An Epitope Common to Gangliosides O-Acetyl-GD$_3$ and GD$_3$ Recognized by Antibodies in Melanoma Patients after Active Specific Immunotherapy

Mepur H. Ravindranath, Donald L. Morton, and Reiko F. Irie

Division of Surgical Oncology, John Wayne Cancer Clinic, and Armand Hammer Research Laboratories, Louis Factor Bldg 9-267, University of California School of Medicine, Los Angeles, California 90024

ABSTRACT

GD$_3$ is a major ganglioside of human melanoma and was shown to be an effective target for passive immunotherapy with murine monoclonal antibodies that were noted earlier that GD$_3$, neither purified nor in melanoma cell vaccine (MCV), could elicit an antibody response in melanoma patients. In this study, we demonstrate that melanomas patients who received MCV had autoantibodies against a derivative of GD$_3$, O-acetylated GD$_3$ (O-AcGD$_3$), a minor ganglioside expressed on human melanoma cells, and that the antibodies cross-reacted with GD$_3$. Thin layer chromatographic immunostaining revealed that all of the sera containing antibodies against O-AcGD$_3$ also reacted to GD$_3$. None of the other sera responded only to GD$_3$, although the MCV contained 7- to 12-fold higher GD$_3$ than O-AcGD$_3$. Furthermore, the antibody activity was completely abolished by absorption with animal erythrocytes expressing either O-acetyl disialogangliosides or GD$_3$, indicating that the antibodies recognize an epitope commonly shared by GD$_3$ and O-AcGD$_3$. The antibodies bound only to the sialyloligosaccharide moiety but not to the ceramide portion of O-AcGD$_3$ after endoglycosidase treatment. The antibodies failed to bind to GD$_3$ after neuraminidase treatment. These results indicate that the sialyloligosaccharides of the gangliosides are important components of the epitope. Periodate oxidation abolished reactivity of the antibodies to GD$_3$ but not to O-AcGD$_3$, revealing that the glycerol side chain of the sialic acids in both GD$_3$ and O-AcGD$_3$ was an important structure of the epitope. The binding of the antibodies to melanoma cell surface gangliosides was confirmed by an assay with a GD$_3$- and O-AcGD$_3$-positive melanoma cell line. These results in the light of previous reports on the inability of GD$_3$ to elicit immune response in humans suggest that anti-GD$_3$ antibodies found in the melanoma patients were induced by immunization with O-AcGD$_3$ and O-AcGD$_3$ present in the MCV would serve as an antigen source for GD$_3$-targeted active specific immunotherapy of melanoma.

INTRODUCTION

Gangliosides are recognized as potential target antigens in immunotherapy of melanoma (1). These glycolipids contain a hydrophobic ceramide portion embedded in the cell membrane and a hydrophilic sialyloligosaccharide moiety exposed (1). Only a small but sequential group of gangliosides is expressed on the melanoma cell surface (1). Normal melanocytes, precursors of melanoma, predominantly contain GM$_3$ (>95%), a hydrophobie ceramide portion embedded in the cell membrane and a hydrophilic sialyloligosaccharide moiety exposed (1).

Since GD$_3$ is prominently expressed on melanoma cell surface, it was used as a target for passive immunotherapy of melanoma using GD$_3$-binding murine monoclonal antibodies (7, 8). Melanoma patients were also immunized with purified GD$_3$ to induce active immunity in patients, but no detectable anti-GD$_3$ antibodies were observed (9). Early attempts at immunization trials utilizing a MCV containing all of the melanoma-associated gangliosides, elicited antibody response against GD$_3$ and GM$_2$ but not against GD$_3$ (10), indicating that GD$_3$ may not be immunogenic in humans. However, autoantibodies against O-AcGD$_3$, an O-acetylated derivative of GD$_3$ (see structure in Fig. 1), was noted in the sera of immunized melanoma patients (5); a unique lectin that specifically binds to O-acetylsialic acids on gangliosides blocked the binding of the antibodies from the sera of the patients to a melanoma cell line (UCLASO-M25) that expresses prominently O-AcGD$_3$ (5). In addition, sera of some of the MCV recipients contained antibodies reacting to purified O-AcGD$_3$ in an ELISA.

