late microalgal growth. Results from the field were consistent with those from the laboratory.

These interspecific differences in food growth stimulation may be understood by considering the interspecific differences in natural history and the energetic cost of mucus production (*12*). *Lottia gigantea* and *C. scabra* invest more energy in mucus per unit of body weight than *C. digitalis* (*13*). Mucus production accounts for approximately 23 percent of the energy budget of an individual *C. scabra*, compared to 20 percent for *C. digitalis* (*13*). This extra investment may be tolerated by *L. gigantea* and *C. scabra* because it brings greater returns by its growth-stimulating action. In order to receive these benefits, however, individuals must be separated and must remain in the same area to allow retracing and ingestion of previously laid trails. *Lottia gigantea* and *C. scabra* meet both conditions. Individual *L. gigantea* defend territories and return at low tide to home scars excavated in the rock (*3*). *Collisella scabra* maintain home scars, move an average of only 12 to 15 cm in a feeding cycle (*13, 14*), and retrace their own trails (*5*). Thus, members of both species may effectively, and often exclusively, benefit from any increase in food caused by their mucus. Production of a nutrient-rich provendering mucus may allow these limpets to restrict their foraging distance to ensure return to their home scars.

In contrast, production of a provendering mucus by *C. digitalis* might not be advantageous because these limpets commonly follow the trails of conspecifics and form large intraspecific aggregations. Aggregations presumably prevent a limpet from exclusively receiving the nutritional benefits derived from nutrient-rich mucus. A "cheater" could produce a low-cost mucus while benefiting from the high-cost mucus produced by others (*12*). *Collisella digitalis* individuals have larger home ranges and are more migratory than the other two limpet species, and thus would not be as effective at retracing their own mucus trails.

These experiments show that pedal mucus, previously thought to be associated primarily with adhesive locomotion, may also play an important role in the feeding biology of herbivorous limpets. The mucus of all species examined acts as an adhesive that traps food species. In addition, a territorial species and a homing species produce mucus that stimulates algal growth; these limpets may be viewed as farming algae for their exclusive use. With the large energetic cost of mucus production in gastropods, it is perhaps not surprising that these secretions serve multiple functions and that some may be specifically tailored to the animal's social and feeding ecology.

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References and Notes

1. M. Denny, *Nature (London)* **285**, 160 (1980); J.-F. Grenon and G. Walker, *Comp. Biochem. Physiol. B* **66**, 451 (1980).
2. P. Calow, *Oecologia* **16**, 149 (1974); M. Denny, *Science* **208**, 1288 (1980).
3. J. Stimson, *Ecology* **51**, 113 (1970); S. B. Haven, *ibid.* **54**, 143 (1973).
4. Many limpet species form snug depressions on the substrate and return to them after each foraging bout. When on this home scar, they are more resistant to desiccation and are more protected from predation, sand scouring, and dislodgment by waves.
5. W. G. Hewatt, *Am. Midl. Nat.* **24**, 205 (1940).
6. P. A. Breen, *Veliger* **15** (No. 12), 133 (1973).
7. Millipore filters were used as the substrate to minimize error during chlorophyll extraction and determination. The use of any other substrate would require microalgal collection by scrubbing the substrate and then filtering the resulting suspension.
8. J. D. H. Strickland and A. Parsons, *Bull. Fish. Res. Board Can.* **167** (1968).
9. The microalgae used in the laboratory experiments were primarily a mixture of diatoms (*Fragilaria, Navicula, Coscinodiscus, Nitzschia, Achnanthes, Skelatonema, Synedra*, and *Melosira*) and two unidentified species of unicellular green algae collected directly from the substrate of the same field sites where the *Collisella* species were collected. Diatoms are the primary food resource of both *Collisella* species, and all the diatom species included in the culture are consumed by the limpets. *Lottia gigantea* ingests diatoms, but also consumes significant amounts of blue-green or encrusting red algae; diet appears to vary with geographic location.
10. Interspecific comparisons of the effect of pedal mucus on microalgal adhesion and growth stimulation are based on an analysis of variance and mean separation (Student-Newman-Keuls multiple range test) performed on logarithmically transformed data.
11. Microalgal biomass was higher after 1 day of exposure than after 7 days. This is because the 1-day experiment was conducted during a phytoplankton bloom and the 7-day experiment was not. Limpets can utilize phytoplankton as an additional food resource.
12. P. Calow, *Am. Nat.* **114**, 149 (1979).
13. V. Connor, thesis, University of California, Davis (1983).
14. R. A. Wells, *J. Exp. Mar. Biol. Ecol.* **48**, 151 (1980).
15. We thank R. Jensen and J. Allen for collecting *L. gigantea* and K. Rice, D. Phillips, J. Crowe, B. Johnson, and two anonymous reviewers for commenting on the manuscript. Supported by research grants from Sigma Xi and the University of California, Davis.
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5 December 1983; accepted 8 June 1984

