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Expression of De-*N*-acetyl-gangliosides in Human Melanoma Cells Is Induced by Genistein or Nocodazole*

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Neuraminic acid is the core structure of most known sialic acids. In natural systems, the amino group at the 5 position of neuraminic acid residues is usually assumed to be acylated. Previously, synthetic de-*N*-acetyl-gangliosides (with free amino groups at the 5 position of neuraminic acids) have been shown to modulate cellular proliferation and tyrosine phosphokinase reactions. While indirect evidence has suggested that traces of these molecules exist naturally in certain tumor cells, further exploration has been hampered by the lack of a system showing consistent expression at an easily detectable level. Using synthetic compounds as antigens, we have developed highly specific monoclonal antibodies against de-*N*-acetyl-G_{M3} and de-*N*-acetyl-G_{D3} that require both the free amino group and the exocyclic side chain of sialic acids for recognition. Cultured human melanoma cells showed low but variably detectable levels of reactivity with these antibodies. The ability of various biologically active molecules to stimulate this reactivity was explored. Of many compounds tested, only the tyrosine kinase inhibitor genistein induced reactivity in a dose-dependent manner. Antibody reactivity with ganglioside extracts from genistein-treated cells was abolished by chemical re-*N*-acetylation and/or truncation of sialic acid side chains by mild periodate oxidation. High performance thin layer chromatography immuno-overlay analysis confirmed the presence of the novel compound de-*N*-acetyl-G_{D3} in these extracts. Several other tyrosine kinase inhibitors tested did not give the same increase in de-*N*-acetyl-ganglioside expression. However, the microtubule inhibitor nocodazole caused a similar accumulation of these molecules, particularly in non-adherent cells expected to be arrested at metaphase. Thus, genistein may induce de-*N*-acetyl-ganglioside expression by virtue of its known ability to arrest cells in the G_2M phase, rather than as a general consequence of tyrosine kinase inhibition. These studies also provide a system in which to analyze the enzymatic basis of de-*N*-acetyl-ganglioside expression and their potential roles as growth regulating molecules.

Gangliosides are structurally diverse amphipathic molecules enriched in the outer leaflet of animal plasma membranes (1–3). They can mediate or influence a variety of biological processes including cell-cell interaction (4–8), immune modulation (9), cell growth, and differentiation (3, 10, 11), formation of neurites (12), and developmental organization (8, 13, 14). Since they coexist with other plasma membrane constituents, gangliosides may modulate the functions of proteins associated with or spanning the membrane bilayer. For example, the ganglioside G_{M3}[1] can inhibit both epidermal growth factor receptor (EGFR) autophosphorylation on tyrosine residues (15, 16) and cell growth. In contrast, insulin receptor-associated cellular proliferation and tyrosine kinase activity are specifically inhibited by α2–3-sialylparagloboside and not by G_{M3} (17). Gangliosides can also modulate cellular interactions mediated by extracellular matrix adhesion receptors (4, 18, 19) and the cell surface expression of certain proteins (20).

The defining feature of gangliosides is the presence of at least 1 residue of a nine carbon, anionic monosaccharide called sialic acid. "Sialic acid" is a generic term for a family of molecules represented by over 25 members, the commonest being *N*-acetyl-neuraminic acid (Neu5Ac). Diversity arising from modification of Neu5Ac can add considerable structural variability to gangliosides (21, 22). We and others have provided indirect but highly suggestive evidence for a naturally occurring ganglioside modification in which the C-5 amino group of sialic acid is unsubstituted, creating "de-*N*-acetyl-gangliosides" (deNAc-gangliosides) (16, 23, 24). Also, in contrast to the suppressive effects of G_{M3}, addition of synthetic deNAc-G_{M3} to various cell lines stimulated proliferation (16) as well as *in vitro* tyrosine and/or serine phosphorylation of the EGFR (24). Thus far, deNAc-gangliosides have been detected in extremely low quantities and are suggested to be preferentially expressed in certain tumors and tumor cell lines (16, 23, 24). In human

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[1] The abbreviations used are: G_{M3}, Neuα2–3Galβ1–4Glcβ1–1′ceramide; Neu5Ac, *N*-acetyl-neuraminic acid; Neu, neuraminic acid; mAb, monoclonal antibody; HPTLC, high performance thin layer chromatography; PBS, phosphate-buffered saline; EGFR, epidermal growth factor receptor; OAc, 7 or 9-*O*-acetyl group; NAc, *N*-acetyl group; G_{D3}, Neu5Acα2–8Neu5Acα2–3Galβ1–4Glcβ1–1′ceramide; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment-mass spectroscopy; BSA, bovine serum albumin; EGF, epidermal growth factor; TLC, thin layer chromatography; HPLC, high performance liquid chromotography; PMA, phorbol myristic acid. In the de-*N*-acetyl (deNAc) forms one or more of the acetyl (Ac) groups of Neu5Ac are missing, giving Neu residues with free amino groups.

melanoma cells in culture, we have found that expression of deNAc-gangliosides is transient and variable,[2] making further analysis difficult. We therefore developed new monoclonal antibodies specifically recognizing deNAc-G_{M3} and deNAc-G_{D3} and used them to assess the ability of various agents to stimulate expression of these molecules in cultured melanoma cells.

EXPERIMENTAL PROCEDURES

Materials—High performance thin layer chromatography (HPTLC) plates (Silica Gel-60 10 × 10 cm) were from Merck. Fluorescene isothiocynate-conjugated goat anti-mouse IgM and IgG were from Sigma and Pierce, respectively. Mouse immunoglobulins and G_{M3} were from Sigma and propidium iodide from Calbiochem. Bovine buttermilk G_{D3} was a kind gift of Dr. Robert Yu, Medical College of Virginia. All other chemical were of reagent grade or better and were from commercial sources.

Cell Lines—The following tumor cell lines were from the sources indicated: G361, VMRC-MELG, HMVTG-Cap, MeWo, CRL1579, B16, and RPMI 1846, Japanese Research Bank, Tokyo; SK-MEL-28 and SK-MEL-37, Dr. R. Ueda, Aichi Cancer Center, Nagoya, Japan; M14, Dr. R. F. Irie, John Wayne Cancer Institute, Santa Monica, CA; Colo 38 and SK-MEL-19, Dr. T. Kageshita, Kumamoto University, Japan, and M12, Dr. H. Mekada, Kurume University, Japan. These cells were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum (Hyclone,). The human melanoma cell lines Melur and M21 (23), were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone, Logan, UT).

Synthesis of deNAc-G_{M3} and deNAc-G_{D3}—De-*N*-acetylation of G_{M3} was achieved by modifications of a previously described method (25). G_{M3} (0.5 mg) was dissolved in 12.95 ml of 1-butanol and heated to 100 °C. Tetramethyl ammonium hydroxide (1.44 ml of 10 M, 1 M final) was added and the reaction continued for 4 h at 100 °C with stirring. Additional water (~5 ml) was added and most of the butanol removed by rotary evaporation with repeated additions of water, making sure the volume never dropped below 15 ml. The remaining mixture was dialyzed for 24 h against water at 4 °C, lyophilized, and analyzed by HPTLC. Under these conditions, ~5–10% of the G_{M3} remained intact, ~50% was converted to deNAc-G_{M3}, and the remaining represented lyso-deNAc-G_{M3}. Synthesis of deNAc-G_{D3} was achieved identically but resulted in a more complex mixture. Reaction times >4 h caused significant hydrolysis of the outer sialic acid residue, causing formation of deNAc-G_{M3} and derivatives thereof.