While demonstrating the direct binding of such antibodies to O-AcGD$_3$ on a TLC, we observed that those sera containing antibodies reacting to O-AcGD$_3$ also cross-reacted with GD$_3$. O-AcGD$_3$ is only a minor fraction of melanoma (rarely exceeding 10% of total gangliosides), whereas GD$_3$ ranges between 20 and 80% of the total gangliosides of melanoma (2-4). Therefore, GD$_3$ would be an excellent target for the active specific immunotherapy of melanoma, if the sources of the antigen responsible for the induction of GD$_3$ antibodies in patients can be identified. Here we report the antibody response against GD$_3$ and have assessed the possibility of the cross-reactivity of anti-O-AcGD$_3$ antibodies with GD$_3$. This study postulates that O-acetylation may render GD$_3$ immunogenic in melanoma patients.

MATERIALS AND METHODS

Antibody Source. The sera of melanoma patients collected before and after immunization were used as the source of antibody. Sera were stored at -80°C and then thawed and centrifuged before being used at 10$^4$ x g at 4°C. We selected 20 of the melanoma patients based on the positivity of their sera to UCLA melanoma cell line M25 in IA (11) and membrane immunofluorescence (12) assays. Fourteen of these patients received MCV consisting of three different melanoma cell lines, after surgery. In addition we have used the sera of three patients that were negative to IA against M25 as a control.

Two kinds of MCVs were given to the patients intradermally (10) or intralymphatically (13) during the course of the clinical trials. Prior to use, the MCV was irradiated with 10$^4$ rad in a 2 s-a-dose/time schedule that maintains cell viability but prevents cell proliferation. For intralymphatic immunization a total of 25 x 10$^6$ cells in 5 ml of lactated Ringer’s solution were distributed equally at one to three sites; generally two sites were used for each immunization. The dorsal aspect of either hands or the feet was the site of lymphatic cannulation. For intralymphatic immunization a total of 75 x 10$^6$ cells were administered at multiple sites on the lower abdomen and lateral chest. Table 1 shows the UCLA melanoma cell lines used in the vaccine and the relative ratio of the gangliosides in the vaccine.

Ganglioside: Source, Purification, and Analysis. The gangliosides of...
M25 melanoma cell line were isolated and purified following the procedure of Ladisch and Gillard (14) and modified by us (5). The gangliosides purified by gel filtration were subjected to TLC. TLC was performed with chloroform:methanol:0.25% CaCl₂ in water (60:30:9, v/v/v). The glycolipids were detected with orcinol-ferric chloride and resorcinol-HCl. The other gangliosides used in this study included bovine brain GM1 (supplied by Dr. Pallman, K. G. 800 Munich 40, West Germany) and bovine brain GD1α (Sigma). The gangliosides are identified by the nomenclature of Svennerholm (15).

Enzymatic Alteration of Gangliosides. The oligosaccharide moiety of G0d1 was enzymatically cleaved from the ceramide portion using a novel enzyme endoglycosylceramidase (GeneZyme, Boston, MA), purified from *Rhodococcus* (16). Fifty µg of G0d1 and 0.5 milliunit of endoglycosylceramidase were incubated in 50 mM sodium acetate buffer (pH 6.0) containing 0.5 mg/ml of sodium taurodeoxycholate (total volume, 50 µl) for 72 h at 37°C. After the incubation, the hydrolysate was diluted with 9 volumes of methanol, evaporated to dryness over N₂, and resuspended in chloroform:methanol (1:1, v/v).

The sialic acids of G0d1 were removed using *Clostridium perfringens* neuraminidase in the presence of sodium taurocholate (5). We have not used O-AcG0d1 for neuraminidase treatment because it is known that O-acetylation renders the sialic acid resistant to C. perfringens neuraminidase. Fifty µg of G0d1 and 150 units of neuraminidase were incubated in 50 mM sodium acetate buffer (pH 5.5) containing 0.5 mg/ml of sodium taurodeoxycholate (total volume, 50 µl). After incubation, the hydrolysate was diluted with 9 volumes of methanol, evaporated to dryness over N₂, and resuspended in chloroform:methanol (1:1, v/v).

Base Treatment of Gangliosides. O-Acetyl groups of O-AcG0d1 were removed by base treatment with concentrated ammonium hydroxide (1 h at 25°C) as described earlier (5). Ammonia was evaporated over N₂, and the gangliosides were washed three times with methanol and evaporated over N₂.