O-Acetylation of Disialoganglioside GD_3 by Human Melanoma Cells Creates a Unique Antigenic Determinant

Abstract. *Monoclonal antibody Mab D1.1 recognizes on human melanoma cells a ganglioside antigen characterized by an alkali-labile* O*-acetylated sialic acid residue. Immunochemical analysis showed that this molecule is an* O*-acetylated product of the neuroectoderm-associated disialoganglioside* GD_3. *Controlled chemical* O*-acetylation of purified* GD_3 *resulted in the generation of this same epitope. Lysates of human melanoma cells were found to contain* O*-acetyltransferase activity capable of generating the antigenic epitope recognized by Mab D1.1. Thus, the addition of a single* O*-acetyl group to a common cell surface–associated ganglioside can create a potentially tumor-specific antigen.*

Gangliosides (sialic acid–bearing glycolipids) on the surface of normal and transformed eukaryotic cells have received attention in recent years because of their putative role in cell surface recognition phenomena (*1*). Monoclonal antibodies have been of great value in defining these carbohydrate structures and in investigating their role as tumor cell markers. Monoclonal antibodies that specifically recognize gangliosides associated with melanoma (*2, 3*), neuroblastoma (*4*), and colon carcinoma (*5*) have been reported. Levine *et al.* (*6*) described the monoclonal antibody Mab D1.1, which recognized a fetal rat neuroectoderm–associated antigen present on a ganglioside. We used Mab D1.1 to screen various human adult, fetal, and tumor tissues and showed that its reactivity in human tissues was restricted to melanomas (*7*).

We now report that the antigen specifically recognized by Mab D1.1 is the alkali-labile *O*-acetylated product of the neuroectoderm-associated disialoganglioside GD_3 (*8*). The first indication that Mab D1.1 recognized an *O*-acetylated ganglioside came from our original studies, indicating that alkali treatment of this melanoma-derived ganglioside caused a decrease in its migration on thin-layer chromatography (TLC) (*7*). To extend these observations, we isolated small amounts of the ganglioside and found that it migrated on TLC as a doublet between the monosialylated ganglioside standards GM_1 and GM_2. When

the purified ganglioside was subjected to increasing periods of alkali treatment, it became—first partially and then completely—converted to a new doublet migrating in the position of the disialoganglioside GD_3 (data not shown). This suggested that this still undefined ganglioside may be structurally related to GD_3. The occurrence of a single downward shift in migration on TLC implied that a single base-labile *O*-acyl group was removed.

The conventional approach to prove the structure of the ganglioside would be to study it by compositional analysis, methylation analysis, and direct-probe mass spectrometry. However, because this ganglioside constitutes a relatively minor (5 percent) and unstable component of total melanoma gangliosides, we used biochemical and immunochemical techniques to confirm its structure. Specifically, to determine the antigenic characteristics of the de-*O*-acetylated GD_3, we used—in addition to Mab D1.1—two monoclonal antibodies, Mab R24 (*3*) and Mab MB3.6 (*9*), prepared against human melanoma cells; these two monoclonal antibodies specifically recognize only native GD_3 and do not cross-react with any other gangliosides. Base-induced de-*O*-acetylation of total gangliosides purified from melanoma cells completely destroyed reactivity with Mab D1.1 (Fig. 1A). In contrast, the same treatment caused a reciprocal increase in the reactivity of Mab R24.