Production of Monoclonal Antibodies (mAbs) SMR36 and SGR37—DeNAc-G_{M3} reaction mixtures were fractionated on an Iatrobeads HPLC column with a 90-min linear gradient from chloroform/methanol/water (63:31:3, v/v/v) to chloroform/methanol/water (52:39:9, v/v/v) and elution monitored by HPTLC, using resorcinol for detection. Fractions corresponding to the previously reported (26) HPTLC migration of deNAc-G_{M3} (relative to G_{M3}) were used to develop mAbs. The unfractionated deNAc-G_{D3} reaction mixture was used to develop mAbs. Seven-week-old female C3H/HeN mice (Japan Clea, Tokyo, Japan) were intravenously immunized on days 0, 4, 7, 11, and 21 with chemically synthesized deNAc-gangliosides (10 μg total/mouse) coated onto *Salmonella minnesota* strain R595 (T. Tomita, University of Tokyo), as described previously (27, 28). Spleen cells were obtained 3 days after the last injection, fused with a myeloma cell (PAI), and hybridomas screened against the immunizing ganglioside(s). Antibody titers in hybridoma supernatants were determined by ELISA (27, 28). Positive hybrids were cloned by limiting dilution, and the antibody isotype determined with a kit (Amersham, United Kingdom).

Fractionation of mAb-reactive Gangliosides—DeNAc-G_{M3} reaction mixtures were suspended in chloroform/methanol/water (1:8:1, v/v/v), injected onto a TSK DEAE 2SW-HPLC column (TosoHaas) (0.5 × 30 cm) equilibrated in the same solvent, eluted isocratically at 1 ml/min for 10 min, followed by a 72-min linear gradient to chloroform/methanol, 1.2 M ammonium acetate (1:8:1, v/v/v). Fractions were monitored by lipid ELISA as described (23), using SMR36 hybridoma supernatant. The monosialogangliosides (identified by a preceding run with authentic melanoma [^{14}C]G_{M3}) were pooled and fractionated on an Iatrobeads HPLC column using a 90-min linear gradient from chloroform/methanol/water (63:31:3, v/v/v) to chloroform/methamol/water (52:39:9, v/v/v), monitoring by lipid ELISA as above. DeNAc-G_{D3} reaction mixtures were fractionated identically, except that elution was monitored with SGR37 hybridoma supernatant. Antibody-reactive fractions were pooled, dried, and characterized by FAB-MS.

[2] E. Sjoberg, R. Chammas, and A. Varki, unpublished data.

Fast Atom Bombardment Mass Spectrometry—FAB mass spectra were obtained using a VG Analytical ZAB-2SE FPD mass spectrometer fitted with a cesium ion gun operated at 20–25 kV. Data acquisition and processing were performed with VG Analytical Opus® software. Monothioglycerol was used as matrix for native and deuteropermethylated samples, in the negative and positive ion mode, respectively. Deuteropermethylation was done as described previously (29).

Extraction of Gangliosides from Cultured Melanoma Cells—Washed cell pellets were extracted as described previously (23, 30, 31). In some experiments, an alternative protocol (32) for extraction was used that seemed to give a better yield and purity. In brief, cells were resuspended in 3 volumes of ice-cold deionized water, homogenized at 4 °C, and the homogenate added dropwise to 10.6 volumes of methanol at room temperature under constant stirring. Chloroform (5.3 volumes) was then added to the suspension. After centrifugation, the supernatant was collected and adjusted to a final chloroform/methanol/water ratio of 4:8:5.6 (v/v/v). After phase separation, gangliosides were enriched in the hydrophilic upper phase, which was dried down, resuspended in methanol, and kept at −20 °C until use.

ELISA Plate Assays for De-N-acetyl-gangliosides—Synthetic deNAc-gangliosides or total ganglioside extracts prepared as above were studied by lipid ELISA as described previously (23, 30, 31), using a 1:1 mixture of SMR36 and SGR37. The effects of mild periodate oxidation or chemical re-*N*-acetylation on reactivity were assessed. Periodate oxidation was done with 2 mM sodium *meta*-periodate in PBS, pH 7.2, at 4 °C for 15 min (33). Re-*N*-acetylation was done at room temperature, using freshly prepared 3.3% acetic anhydride in saturated sodium bicarbonate, applied for three successive periods of 15 min each (34). Sham incubations with buffer alone (PBS or saturated bicarbonate) were run in parallel to each treatment. All subsequent incubations were done at room temperature. After treatment, the plates were extensively washed with PBS, blocked with 2% BSA in PBS for 1 h, and then incubated with the mAb mixture for 2 h. After washing three times with 1% BSA, a mixture of horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-mouse IgM antibodies were added (each at 1:1000 dilutions) for 1 h. After washing, the reaction was developed as described (30). To compare the amount of deNAc-gangliosides between control and genistein-treated Melur cells, reactivity with SMR36/SGR37 was adjusted to the overall content of G_{D3} in the ganglioside extracts used, as determined by reactivity with mAb 3.6 (33). Background levels determined with the secondary antibody alone were subtracted in all cases.

HPTLC Immuno-overlays of Gangliosides—Gangliosides were separated on aluminum-backed Silica Gel-60 HPTLC plates, the plates plasticized, and overlaid with antibodies as described previously (30). For *in situ* reactions with periodate or acetic anhydride, the plates were cut into strips prior to antibody overlay. Strips were soaked in 10 ml of either 2 mM sodium metaperiodate in PBS or PBS alone for 20 min at 4 °C. Alternatively, they were soaked in 10 ml of 0.5 M sodium bicarbonate (with or without 500 μl of freshly added acetic anhydride) for 20 min at room temperature. The strips were then washed extensively in PBS, blocked in 5% BSA for 15 min at room temperature, washed with 1% BSA, and incubated with hybridoma supernants overnight at 4 °C. After washing three times in PBS the strips were reacted with horseradish peroxidase-conjugated goat anti-mouse IgG (for SGR37) or horseradish peroxidase-conjugated goat anti-mouse IgM (for SMR36) at 1:1000 dilutions for 1 h at 4 °C. The strips were then washed and developed as described (30).

HPTLC Analysis of Endogenous Melanoma Gangliosides—Gangliosides from genistein-treated or control cells (1.6 × 10^7 cells each) were extracted as described (23) and fractionated by DEAE-HPLC into monosialogangliosides (fractions 13–33) and disialogangliosides (fractions 34–50). These were further resolved by HPTLC and overlaid with SMR36 and SGR37 (30). Biotinylated secondary antibodies recognizing either mouse IgG 1:2000 (SGR37) or mouse IgM 1:500 (SMR36) were reacted with the plate for 1 h at 4 °C. After washing, plates were incubated with 5 μCi/25 cm^2 of [^{35}S]streptavidin (Amersham, 920 Ci/mmol) for 1 h at 4 °C. The plates were washed extensively, monitored by Geiger counter until further radioactivity could not be removed, sprayed with En^{3}Hance (DuPont NEN), dried, and exposed to x-ray film.

Treatment of Melanoma Cells with Biological Modulators—Melur melanoma cells were grown to approximately 90% confluence in 6-well 30-mm plates, and fresh media containing the following were added (concentrations chosen based upon prior literature): 0.5 μM phorbol myristic acid (PMA), 2 μM okadaic acid, 1 μg/ml staurosporine, 1 mM dibutyryl cAMP, 100 μg/ml genistein, or 10% NuSerum, containing added epidermal growth factor and steroids. Control cells were sham-

treated with solvent alone (0.5% Me_2SO or 0.1% ethanol, v/v). After 15 h, cells were examined by phase contrast microscopy. Okadaic acid-treated cells no longer adhered to the plate, indicating some loss of viability: the other compounds did not have this effect. For time course studies with genistein, Melur cells were grown to ~90% confluence in 6-well 30-mm plates, spent media replaced with 2 ml of fresh media, and 11.2 μl of 32 mM genistein (200 μM final) in Me_2SO (mixed with 2 μl of ethanol for maximum solubility) added. Control cells were treated with Me_2SO and ethanol alone. For a dose-response analysis, control and genistein-treated cells were incubated with identical solvent volumes for each genistein concentration. In other experiments, the effects of additional tyrosine kinase inhibitors were tested, including herbimycin A (Life Technologies, Inc., 0.4 μM), 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid (Life Technologies, Inc., 0.44 μM final concentration), tyrphostin (Life Technologies, Inc., 150 μM final concentration), lavendustin A (Life Technologies, Inc., 0.2 μM final concentration), and *methyl*-2,5-dihydroxycinnamate (Life Technologies, Inc., 5 mM final concentration). All these incubations were for 15 h unless otherwise indicated.