Periodic Acid Oxidation. To cleave the glycols in the glycerol side chain of G0d1, the gangliosides were incubated in 5 mM sodium metaperiodate in PBS for 1 h at 37°C. The periodate-oxidized gangliosides were then chromatographed and tested for antibody binding.

IA. The IA, performed as described by Irie et al. (11), is a sensitive method for detecting antibody bound to human tumor cells. In this assay target cells were coated with antibodies by incubating the cells with varying dilutions of antibodies. After 90 min of incubation at 37°C, the cells were washed and incubated with a guinea pig fresh serum (complement) (diluted 1:40) for 10 min. After complete settling of the mixture of melanoma cells and erythrocytes (20 min), erythrocyte rosetting around target cells were examined. The percentage of rosette-forming tumor cells is proportional to the antibody titer in the sera. In this study UCLASO-M25 melanoma cells were used as the target cells because of the cell surface expression of O-AcG0d1 by the cell line (5).

Enzyme-linked Immunosorbent Assay (ELISA). We performed the ELISA for anti-ganglioside antibodies to detect anti-G0d1 antibodies in the patient's sera, following a method empirically assessed in our laboratory for the monitoring of anti-ganglioside antibodies in cancer patients (19). Assay wells were coated with 4 µg of bovine brain GM3. The gangliosides were diluted to the desired concentration in absolute ethanol. Aliquots (50 µl) were pipetted into each microtiter well and evaporated to dryness in a vacuum. Peroxidase-conjugated goat anti-human IgG or IgM was used as second antibody.

A total of 23 serum specimens obtained from melanoma patients (see "Materials and Methods") were diluted at 1:400 or 1:250 and tested by the TLC immunostaining. Most of the sera reacted to a doublet migrating at a position corresponding

### RESULTS

Detection of Anti-O-AcG0d1 Antibodies in Patients' Sera. Gangliosides extracted from the M25 human melanoma cell line were used for the TLC radioimmunostaining to detect anti-O-AcG0d1 antibodies in the patients' sera. Earlier we have used an O-acetyl sialic acid-specific lectin to show that M25 human melanoma expresses a higher level of O-AcG0d1 than any other cultured melanoma cell line tested (5). Fig. 2 shows that the major gangliosides of M25 melanoma cells are GM3 and G0d1, constituting 21 and 60% of the total gangliosides, respectively, and O-AcG0d1 constitutes about 7%, whereas GM2 and GD1 are 3 and <6% of the total gangliosides, respectively. The O-AcG0d1 migrated as a doublet almost in the same position of GM2 (<2%). The O-AcG0d1 isolated from the total gangliosides, migrated to a position identical to G0d1, on TLC after base treatment, which removes O-acetyl groups from G0d1 (for figure see Ref. 5).

A total of 23 serum specimens obtained from melanoma patients (see "Materials and Methods") were diluted at 1:400 or 1:250 and tested by the TLC immunostaining.

### Table 1

<table>
<thead>
<tr>
<th>Gangliosides</th>
<th>MCV I (M14, M12, M7)</th>
<th>MCV II (M14, M20, M7)</th>
<th>% of total gangliosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM3</td>
<td>40</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>GD1</td>
<td>36</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>GM2</td>
<td>10</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>GD3</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>O-AcG0d1</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

*Identified by Rₐ, alkali lability, and comparison with the Rₐ of O-AcG0d1 purified and isolated from M25 cells.
ganglioside antibodies known to occur in some normal individuals (20). As noted earlier, all sera reacted strongly with the fractions corresponding to G_{M3}. Immune sera reacted more intensely than the preimmune sera to this fraction. All the immune sera showed distinct reactivity to O-AcG_{D1} and G_{D3} (Fig. 3). The sera of Patient D-1-VA reacted to G_{D2} and G_{T1} as well.