To gain direct evidence that Mab D1.1 recognizes an acetylated form of GD_3, both total melanoma gangliosides and the purified ganglioside recognized by Mab D1.1 were analyzed in parallel on TLC, with or without prior base treatment, and directly immunostained with monoclonal antibodies (Fig. 1B). Base treatment of the purified ganglioside recognized by Mab D1.1 had three effects: (i) it caused loss of reactivity with Mab D1.1; (ii) it brought about a decrease in mobility on TLC resulting in migration with purified GD_3; and (iii) the base-treated ganglioside reacted exclusively with Mab R24 and Mab MB3.6, which are specific for nonacetylated GD_3.

We had observed earlier that one of two labeled sialic acid residues of this ganglioside chromatographed with 9-*O*-acetyl-*N*-acetyl neuraminic acid (*7*). To determine whether the *O*-acetyl group was located on the 9-position of the terminal sialic acid residue, we performed periodate oxidation on gangliosides isolated from melanoma cells. The conditions used for periodate oxidation were chosen so that oxidation would occur only between the exocyclic 7-, 8-, and 9-hydroxyl positions of terminal, unsubstituted sialic acid residues (*10*). In this regard, it was earlier shown that an acetyl group in the 9-position sterically hinders this oxidation, whereas substitutions on the 7- or 4-positions do not (*11*). Substitution at the 8-position is extremely rare because of rapid migration to the 9-position (*12*). Figure 1C shows that