SMR36/37 Staining of Melanoma Cells for Flow Cytometry Analysis—Melur cells were harvested by scraping into media, pelleted, resuspended in 1 ml of trypsin ATV for 5 min at 15 °C, and then repelleted and washed in media containing serum to inactivate residual trypsin. These processing conditions yielded optimal fluorescence in flow cytometry experiments with ganglioside-specific antibodies. Single cell suspensions were transferred to 96-well plates and incubated with a 1:1 mixture (200 μl of total volume) of SMR36 and SGR37 hybridoma supernatants for 1 h on ice. Control cells were incubated with similar amounts of mouse IgG_3 or IgM in 1% BSA in PBS. Cells were then washed three times with 1% BSA in PBS, incubated with a mixture of fluorescene isothiocynate-conjugated goat anti-mouse IgG and fluorescene isothiocynate-goat anti-mouse IgM (each at 1:50, 100 μl total volume) for 40 min on ice, washed three times in PBS, fixed in 2% paraformaldehyde in PBS, and analyzed by flow cytometry using a Becton Dickinson FACScan machine. Intact cells were gated on by their forward and 90° light-scattering characteristics.

Propidium Iodide Staining of Melanoma Cells and Flow Cytometry Analysis of Cell Cycle Status—Aliquots of cultured cells were washed, fixed with 70% ethanol for 2 h, and then incubated in hypotonic fluorochrome solution (propidium iodide (Life Technologies, Inc., 20 μg/ml) containing RNase A (Boehringer Mannheim, 40 μg/ml) in 0.1% sodium citrate and 0.1% Triton X-100) for 30 min. All subsequent procedures were done at 4 °C. The DNA content was measured using FACScan (Lysis program, Becton Dickinson). Two-color double-staining of cells for reactivity with anti-ganglioside antibodies and propidium iodide was not possible because the ethanol and Triton required for optimal staining with the latter resulted in loss of anti-ganglioside reactivity. Therefore, parallel aliquots of each batch of cells were stained separately with propidium iodide or with SMR36/SGR37 antibodies as above. To arrest cells in mitosis, the microtubule inhibitor nocodazole was used at a final concentration of 0.1 μg/ml, for 8 h. Non-adherent cells were collected and analyzed separately from adherent cells, which were harvested as described above. Both adherent and non-adherent cells were treated with trypsin under the same conditions and analyzed regarding DNA content and reactivity with anti-deNAc-gangliosides, as above.

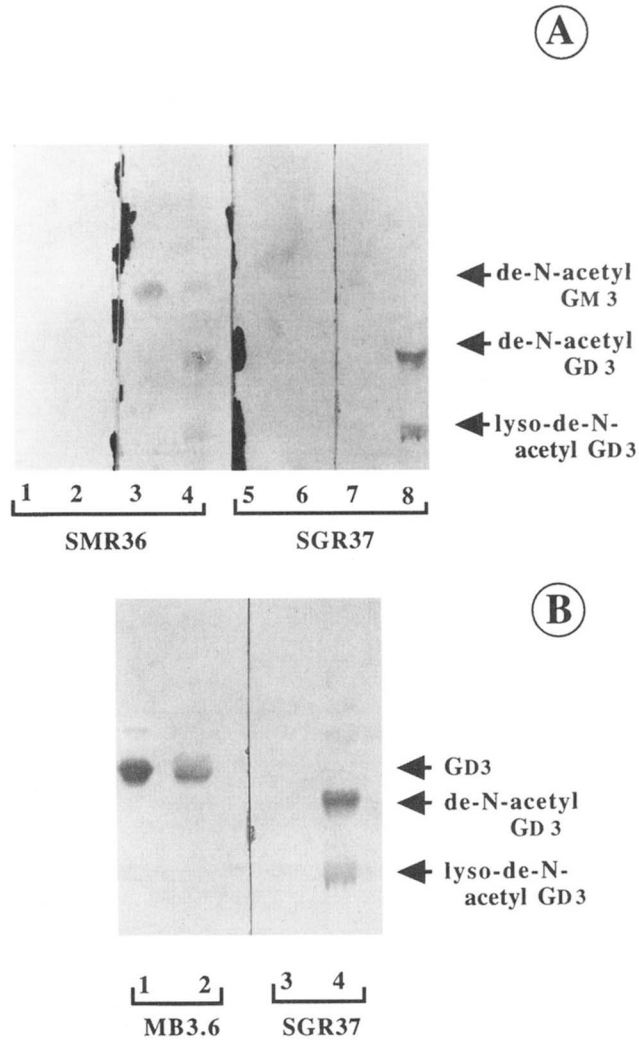

FIG. 1. **mAb reactivity by HPTLC immuno-overlay and the effect of mild periodate oxidation.** *Panel A*, purified synthetic deNAc-G_{M3} (1 μg, *lanes 1, 3, 5,* and *7*) and deNAc-G_{D3} reaction mixture (2 μg, *lanes 2, 4, 6,* and *8*) were loaded onto HPTLC plates and developed using (50:40:10, v/v/v) chloroform/methanol, 0.02% $CaCl_2$. *Lanes 1* and *2* and *5* and *6* were cut out and treated with mild periodate while *lanes 3* and *4* and *7* and *8* were incubated with PBS alone as described under "Experimental Procedures." The plates were then probed with SMR36 or SGR37 as indicated. *Panel B*, the synthetic deNAc-G_{D3} reaction mixture (2 μg, *lanes 2* and *4*) or the G_{D3} starting material (1 μg, *lanes 1* and *3*) was loaded onto a HPTLC plate and developed as in *panel A*. After development, the plate was split in half and overlaid with either SGR37 (*lanes 3* and *4*) or an antibody directed against G_{D3}, MB3.6 (*lanes 1* and *2*).

RESULTS

Production and Characterization of SMR36 and SGR37 Antibodies—In spite of previous indirect evidence for the presence of deNAc-G_{M3} and deNAc-G_{D3} in human melanoma cells (23, 24), low quantities and variable expression has impeded further analysis.[2] We therefore synthesized deNAc-G_{M3} and deNAc-G_{D3} by modifications of described methods and used them to generate specific mAbs. Base hydrolysis of G_{M3} yielded two products with HPTLC migration retarded relative to G_{M3} (data not shown). Characterization of the mixture by FAB-MS showed that the most abundant signals in the molecular ion region were 1219 ($[M-H]^-$ for de-*N*-acetyl G_{M3}), and m/z 871($[M-H]^-$ for lyso-de-*N*-acetyl G_{M3}, lacking the fatty acyl chain). Their *N*-acetylated counterparts gave minor signals at m/z 913 (lyso-G_{M3}) and 1261 (G_{M3}), together constituting less than 10% of the total molecular ion abundance. The mixture was fractionated by Iatrobeads HPLC and fractions with HPTLC migration similar to the reported position of deNAc-G_{M3} used as antigen for immunization and screening of hybridomas. The resulting mAb SMR36 reacted with the two major HPTLC bands in the mixture corresponding to deNAc-G_{M3} and lyso-deNAc-G_{M3}, both of which are retarded relative to G_{M3}, which is not reactive (detailed data not shown, see Fig. 1*A* for example showing reactivity with deNAc-G_{M3}). The mixture was then fractionated by DEAE-HPLC, and the SMR36-reactive fractions studied by FAB-MS, which showed mass ions corresponding to deNAc-G_{M3} or lyso-deNAc G_{M3} (data not shown). This suggests that SMR36 reactivity requires the free amino group at the C-5 position of sialic acid on G_{M3}, but that the ceramide fatty acyl chain is not relevant. As Fig. 2*A* demonstrates, deNAc-G_{M3} derivatives coelute with G_{M3} in anion-exchange chromatography (*i.e.* the amino group has no effect on elution). *In situ* re-*N*-acetylation of the deNAc-G_{M3} synthetic mixture with acetic anhydride completely abrogated SMR36 binding, confirming that a free amino group at the C-5 position