Demonstration of Specificity of the Antibodies for O-AcG_{D1} and G_{D3}. In order to assess the specificity of the antibodies to G_{D3} and O-AcG_{D1}, the positive sera were tested against the M25 melanoma gangliosides after absorption with erythrocytes containing these gangliosides. We selected three different kinds of erythrocytes; their major ganglioside profiles are defined in Table 2. Ox erythrocytes contained only those gangliosides with the terminal Sia{\textsubscript{2}}-3Gal sequence characteristic of G_{M3} (21), whereas the pig erythrocytes contained both G_{M3} and G_{D3} in a ratio of 1:1 (22). About 50% of the ox and pig erythrocyte gangliosides contained N-glycolylneuraminic acid as terminal sialic acid (21, 22). Neither of these erythrocytes contained O-acetylsialic acids. Rat erythrocytes, on the other hand, contained O-acetyl sialic acids in a disialoganglioside (23). Rat erythrocytes do not contain any detectable quantity of G_{D3}, and none of the erythrocytes tested contains G_{D3} (Table 2).

Fig. 4 presents the results of antisera reactivity on TLC before and after preabsorption. While the sera preabsorbed by ox erythrocytes continued to react with the doublets corresponding to G_{D3}, O-AcG_{D1}, and G_{D2}, the sera preabsorbed to pig and rat erythrocytes failed to react with both G_{D3} and O-AcG_{D1}. These results confirmed that immunostaining to areas of G_{D3} and O-acetylated gangliosides on TLC was in fact due to the antibody reactivity against these ganglioside antigens. Pig erythrocytes that contained G_{D3} but not any O-acetylgangliosides, and the rat erythrocytes that contained O-acetyldisialoganglioside but not G_{D3} abolished the reactivity of the sera to both O-AcG_{D1} and G_{D3} (Fig. 4), indicating the cross-reactivity of the antibodies reacting to both the gangliosides. The antibodies

Table 2 Major gangliosides of the erythrocytes used for absorption (based on Refs. 21 to 23)

<table>
<thead>
<tr>
<th>Gangliosides</th>
<th>Ox*</th>
<th>Pig</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>G_{M3}</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>G_{D3}</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>G_{D2}</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O-AcG_{D1}</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* All the gangliosides contain terminal sequence characteristic of G_{M3}, with acetylsialic acid and glycolyneuraminic acid in a ratio of 1:1. +, relative abundance of the gangliosides on the erythrocytes; -, their absence.

Fig. 4. Autoradiography showing immunoreactivity of antibodies with G_{D3} and O-AcG_{D1} in the serum of an immunized patient (Lane B) compared with a preimmune serum (Lane A), the immunized serum adsorbed with G_{D3}-positive (ox; Lane C1), G_{D3}-rich (pig; Lane C2), and G_{D3}-negative but O-acetyldisialoganglioside-rich (rat; Lane C3) erythrocytes. * corresponds to position of G_{M3}.

Fig. 3. Autoradiography showing immunoreactivity of antibodies with G_{D3} and O-AcG_{D1} in the sera of three melanoma patients before (Lane A) and after (Lane B) immunization with MCV. The sera were tested at a dilution of 1:250. *= corresponds to position of G_{M3}.

to G_{M3}. The immunological and epitope specificity of the G_{M3} reactivity of the antibodies is under investigation in our laboratory. However, similar antibodies have been identified previously as human erythrocyte autoantibodies (20). Eight of the 23 sera reacted to O-AcG_{D1} doublets. The same sera that reacted to O-AcG_{D3} also reacted to G_{D3}. Interestingly none of the sera was positive to G_{D3} alone.

Differences in Reactivity between Preimmune and Immune Sera. To examine whether the immunization of patients with MCV elicited antibody response against O-AcG_{D1} and G_{D3}, we compared the reactivity of the sera before and after the administration of the MCV. The MCV contained both G_{D3} (20 to 36%) and O-AcG_{D1} (3%) (Table 1). The G_{D3} content of MCV is lower than the level of G_{D3} seen in most of the melanoma biopsies and cultured cell lines. Three patients whose immunized sera showed a high reactivity on to G_{D3} and O-AcG_{D1} on TLC were examined. Both pre- and postimmunized sera were tested at a dilution of 1:250 and the results are presented in Fig. 3. None of the preimmune sera reacted to O-AcG_{D3} or G_{D3}, in contrast to the positive reactivity in the sera obtained from immunized sera. Therefore, the G_{D3}/O-AcG_{D1} positivity might not be due to cross-reactivity of human erythrocyte ant antigens.