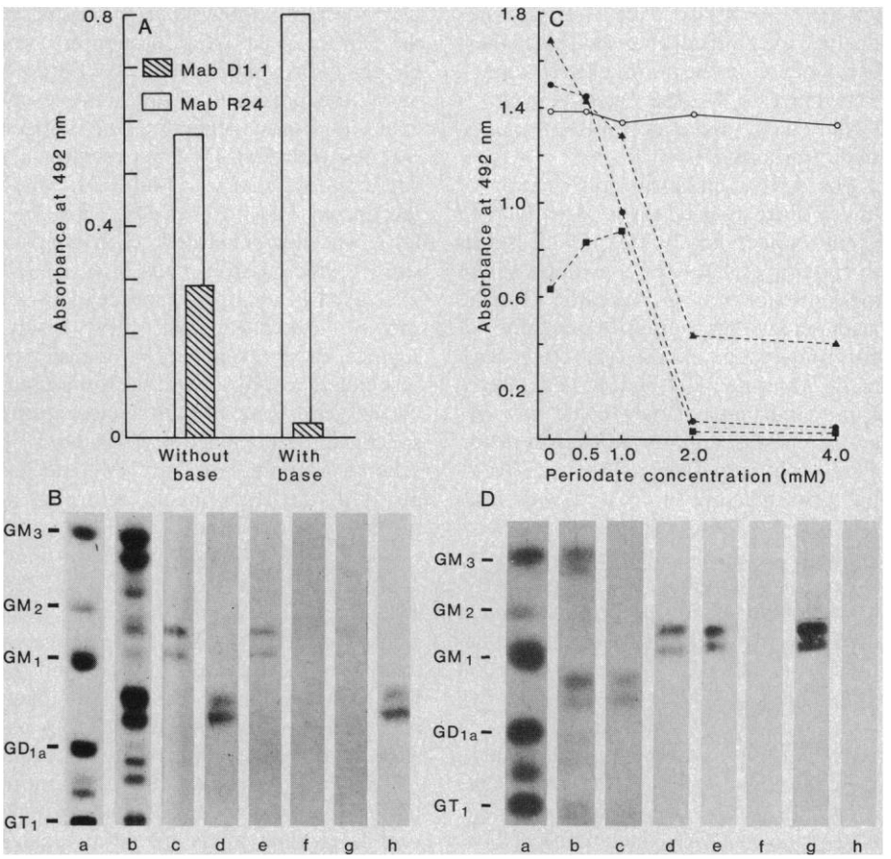

Fig. 1. (A) Reactivity of monoclonal antibodies on total melanoma gangliosides. Gangliosides isolated from M14 melanoma cells and used as target antigens (*15*) in a solid-phase enzyme-linked immunosorbent assay (ELISA) (*7, 15*) were reacted with Mab D1.1 or Mab R24 with or without prior alkali-induced de-*O*-acetylation. (B) Immunoreactivity of Mab D1.1 and Mab R24 with total melanoma gangliosides and purified D1.1 ganglioside. Melanoma gangliosides separated by TLC were directly visualized by resorcinol spray or ELISA immunostaining as described (*7*). (Lane a) Ganglioside standards visualized with resorcinol; (lane b) total gangliosides isolated from M14 melanoma cells and visualized with resorcinol; (lanes c and d) immunostaining pattern of total M14 gangliosides; (lanes e and f) immunostaining pattern of the purified ganglioside recognized by Mab D1.1; (lanes g and h) immunostaining pattern of this same ganglioside after alkali-induced de-*O*-acetylation (*7*). Lanes c, e, and g were immunostained with Mab D1.1. Lanes d, f, and h were immunostained with Mab MB3.6. (C) Effect of periodate oxidation on ganglioside antigenicity. Melanoma gangliosides were dried onto microtiter plates as described above. Sodium *m*-periodate was added to phosphate-buffered saline (PBS) containing 1 percent bovine serum albumin (BSA), *p*H 7.2, to attain a final concentration of 0 to 4 m*M*. This solution (50 μl) was added to microtiter wells and incubated at 4°C for 45 minutes. The plates were washed three times (150 μl per well) with cold PBS containing 1 percent BSA, and the lipid ELISA's were carried out with the primary monoclonal antibodies Mab R24 (●), Mab MB3.6 (■), Mab 126 (▲), or Mab D1.1 (○), as described above. (D) Immunoreactivity of Mab D1.1 with chemically acetylated GD_3. Gangliosides separated by TLC were visualized with resorcinol or ELISA immunostaining on TLC plates. (Lane a) Ganglioside standards; (lane b) total gangliosides isolated from M14 melanoma cells; (lane c) purified melanoma GD_3 visualized with resorcinol; (lane d) total melanoma gangliosides immunostained with Mab D1.1; (lanes e to h) purified GD_3 chemically *O*-acetylated and immunostained with Mab D1.1. For chemically induced acetylation, base-treated purified GD_3 in 20-μg portions was thoroughly dried and then dissolved in dry pyridine at 50°C for 30 minutes. *N*-Acetylimidazole was added to obtain up to fivefold molar excess, and the mixture was heated at 50°C for 16 hours and taken to dryness. Portions (5 μg) of each were spotted on TLC, with or without intermediate alkali treatment, developed and immunostained as in Fig. 1B. Lanes e and f and lanes g and h represent GD_3 acetylated with a 2.5- and a 5.0-fold molar excess of *N*-acetylimidazole, respectively. The materials shown in lanes f and h were treated with base, whereas those shown in lanes e and g were not. Treatment with pyridine alone had no effect (data not shown).

controlled periodate oxidation of total melanoma gangliosides completely abolished their antigenic reactivity with Mab R24 and Mab MB3.6, which specifically recognize nonacetylated GD_3, as well as reactivity with Mab 126, which reacts specifically with GD_2 (*9*). In contrast, antigenic reactivity with Mab D1.1 remained unchanged even at the highest periodate concentration used (4 m*M*). This suggests that the *O*-acetyl group is located at the 9-position of the terminal sialic acid of GD_3.

For further chemical confirmation of this finding, we used the mild acetylating agent *N*-acetylimidazole, which selectively *O*-acetylates the 9-position of the methyl ester–methyl glycoside of sialic acid under appropriate conditions of controlled molar excess (*13*). Such treatment of purified GD_3 indeed generated a compound that not only could now migrate in the appropriate position on TLC—that is, between GM_1 and GM_2—but also specifically reacted with Mab D1.1 (Fig. 1D). As expected, base treatment of chemically acetylated GD_3 resulted in the complete loss of its reactivity with Mab D1.1. A control experiment involving the identical chemical acetylation of gangliosides GM_1 and GT_{1b} did not yield a product that was reactive with Mab D1.1 (data not shown).