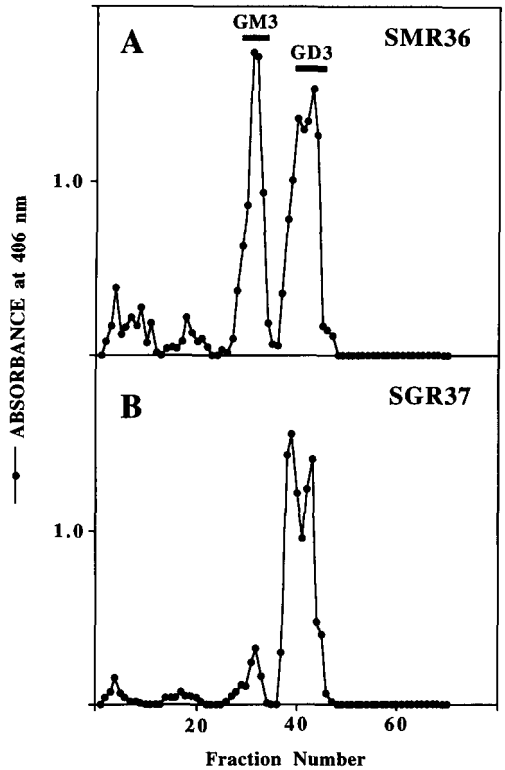

FIG. 2. **DEAE-HPLC profile of SMR36 and SGR37 reactive products.** Synthetic deNAc-G_{M3} (1 μg) and deNAc-G_{D3} (2 μg) were fractionated on a TSK-DEAE-HPLC column as described under "Experimental Procedures." Aliquots (100 μl) of fractions were dried in 96-well plates and analyzed by ELISA using hybridoma supernatants from SMR36 (*panel A*) or SGR37 (*panel B*). The elution positions of G_{M3} and G_{D3} (indicated by *bars*) were established using known ^{14}C-labeled gangliosides from Melur cells.

of sialic acid on G_{M3} is required for recognition (data not shown). SMR36 also reacts with two species in deNAc-G_{D3} synthetic mixtures (Fig. 1*A*) characterized subsequently as deNAc-G_{D3} and lyso-deNAc-G_{D3}, with free amino groups on either the inner or the outer sialic acid residue (see below).

Base hydrolysis of G_{D3} gave a more complex array of products (by resorcinol staining, not shown). However, SGR37 reacts only with two major products migrating slower than G_{D3} (Fig. 1*B*) that were subsequently identified as deNAc-G_{D3} and lyso-deNAc-G_{D3}. On DEAE-HPLC these SGR37 reactive products elute in the region expected for disialogangliosides (Fig. 2*B*, again the free amino groups do not alter DEAE elution). These were further fractionated by Iatrobeads HPLC and elution of SGR37-reactive products monitored by HPTLC immuno-overlay. A single SGR37 reactive fraction was observed and characterized by FAB-MS in the negative ion mode, yielding a major cluster of signals shifted from G_{D3} by 42 mass units, *i.e.* de-*N*-acetylated G_{D3}. The most abundant ions were at *m/z* 1457, 1471, 1485, 1499, and 1513 corresponding to de-NAc-G_{D3} containing fatty acyl chain lengths C20:0, C21:0, C22:0, C23:0, and C24:0 respectively, with the last three being most prominent (data not shown). Deuteropermethylation of the SGR37-reactive fraction afforded major molecular ions at *m/z* 1827, 1841, and 1855 (Fig. 3), corresponding to fully deuteromethylated mono-de-NAc-G_{D3} species having the fatty acyl chain lengths of the most abundant components previously defined by the FAB experiments on the native G_{D3}. Fragment ions at *m/z* 359 (*m/z* 394 minus deuteromethanol), 369 (de-*N*-acetylNeuAc$^+$), 394 (NeuAc$^+$), and 745 (de-*N*-acetylNeuAc-NeuAc$^+$ or NeuAc-de-*N*-acetylNeuAc$^+$) indicate that mono-deNac-G_{D3} is the major component in the reactive fraction with deacetylation on either the inner or the outer Neu5Ac residue. Thus, SGR37 reacts with de-*N*-acetylG_{D3} having a single free amino group on the inner and/or the outer sialic acid residue (since these two isomers do not separate by HPTLC or Iatrobeads, we cannot resolve this issue). In earlier experiments the highly retarded lyso form of deNAc-G_{D3} also eluted very late from the column (confirmed by FAB-MS, data not shown) and reacted with the antibody, showing that reactivity is not dependent on the presence of the ceramide fatty acyl chain. Re-*N*-acetylation of de-NAc-G_{D3} reaction mixtures with acetic anhydride abolished all reactivity with SGR37 confirming the requirement for the free amino group of neuraminic acid (data not shown). Thus, in contrast to SMR36, which reacts with both de-*N*-acetyl G_{M3} and de-*N*-acetylG_{D3}, SGR37 is relatively specific for the latter, cross-reacting very weakly with de-*N*-acetylG_{M3} (Fig. 1*A*). If it is assumed that SMR36 cross-reacts with the de-NAc-G_{D3} species having the free amino group on the inner residue (*i.e.* similar to de-NAc-G_{M3}), then substitution by the outer sialic acid residue at the C-8 position of the inner one does not adversely effect recognition. Although we examined the synthetic mixture for the presence of di-de-NAc-GD3, the complexities of the ceramide fatty acyl chain heterogeneity made it difficult to be certain if this derivative is present. Therefore, we cannot rule out the possibility that SGR37 also reacts with di-de-NAc-G_{D3} (although the latter would be expected to have a slower migration on HPTLC).

Mild periodate treatment selectively truncates the unsubstituted exocyclic side chain of terminal Neu5Ac, forming C-7 and C-8 derivatives without affecting the underlying oligosaccharide structure (35–37) and can abolish mAb reactivity with some gangliosides (33). Such treatment abrogates recognition of deNAc forms of G_{M3} by SMR36 and deNAc forms of G_{D3} by both SMR36 and SGR37 (Fig. 1*A*). Identical treatment of a mild periodate-resistant ganglioside, 9-*O*-acetylatedG_{D3} (33), did not affect recognition by the specific antibody JONES (38), indicating that the *in situ* periodate treatment did not nonspecifically affect colorimetric detection on the plate (data not shown). Thus, both SMR36 and SGR37 require intact exocyclic side chains on terminal sialic acids of the molecules they recognize. The data raise the possibility that when SMR36 recognizes deNAc-G_{D3}, it may be recognizing the isomer with the outer sialic acid de-*N*-acetylated. Alternatively, oxidation of the side chain of the outer residue may change the conformation of the molecule such that an inner residue can no longer be recognized.

In summary, SMR36 recognizes deNAc-G_{M3}, deNAc-G_{D3}, and their lyso derivatives, while SGR37 reacts only with deNAc-G_{D3} and its lyso derivative. Recognition by each antibody shows an absolute requirement for a free amino group at the C-5 position of sialic acid and an intact exocyclic sialic acid side chain on the terminal sialic acid residue. This dual requirement indicates that these mAbs can be used as highly specific probes for detection of deNAc-gangliosides in melanoma cells. Furthermore, mild periodate oxidation and re-*N*-acetylation can be used in appropriate situations to confirm the specificity of antibody reactivity.

