cells (56–58, 73, 74). Likewise, the bulk of lactosylceramide in neutrophils is reported to be in internal membrane compartments (75). There is also evidence for recycling of gangliosides from the cell surface to Golgi, where they may be remodeled (76), or to lysosomes, where degradation takes place (1, 63). On the other hand, gangliosides are also shed (77) and may be found deposited on the extracellular matrix (78) or associated

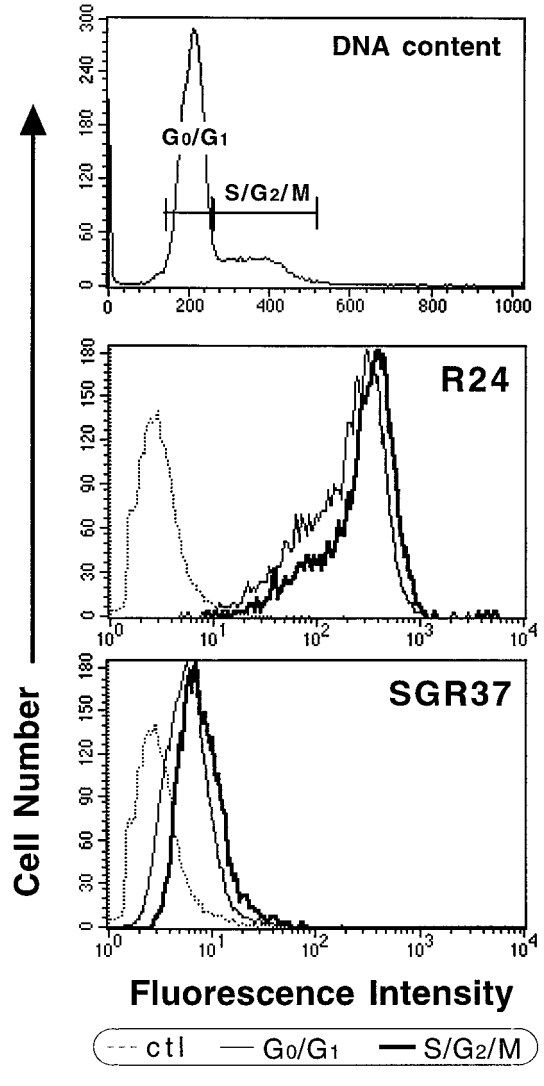

Fig. 9. Accumulation of the SGR37 antigen on the cell surface is not cell cycle dependent. Simultaneous analysis of cell cycle and ganglioside accumulation on the cell surface. Ganglioside staining was analyzed in cells in G_0-G_1 and S-G_2-M phases. No significant differences were observed in the different phases, indicating that accumulation of SGR37 is associated with cell shape and adhesion state but not with cell cycle stage.

with some secreted proteins (79, 80). Relatively little is known about the dynamics and intracellular trafficking of glycosphingolipids within the different subcellular compartments (72).

Here we show that the subcellular distribution of G_{D3} and its derivatives (9-*O*-acetyl-G_{D3} and de-*N*-acetyl-G_{D3}) at the steady state varies according to the de-*N*-acetylation state of the Sia residue. G_{D3} and *O*-acetylated G_{D3} are present in essentially the same subcellular compartments, the plasma membrane and lysosomes. In striking contrast, the de-*N*-acetyl-G_{D3} is distributed diffusely throughout the cytosol. It will be very interesting to explore what drives the markedly differential subcellular localization of de-*N*-acetyl-$G_{D3,}$ which differs from G_{D3} solely by the presence of a free amino group (absence of the *N*-acetyl group). In this regard, our data do indicate that de-*N*-acetylated G_{D3} is not simply a terminal degradation product of G_{D3}. The absence of SGR37 antigen from lysosomes and its accumulation in other elements during adsorptive endocytosis suggest that these molecules are recycled. This also fits well with our early studies in this system, where we had noted the presence of a re-*N*-acetylase activity, which could restore G_{D3} from endogenous de-*N*-acetyl-G_{D3} (30).

The functional consequences of de-*N*-acetyl-G_{D3} expression also need to be explored. We have noted that during a shape change from flattened to rounded state, the de-*N*-acetyl-G_{D3} became enriched on the cell surface. In this regard, others have also shown that exogenously added de-*N*-acetylated gangliosides can block adhesion of cultured cells on extracellular matrix proteins (81). Also, replacement of the Sia *N*-acetyl group by *N*-propionyl or other acyl chains, achieved by feeding cells with mannosamine analogues, led to loss of cell growth control and loss of contact inhibition (82). Interestingly, in the course of our experiments, a subclone of nonadherent Melur cells arose spontaneously. This subclone presented higher cell surface reactivity to SGR37 as compared with the parental cell line. After a short number of passages, the clone became growth arrested and started producing a melanin-like pigment (data not shown). Because this subclone could not be maintained in culture, this issue was not pursued further. It is also of interest that G_{D3} accumulation was recently associated with apoptosis of Fas-induced T-cell lymphomas (83). The mechanism for this novel G_{D3} function is still unclear, and it is not known if this apoptosis pathway is present in all cell types. The opposing effects of *in vitro* addition of G_{M3} (growth suppression) and de-*N*-acetyl-G_{M3} (growth enhancement) to cells in culture reported by others (29, 38) may provide a conceptual framework for continuing these studies. For example, will de-*N*-acetyl-G_{D3} antagonize G_{D3} function in promoting apoptosis?

The recent discovery by Mitsuoka *et al.* (84) that neuraminic acids can be converted into intramolecular lactams adds further complexity to the study of de-*N*-acetyl-gangliosides. Many other questions regarding the mechanisms of biosynthesis, trafficking, and function of these novel gangliosides remain to be elucidated. Meanwhile, the very low levels of the SGR37 antigen in normal tissues, the high levels in melanomas and some lymphomas, its selective endocytosis from the cell surface, and its high expression on rounded-up/nonadherent cells suggest its exploration in diagnostic or therapeutic approaches to these diseases. Indeed, animal and clinical studies provide encouragement for the concept of using unusual tumor gangliosides as targets for immunotherapy (20–24).

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