To determine whether human melanoma cells contain an acetyltransferase responsible for the acetylation of a sialic acid residue of GD_3, melanoma cell-derived gangliosides were de-*O*-acetylated, incubated with lysates of melanoma cells, and assayed for synthesis of the antigen specifically recognized by Mab D1.1. Reactivity with this antibody was generated in this manner—as a function of protein concentration (Fig. 2A) and of time (data not shown). Prior heating of the extract at 80°C for 20 minutes completely abolished this reactivity. Furthermore, the presence of the putative enzyme not only generated the antigen reactive with Mab D1.1 but reciprocally decreased reactivity of Mab R24, which recognizes GD_3. Reactivity with Mab D1.1 was not generated when gangliosides extracted from cells lacking GD_3 were used as a substrate source (data not shown). Taken together, these results suggest the presence of a melanoma-associated acetyltransferase that is capable of generating the antigenic epitope specifically recognized by Mab D1.1. A scheme outlining the probable mechanisms of the experiments peformed in this study is shown in Fig. 2B.

The results of our experiments indicate that the ganglioside recognized by Mab D1.1 differs from GD_3 only by a single *O*-acetyl ester at the 9-position of the terminal sialic acid residue. Although 9-*O*-acetylated sialic acids have been previously reported in other human tissues such as the colon (*14*) and brain (*12*), Mab D1.1 does not react with these tissues (*7*), presumably because these *O*-acetylated sialic acids are on molecules other than GD_3. Thus, the generation of the epitope recognized by Mab D1.1 requires two independent factors: first, the synthesis of the disialoganglioside GD_3, and second, the presence of an *O*-acetyltransferase capable of acetylating the 9-hydroxyl position of the terminal sialic acid residue. Although each of these factors may be individually present in other normal tissues and tumors, it is the combination of the two in human melanoma cells that results in the generation of an apparently tumor-specific antigen.

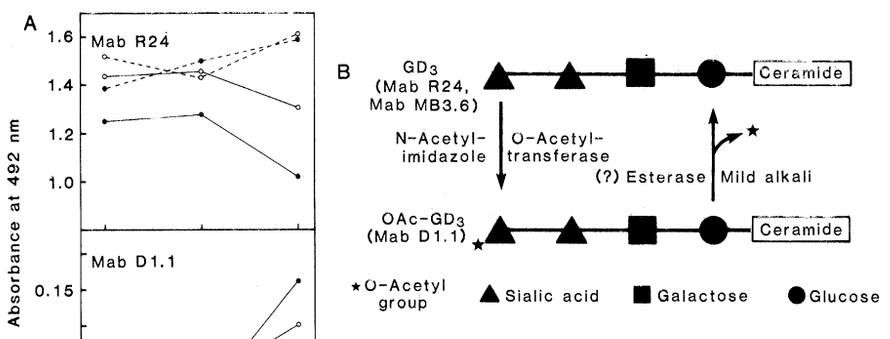

Fig. 2. (A) Demonstration of *O*-acetyltransferase activity in human melanoma cells. Melur melanoma cells were suspended at 5×10^7 cells per milliliter in 0.1*M* phosphate buffer, *p*H 7.0, containing 1 m*M* EDTA and 1 m*M* mercaptoethanol (*12*). This cell suspension was sonicated in the cold for 10 seconds (●) or 60 seconds (○) [probe sonicator (Branson) used at 40 W]. The cell lysates were centrifuged at 100,000*g* at 4°C for 1 hour and the supernatant was used as the enzyme source (——). Controls were cell lysates previously heated to 80°C for 20 minutes (- - - -). Melanoma gangliosides were attached to microtiter wells (*15*), treated with base as described (*7*), and used as the substrate. Enzyme (25 μl) (or a buffer blank) was added to appropriate wells and incubated for 90 minutes at 37°C, after which the wells were washed three times with PBS containing 1 percent BSA, *p*H 7.2 (150 μl per well). A lipid ELISA was then performed with Mab D1.1 or Mab R24 as described above. (B) Scheme for the postulated relation between GD_3 and *O*-acetylated GD_3. The effects of *N*-acetylimidazole and mild alkali treatment are indicated. The *O*-acetyltransferase and esterase depicted in this scheme are postulated to catalyze reactions that occur in vivo.