Expression of DeNAc Gangliosides on Melur Melanoma Cells Is Induced by Genistein—We initially screened several melanoma cell lines from human, murine, and hamster sources (see listing under "Experimental Procedures") with SMR36 and SGR37 by flow cytometry analysis and HPTLC immuno-overlay of extracted lipids using colorimetric detection. In each case, we failed to consistently detect deNAc-gangliosides, although some cell lines occasionally displayed a small population of positive cells (1–2% by flow cytometry, data not shown). However, if deNAc-gangliosides are transiently expressed in

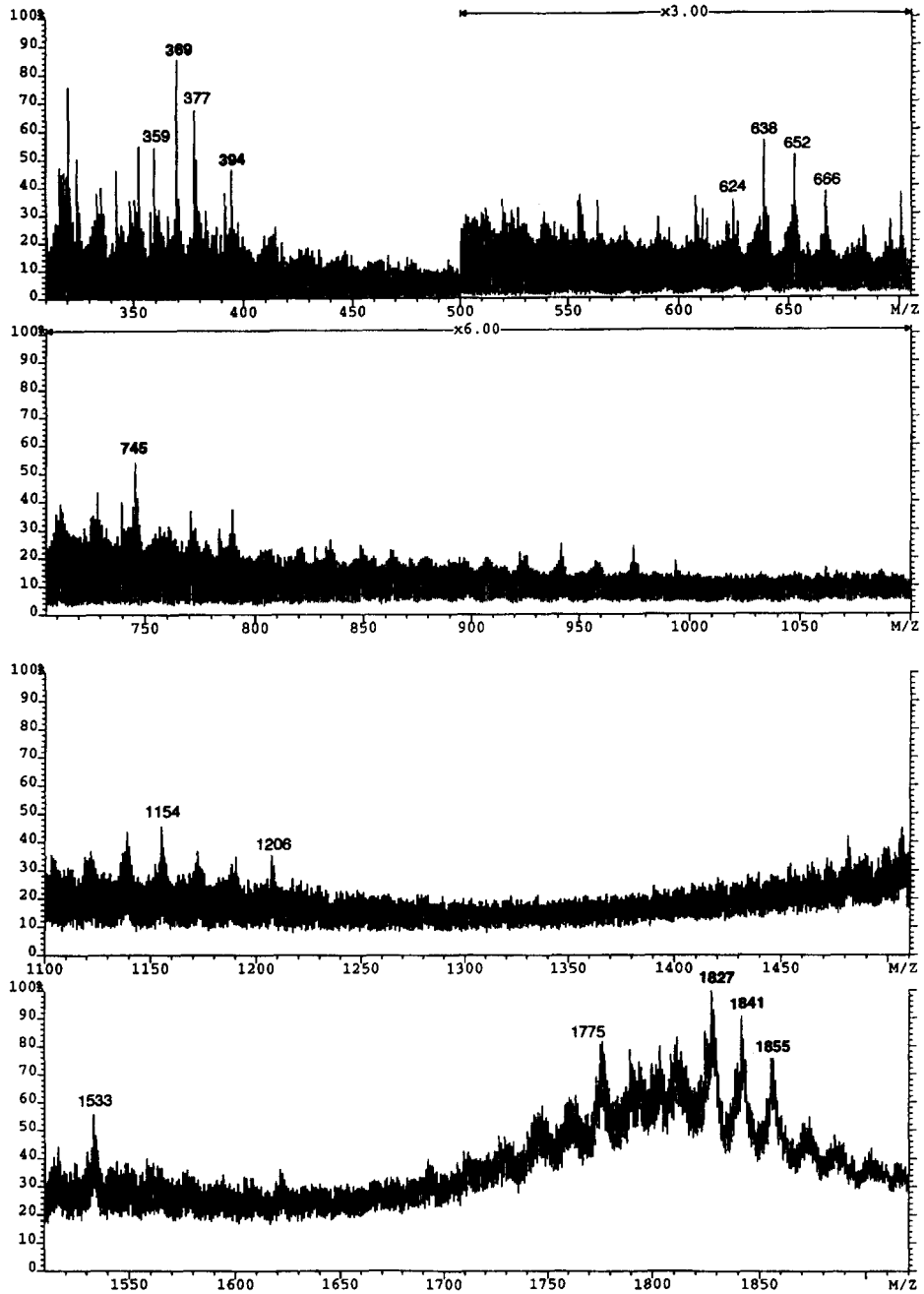

FIG. 3. **Characterization of SGR37 reactive fraction by fast atom bombardment-mass spectrometry.** SGR37 reactive material was purified and deuteropermethylated and the products analyzed by positive FAB-MS. The signals separated by increments of 14 mass units from m/z 624 to 666 are derived from the ceramide. The signal at m/z 377 is probably a b-cleavage ion derived from a penultimate NeuAc residue. Other signals attributable to de-*N*-acetyl G_{D3} are described in the text. Minor unassigned signals above m/z 900 are probably derived from contaminants.

small quantities, *e.g.* as growth regulating molecules or with cell cycle-dependent expression, they may not be consistently detectable under these conditions. We therefore tried to induce stable expression of these molecules, using various compounds known to affect cellular growth and/or differentiation. We chose the Melur cell line for further analysis since it is known to express the gangliosides G_{M3}, G_{D3}, and *O*-acetylated G_{D3}, and indirect evidence had suggested expression of deNAc-G_{M3} and deNAc-G_{D3} (23). Cells were grown for 15 h in the presence of staurosporine, okadaic acid, PMA, Nuserum with EGF and steroids or genistein, and a mixture of SMR36 and SGR37 were used to detect the cell surface expression of deNAc-gangliosides by flow cytometry. Of these, only PMA (a protein kinase C activator) and genistein (a tyrosine kinase inhibitor) induced increased fluorescence relative to control cells (Fig. 4). Over a 15-h time period, PMA is probably desensitizing protein kinase C rather than activating it (39, 40). Regardless, since induction by PMA was lower and less consistent than with genistein, this matter was not pursued further. Genistein treatment consistently yielded "bright" cells representing 15–30% of the population analyzed. This treatment also altered the morphology of the cells to a spindly neuron-like appearance and significantly inhibited further growth (data not shown). However the increased staining is not a nonspecific consequence of the morphological alteration, since cells were treated with trypsin prior to analysis, resulting in a uniform round shape. Additionally, genistein-treated cells stained with isotype-matched immunoglobulins did not show fluorescence greater than that seen with non-treated control cells stained by SMR36/SGR37 (Fig. 4).

Induction of SMR36/SGR37 Cell Surface Staining by Genistein Is Dose-dependent—To further characterize the genistein effect, concentrations ranging from 5–400 μM were tested. As illustrated by the examples in Fig. 5, maximal induction occurred at 200 μM. Genistein is known to inhibit EGFR autophosphorylation in isolated membranes with an IC_{50} of 20 μM (41) and, in intact cells, at a 40 μM concentration

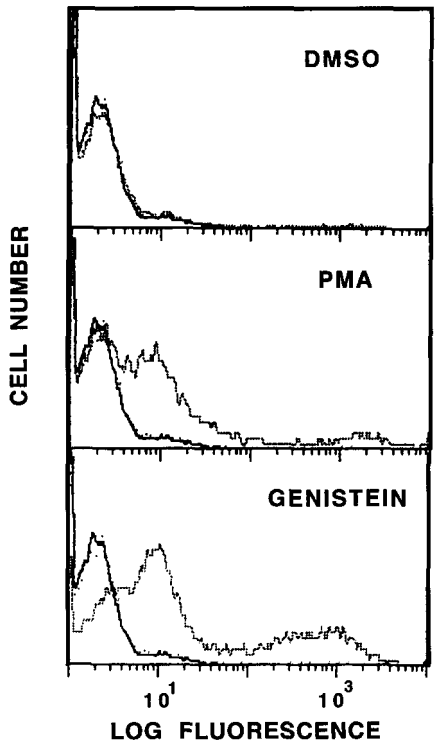

FIG. 4. **The effect of various compounds on deNAc-ganglioside expression.** Melur cells were grown with added Me_2SO solvent (2 μl), PMA (0.5 μM), or genistein (100 μg/ml) as indicated. After 15 h, the cells were harvested and stained with SMR36 and SGR37 (1:1) as described under "Experimental Procedures." *Dotted lines*, isotype stained non-treated cells; *dashed lines*, SMR36/SGR37 stained cells treated with the indicated compound; *solid lines*, non-treated control cells stained with SMR36/SGR37.

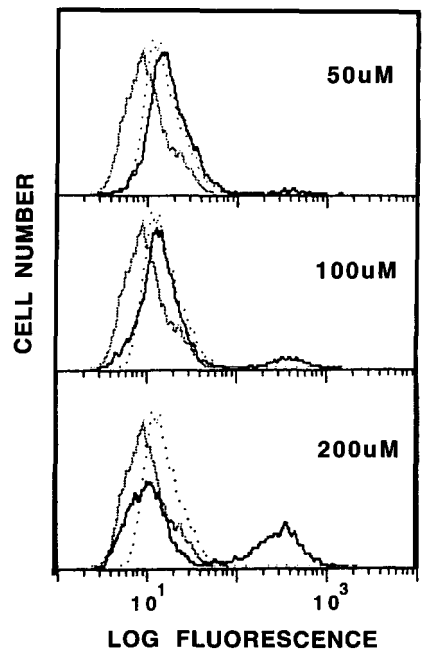

FIG. 5. **Dose dependence of genistein effect on deNAc-ganglioside expression.** Melur cells were grown in the presence of genistein at the concentrations indicated, for 15 h, the cells harvested, and stained with SMR36 and SGR37 (1:1 v/v) as described under "Experimental Procedures." *Dotted lines*, SMR36/SGR37 stained non-treated cells grown with equivalent solvent volumes; *dashed lines*, genistein-treated cells stained with isotype matched antibodies; *solid lines*, genistein-treated cells stained with SMR36/SGR37.

(42). Since we used genistein on intact cells rather than on purified membrane preparations, it is not surprising that maximal induction of deNAc-gangliosides occurred at a 10-fold greater concentration. We also examined the time course of induction by genistein, fixing the concentration at 200 μM. Several experiments were performed, analyzing time points from 1 to 24 h. The results showed a highly variable onset and tempo of deNAc-ganglioside induction, peaking at 6–15 h (data not shown). This may indicate that the inductive effect of genistein requires up-regulation of new biosynthetic machinery required for deNAc-ganglioside biosynthesis. Alternatively, the variability could be due to differences in the growth status of the starting cells. Indeed, we have noted that cells that had been in extended culture sometimes did not show as strong a response.[2] As discussed below, this variability may be due to the fact that genistein can specifically block cell cycle progression at the G_{2M} phase (43).

Antibody Reactivity with Gangliosides from Melanoma Cells Is Abrogated by Mild Periodate Oxidation or Re-N-acetylation—Although SMR36 and SGR37 appear to be highly specific for deNAc-gangliosides, the increased staining could be due to a cross-reacting molecule that is up-regulated in response to genistein. Alternatively, deNAc-gangliosides may be cryptically expressed in untreated cells due to "masking" by co-expression of a cell membrane component that is down-regulated by genistein. Total lipids were therefore extracted from control and genistein-treated cells and studied for deNAc-ganglioside expression by lipid ELISA. As shown in Fig. 6, extracts from control cells showed barely detectable antibody reactivity, while those from genistein-treated cells showed a clear increase. In each case this reactivity was markedly abrogated by treatment of the plate-bound lipids with either mild periodate oxidation (which would selectively truncate sialic acid side chains) or with chemical re-*N*-acetylation (which would acetylate the free amino group required for reactivity) (see Fig. 6). The loss of binding with these two treatments confirms that the reactivity was due to the presence of deNAc-gangliosides and not some unknown cross-reactive lipid component. Taken together, these results indicate that genistein induces an increase in deNAc-ganglioside synthesis and not only a redistribution of existing molecules between distinct subcellular compartments, *e.g.* plasma membrane and Golgi.

DeNAc-G_{D3} and Not DeNAc-G_{M3} Is Detected in Genistein-treated Cells—Preliminary experiments indicated that even in the presence of genistein, deNAc-ganglioside expression levels were too low to allow purification of sufficient quantities for definitive structural identification by FAB-MS. Therefore, we analyzed the extracts by HPTLC immuno-overlay assays using SMR36 and SGR37. Total lipid extracts could not be loaded onto HPTLC plates in quantities sufficient to detect deNAc-gangliosides, even when detected by the more sensitive [^{35}S]streptavidin method (data not shown). Therefore, we fractionated the extracts by DEAE-HPLC, pooled areas representing neutral lipids monosialo- or disialogangliosides, and analyzed them (Fig. 7). The antibodies clearly recognize a band in the disialoganglioside fraction of genistein-treated cells that migrates slightly below standard synthetic deNAc-G_{D3}. This relative retardation is an artifact caused by overloading with unmodified G_{D3}. A visible shadow corresponding to excess G_{D3} can be seen (also in the control lanes), which forces the antibody-reactive band to migrate with retarded mobility relative to standard deNAc-G_{D3}. The SMR36/SGR37-positive band is undetectable in the disialoganglioside fraction of control cells. In comparison to known standards, the deNAc-G_{D3} detected by HPTLC immuno-overlay corresponds to ~50–75 pmol (*i.e.*~ 25–35 pmol/5 × 10^6 cells or 500,000 molecules of deNAc-G_{D3}/cell). Notably, although the Melur cell line expresses only

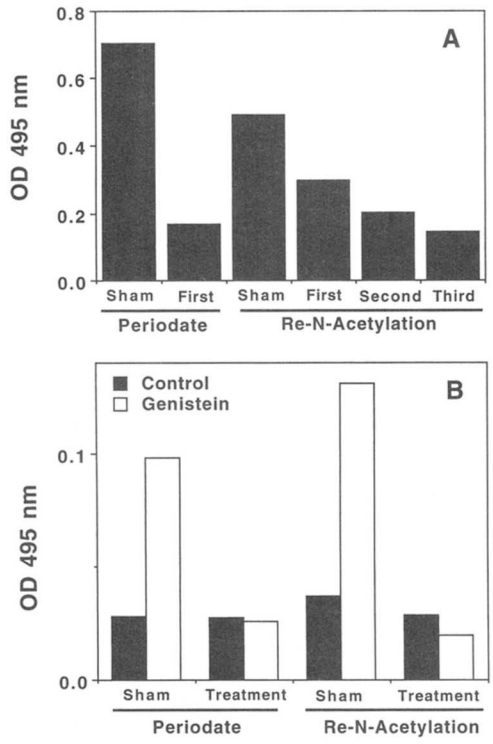

FIG. 6. **ELISA plate assay for detection of deNAc-ganglioside expression in Melur melanoma cells treated with genistein effects of mild periodate and de-*N*-acetylation.** Synthetic deNAc-G_{D3} (*panel A*) and ganglioside extracts of either control or genistein-treated Melur cells (*panel B*) were studied for reactivity with a 1:1 mixture of SMR36 and SGR37 in ELISA plates. Mild periodate oxidation and re-*N*-acetylation destroyed the epitope after one or three successive rounds of reaction, respectively, as shown in *panel A*. *Panel B* shows the effect of genistein on deNAc-ganglioside expression in Melur cells. The specificity of the reaction was confirmed by its abrogation after periodate or re-*N*-acetylation treatment. Reactivity in *panel B* was adjusted for the amount of G_{D3} present in each sample, determined in parallel by ELISA with an anti-G_{D3} antibody.

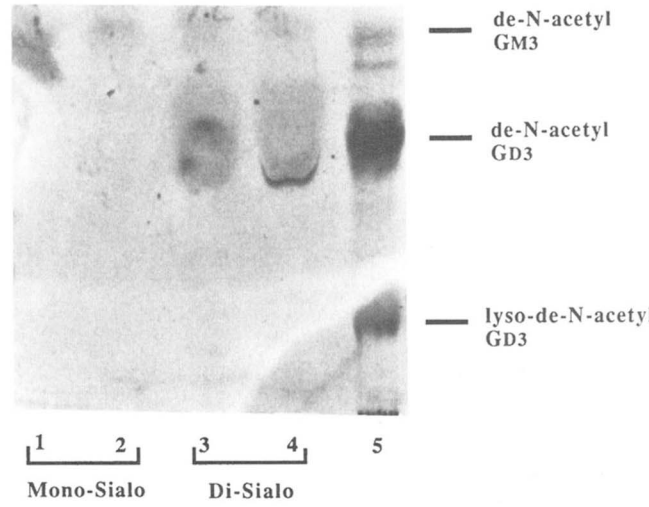

FIG. 7. **SMR36/SGR37 immuno-overlay of endogenous Melur gangliosides treated with and without genistein.** Melur cells (2 × 150-mm plates) were grown in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of 200 μM genistein for 15 h. The cells were harvested, and total lipids were extracted and fractionated by DEAE-HPLC into mono- and disialogangliosides. The fractions were loaded onto HPTLC plates and developed using chloroform/methanol, 0.02% $CaCl_2$ (50:40:10, v/v/v). The plates were overlaid with SMR36 and SGR37 and deNAc-gangliosides detected using [^{35}S]streptavidin as described under "Experimental Procedures." *Lane 5* is a 2-μg aliquot of the deNAc-G_{D3} reaction mixture.