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References and Notes

1. C. C. Blackburn and R. L. Schnaar, *J. Biol. Chem.* **258**, 1180 (1983); W. E. Van Heyningen, *Nature (London)* **249**, 415 (1974); R. M. Perkins, S. Kellic, B. Patel, D. R. Critchley, *Exp. Cell Res.* **141**, 231 (1982).
2. E. Nudelman *et al.*, *J. Biol. Chem.* **257**, 12752 (1982).
3. C. S. Pukel, K. O. Lloyd, R. Travassos, W. G. Dippold, H. F. Oettgen, L. J. Old, *J. Exp. Med.* **155**, 1133 (1982).
4. G. S. Eisenbarth, F. S. Walsh, M. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4913 (1979).
5. H. Koprowski, Z. Steplewski, K. Mitchell, M. Herlyn, P. Fuhrer, *Somat. Cell Genet.* **5**, 957 (1979).
6. J. M. Levine, L. Beasley, W. B. Stallcup, *J. Neurosci.* **4**, 820 (1984).
7. D. A. Cheresh, A. P. Varki, N. M. Varki, W. B. Stallcup, J. Levine, R. A. Reisfeld, *J. Biol. Chem.* **259**, 7453 (1984).
8. Ganglioside nomenclature as described by L. Svennerholm [*J. Neurochem.* **10**, 613 (1963)].
9. D. A. Cheresh, J. R. Harper, G. Schulz, R. A. Reisfeld, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
10. L. Van Lenten and G. Ashwell, *J. Biol. Chem.* **246**, 1889 (1971); R. L. McLean, M. Suttajit, J. Beidler, R. J. Winzler, *ibid.*, p. 803.
11. A. Varki and S. Kornfeld, *J. Exp. Med.* **152**, 532 (1980); A. K. Shukla and R. Schauer, *Hoppe-Selyer's Z. Physiol. Chem.* **363**, 255 (1982).
12. R. Schauer, Ed., *Sialic Acids: Chemistry, Metabolism, and Function* (Springer-Verlag, New York, 1982).
13. J. Haverkamp, R. Schauer, M. Wember, J. P. Kamerling, J. F. G. Vliegenthart, *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 1575 (1975).
14. P. E. Reid, W-C Tsang, C. W. Ramey, M. G. Clay, *Can. J. Biochem.* **53**, 388 (1975); P. E. Reid and W. L. Dunn, *J. Histochem. Cytochem.* **21**, 473 (1973); C. F. A. Culling and W. L. Dunn, *ibid.* **24**, 1225 (1976).
15. W. W. Young, E. M. S. MacDonald, R. C. Nowinski, S. Hakomori, *J. Exp. Med.* **150**, 1008 (1979).

16. We thank W. B. Stallcup (Salk Institute, La Jolla, Calif.) for the Mab D1.1, S. I. Hakomori (Fred Hutchinson Cancer Research Center, Seattle, Wash.) for the purified GD_3, and K. O. Lloyd (Sloan-Kettering Cancer Research Center, New York, N.Y.) for the Mab R24. We thank B. P. Filiault for manuscript preparation and B. Durango for excellent technical assistance. Supported by NIH grants CA 28420, GM32373, and CA 07544, by NIH fellowship award CA 07544 (to D.A.C.) and by a Hartford Foundation fellowship (A.P.V.). This is publication number 3345-Imm from the Scripps Clinic and Research Foundation, La Jolla, Calif. 92037.

2 February 1984; accepted 25 June 1984