TABLE I
DeNAc-ganglioside expression is increased in the S/G_2M cell cycle phases

Following various treatments, cells were analyzed regarding deNAc-ganglioside expression and DNA content, as described under "Experimental Procedures." Reactivity with SMR36/SGR37 is reported as percent of total cells that were positive under the different experimental conditions. The summed percentage of cells in S, G_2 and M phases are presented, based on DNA content analysis of parallel aliquots. See Figs. 8 and 9 for examples of the data from which this table is derived.

	Melur cells		M21 cells	
	deNAc-gangliosides	S/G_2/M phase	deNAc-gangliosides	S/G_2/M phase
	% positive	*% of total*	*% positive*	*% of total*
Experimental condition				
Control	2.3	31.1	3.1	37.1
Genistein	12.2	69.5	18.7	61.9
Herbimycin A	2.1	28.4	3.7	33.4
Nocodazole, non-adherent	20.8[a]	88.2	18.4	87.5
Nocodazole, adherent	7.8	74.7	7.8	85.9

[a] In this instance, the majority of the remaining cells showed a low level of positivity compared with the control (see Fig. 9 for an example).

~3 times more G_{D3} than G_{M3}, we did not detect deNAc-G_{M3} even in genistein-treated cells. Attempts to improve the esthetic quality of this result by purifying the deNAc-G_{D3} away from the excess G_{D3} have not been successful because of the small quantities of the former present and substantial losses that occur during additional chromatographic procedures (note that antibodies cannot be used to purify gangliosides because of mixed micelle formation in aqueous conditions). Likewise, attempts at direct chemical treatments of gangliosides on the TLC plates have been limited by increase in background resulting from such manipulations. Regardless, taken together with the ELISA data presented above, the expression of de-NAc-gangliosides in genistein-treated cells is reasonably well confirmed.

Induction of De-N-acetyl Ganglioside Expression by Genistein Is Also Found in M21 Melanoma Cells, But Is Not Caused by Other Tyrosine Kinase Inhibitors—The effects of some other tyrosine kinase inhibitors including 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid, tyrphostin (44), lavendustin A (45), and *methyl*-2,5-dihydroxycinnamate (46) were tested in the Melur melnoma cell line. Lavendustin and *methyl*-2,5-dihydroxycinnamate variably but marginally increased expression of deNAc-gangliosides (3–5% positive cells), while the other two compounds were ineffective (data not shown). Induction of deNAc-ganglioside expression by genistein was also found in the human melanoma cell line M21 (Table I). To confirm the selective effect of genistein, both M21 and Melur melanoma cells were studied again, after treatment with either genistein or herbimycin A (47), another well-recognized tyrosine kinase inhibitor. As shown in Table I and Fig. 8, treatment of M21 or Melur cells cells with herbimycin A did not give any increase in cell surface expression of deNAc-gangliosides, whereas a significant increase was observed in both cell types with genistein treatment. Since the concentrations of each of the other tyrosine kinase inhibitors tested were at least 10 times their respective IC_{50} values for inhibition of EGFR phosphorylation, it is unlikely that conditions were suboptimal for deNAc-ganglioside induction (of course, we cannot rule out that in some instances, access of the compounds to the interior of the cell may have been limiting). Alternatively, genistein may be selectively inhibiting specific tyrosine kinases that are not affected by the other inhibitors (41), explaining the selective induction.

Induction of De-N-acetyl-ganglioside Expression by Nocodazole Indicates That Genistein May Act by Arresting Cells in the G_2M Phase of the Cell Cycle—Recent reports have indicated

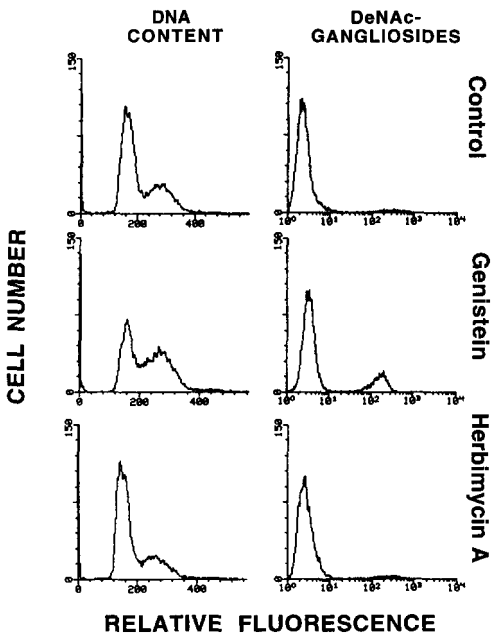

FIG. 8. **Comparative effects of genistein and herbimycin A on de-NAc-ganglioside expression in M21 melanoma cells.** M21 cells were grown in the presence of herbimycin A (0.4 μM) or genistein (200 μM), for 8 h, and cells were harvested and analyzed separately for DNA content and expression of deNAc-gangliosides with SMR36 and SGR37, as described under "Experimental Procedures."

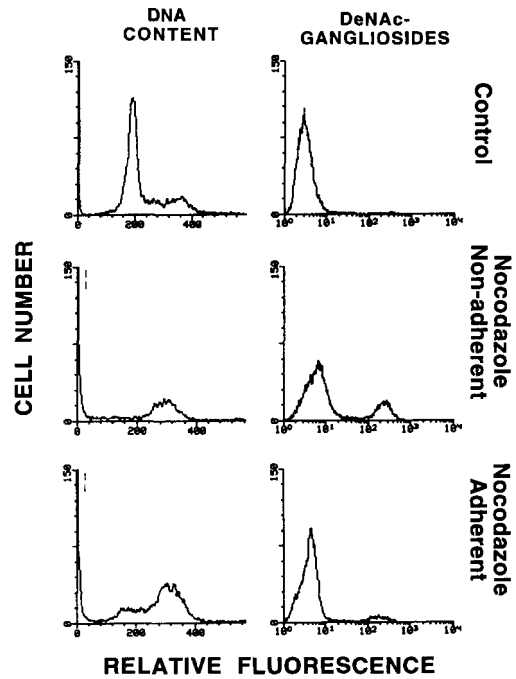

FIG. 9. **Induction of de-NAc-ganglioside expression in melanoma cells by nocodazole.** Melur melanoma cells were grown in the presence of nocodazole (0.1 μg/ml), for 8 h, adherent and non-adherent cells were harvested and analyzed separately for DNA content and expression of deNAc-gangliosides with SMR36 and SGR37, as described under "Experimental Procedures."

that genistein can specifically blocks cell cycle progression at the G_2M phase (43). Interestingly, high concentrations of PMA which also induced some deNAc-ganglioside expression (see above) are also known to cause a similar block in cell cycle progression (48). To explore the possibility that these effects were related, we treated M21 and Melur melanoma cells with nocodazole, a microtubule inhibitor (49) that selectively arrests cells in mitosis (M). After nocodazole treatment, two cell populations can be easily identified and separated by differential harvesting. Non-adherent cells that are round in morphology float in the culture medium and are readily collected and then trypsinized for staining, while adherent cells need to be harvested with trypsin-EDTA. The non-adherent cells typically represented 40–50% of the population, with a viability of 90–95% as determined by Trypan blue exclusion. These two subpopulations (from either Melur or M21 cell cultures) were studied regarding both DNA content and deNAc-ganglioside expression on the cell surface (see Fig. 9 and Table I). Overall, the non-adherent cells expressed higher levels of deNAc-gangliosides on the cell surface. A small but definite shift in the overall profile to the right (see Fig. 9) indicates a general increase of deNAc-gangliosides in these cells. A second population (approximately 20% of these cells) showed a higher expression of deNAc-gangliosides, with the level of reactivity similar to that of genistein-treated cells. As seen in Fig. 9, most of the non-adherent cells (which are expected to be in metaphase) had a two times DNA content. On the other hand, adherent cells did not display the overall general increase in staining, and the percentage of cells considered high expressors were only about 7.8%. For technical reasons (see "Experimental Procedures"), it was not possible to due simultaneous double-color analysis of cell cycle status and deNAc-gangliosides in the same cells. However, the correspondence between the fractional increase in cells in the G_2M phase (see Table I) and the increase in antibody-reactive cells is highly suggestive of this association.

DISCUSSION

Although the existence and potential growth regulatory properties of deNAc-gangliosides were suggested several years ago, analysis of these interesting molecules has proven to be difficult because they are expressed transiently and in very small quantities. For example, B16 melanoma cells, wherein deNAc-G_{M3} was first reported, were estimated to contain ~1 pmol of deNAc-G_{M3} for every 5×10^6 cells even though G_{M3} is the major ganglioside expressed in this cell line (16). Our own previous studies using sensitive double-label pulse-chase analyses provided indirect evidence for expression of deNAc-G_{M3} and deNAc-G_{D3} in human melanoma cells (23). Subsequently, we noted that this evidence for deNAc-gangliosides was variably seen, even between batches of otherwise apparently identical cells.[2] This complicated further analysis but was reminiscent of other transiently expressed molecules known to regulate cellular proliferation or signal transduction (50–52). We therefore attempted to stimulate synthesis of deNAc-gangliosides using various growth altering and biologically active compounds.

To permit rapid screening, we have developed mAbs highly specific for deNAc-gangliosides. Targeted generation of mAbs with purified ganglioside antigens is generally difficult, and most ganglioside-specific antibodies have been derived fortuitously by injection of whole cells into mice (53–55). However, one of us (T. T.) has shown that certain murine strains provide more reliable host responses to purified gangliosides (27, 28, 56). Using this and other refinements, we have reported success in generating sets of mAbs specific for ganglio-series gangliosides of the a, b, and α-pathways, and for NeuGc-containing gangliosides (27, 28, 57, 58). Exploiting this knowledge, we produced two mAbs against purified deNAc-gangliosides (one of them is an IgG). Of several compounds initially screened, only genistein, a tyrosine kinase inhibitor, consistently stimulated cell surface expression of deNAc-gangliosides to a substantial degree. This was confirmed by whole cell lipid ELISA

assays, and by HPTLC immuno-overlays, in which a band corresponding to deNAc-G_{D3} was detected in genistein-treated cells but not in control cells. Ultimately, it will be necessary to isolate sufficient quantities of this material to allow complete structural characterization by FAB-MS, which should also allow for the precise localization of the free amino group on the inner and/or the outer sialic acid residue of G_{D3}. This will require improvements in our current methods for derivatization and detection of gangliosides by FAB-MS. Such efforts are currently under way.

While the transient and variable nature of endogenous deNAc-ganglioside expression in human melanoma cells has hindered careful analysis, this may also suggests the involvement of these molecules in temporally regulated biological processes. Understanding the mechanism by which genistein induces deNAc-ganglioside expression may help to elucidate these matters. Prior studies have provided conflicting results regarding the direction of modulation of EGFR activity by addition of synthetic deNAc-G_{M3} to intact cells and isolated membranes (16, 24, 59, 60). Regardless, these studies all provide evidence linking deNAc-gangliosides to tyrosine kinase related signal transduction. In this regard, the fact that genistein is a known inhibitor of tyrosine kinases in intact cells (41, 46) is of obvious interest. However, while some other tyrosine kinase inhibitors slightly induce de-*N*-acetyl-ganglioside expression, none stimulate expression to the extent achieved by genistein. Since genistein has recently been shown to impede cell cycle progression beyond G_2M (43), it is possible that it exerts its effect on deNAc-ganglioside expression at least partly by blocking cell cycle progression. If the expression of deNAc-gangliosides is normally increased during this particular phase of the cell cycle, blocking further progression would lead to increased expression of deNAc-gangliosides. The cdc2 kinase is a component of the maturation promoting factor that initiates mitosis. Specific tyrosine residues on cdc2 are phosphorylated from the beginning of DNA synthesis to the G_2 phase of the cell cycle (61–63). The activity of maturation promoting factor is then induced by dephosphorylation of tyrosine residues of cdc2 kinase to initiate mitosis (62). A previous study suggested that genistein may alter the tyrosine phosphorylation/dephosphorylation process of cdc2 kinase, thereby blocking the cell cycle at G_2M. Our data indicate that when melanoma cells are treated with genistein over a 15-h time period, the number of cells in G_2M is also increased relative to non-treated control cells. As alternative evidence that this may be the operative mechanism, we have shown that the mitotic spindle inhibitor nocodazole also causes a buildup of deNAc-ganglioside expression. Indeed, the selective buildup in expression in the non-adherent cells in this case indicates a close association with metaphase. This hypothesis is also attractive because it would help to explain the highly variable expression of deNAc-gangliosides found in human melanoma cells in different experiments under slightly varying culture conditions.

While the association of deNAc-ganglioside expression with the G_2M phase of the cell cycle is interesting, no cause-and-effect conclusions can be reached because pharmacological agents were used. Alternatively, it is well documented that pharmacologic intervention in cellular processes can be counteracted by the cell to maintain homeostasis, *e.g.* adrenergic receptor blockade over long periods of time result in up-regulation of adrenergic receptors (64). This could also explain why the maximal inductive effect of genistein on deNAc-ganglioside expression does not occur until 6–15 h after addition of the compound. Finally, it is possible that genistein is working by a mechanism independent of its effects upon tyrosine kinases (65, 66). Studies are currently underway to explore these possibilities further.

The *N*-acetyl group of Neu5Ac originates from conversion of $GlcNH_2$-6-P to GlcNAc-6-P (67–69). GlcNAc-6-P is converted via several steps to CMP-Neu5Ac, which is the donor for sialyltransferases that synthesize gangliosides (21, 22, 67, 68). Since the de-*N*-acetylated form of sialic acid, neuraminic acid, is unstable in its free unbound form, it is reasonable to assume that the *N*-acetyl group remains covalently attached throughout these steps. However, glycosidically bound neuraminic acid is at least as stable as its *N*-acetylated counterpart (70). Thus, the most plausible explanation for deNAc-gangliosides is a specific de-*N*-acetylase working on the intact ganglioside. These *N*-acetyl groups could also be rapidly replaced by an *N*-acetyltransferase. In fact, we have previously presented pulse-chase data suggesting such a de-*N*-acetylation/re-*N*-acetylation process in Melur cells (23). Thus, the delayed onset of the genistein effect on deNAc-ganglioside expression could imply a feedback loop which may cause up-regulation of a de-*N*-acetylase, or inhibition/down-regulation of *N*-acetyltransferase. We are currently searching for such enzyme activities. Further understanding of these systems may also allow manipulations that permit accumulation of sufficient quantities of deNAc-gangliosides for structural analysis by methods such as FAB-MS or NMR.

In summary, this work provides a new system in which to study deNAc-ganglioside biosynthesis and function and lends further support for the involvement of deNAc-gangliosides in growth regulation. Additionally, this report provides the first evidence for the existence of deNAc-G_{D3} in any cell type and raises the possibility that deNAc-gangliosides may play a role in the regulation of the cell cycle. This report also raises many new questions. For instance, what is the mechanism of deNAc-ganglioside biosynthesis? Is the genistein effect due to new ganglioside synthesis or to de-*N*-acetylation of existing gangliosides? While the subcellular site of re-*N*-acetylation appears to be in the Golgi apparatus or Golgi-like elements (23), where is the site of de-*N*-acetylation? Is deNAc-G_{D3} involved in tyrosine kinase signal transduction mechanisms as has been suggested for deNAc-G_{M3}? Is the genistein effect specific for de-*N*-acetylation of G_{D3}, or is deNAc-G_{M3} also induced at lower levels? Which isomer of mono-deNAc-G_{D3} predominates, and does di-deNAc-G_{D3} exist at all? Finally, is deNAc-ganglioside expression indeed cell cycle dependent, and if so, does it play an active role in the regulation of this vital biological process? The present work has set the stage for exploration of many of these questions.

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