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</tr>
<tr>
<td>G_{D3}</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>G_{D2}</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>O-AcG_{D1}</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
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reacting to \( G_D \), were unaffected by erythrocyte absorption treatments, suggesting that the \( G_D \)-reacting antibodies are distinct from the anti-\( G_D \) antibodies. Erythrocyte-absorbed sera showed a decrease in reactivity to the doublet corresponding to \( G_M \), which requires further investigation with regard to antibody specificity.

Epitope Analysis of \( G_D \) and \( O-AcG_D \)-Reactive Antibodies. Gangliosides consist of hydrophilic (carbohydrate portion) and hydrophobic (ceramide, including the sphingosine and fatty acid portions) regions (Fig. 1). The ceramide portion is embedded into the bilayered lipid membrane of the cell, whereas the carbohydrate portion is exposed outside the membrane. Antibodies binding to the ceramide portion have no immunotherapeutic potential, inasmuch as the ceramide portion is not exposed on tumor cells. To exclude the possibility that the antibody cross-reactivity between \( G_D \) and \( O-AcG_D \) is due to a common antigen on the ceramide portion of both gangliosides, the oligosaccharide moiety and ceramide of \( G_D \) were tested independently by cleaving the linkage between them with a novel enzyme called endoglycosylceramidase (16). On TLC plates, the ceramide (stained brown in orcinol-FeCl\(_3\)) migrated faster than \( G_D \) and the oligosaccharide moiety (\( \text{NeuAc}-2-8\text{NeuAc}-2-3\text{Gal}-1-4\text{Glc}^- \) (stained purple in orcinol-FeCl\(_3\)), remained just above the origin in the solvent system used. The antibodies reacting to \( G_D \) bound only to the oligosaccharide portion of the gangliosides but not to the ceramide portion, suggesting that the epitope of the antibodies is located on the oligosaccharide moiety of \( G_D \). The antibody binding may require all the sugar residues in the carbohydrate portion or only the terminal of the oligosaccharide. We tested the reactivity of the antibodies by cleaving the disialyl residues of the terminal portion of the oligosaccharide with \( C. \ perfringens \) neuraminidase (Sigma type X). The antibodies did not bind to the asialo-\( G_D \), indicating the specific affinity of the antibodies to the disialic acid residues characteristic of \( G_D \) and \( O-AcG_D \).

We have further characterized the epitope determinants of the antibodies in the following experiments. We have subjected M25 gangliosides to two kinds of chemical treatments, namely base treatment and periodate oxidation. We have previously shown base treatment of \( O-AcG_D \), removes the \( O \)-acetyl groups from the sialic acids by cleaving the gangliosides (5). If alkali treatment eliminates the TLC immunostaining of the \( O-AcG_D \) band, then it is evident that the antibodies are directed against the \( O-AcG_D \) structure. The results presented in Fig. 5 clearly demonstrate that alkali treatment eliminated the TLC immunostaining of the \( O-AcG_D \) band indicating that the antibodies are in fact directed against \( O-AcG_D \).

Periodate treatment gives additional proof of the antibody specificity for both \( O-AcG_D \), and \( G_D \). If the glycerol side chain of sialic acids are involved in antibody binding then cleaving the glycols in the glycerol side chain should abolish immunostaining of \( G_D \); however, periodate oxidation will not abolish the immunostaining of \( O-AcG_D \), because the substitution of \( O \)-acetyl groups at C-9 would prevent oxidation of glycols. The results obtained with periodate oxidation is more interesting in that none of the gangliosides except \( O-AcG_D \) was recognized by the antibody, demonstrating the importance of the glycerol side of the terminal sialic acid of \( G_D \), and \( O-AcG_D \) in antibody recognition.

Demonstration of the Binding of Anti-\( G_D \) or Anti-\( O-AcG_D \) Antibodies to the Cell Surface of Melanoma Cells. The binding ability of the patients' serum anti-\( G_D \) or \( O-AcG_D \) antibodies to melanoma cell surface was assessed by absorption of the antibodies with M25 melanoma cells that contained \( G_D \) and

\[ O-AcG_D \] in a ratio of 9:1. Reduction of antibody reactivity to purified \( G_D \) would be indicative of the binding of the antibodies to the cell surface antigens. Sera were tested for both IgM and IgG antibodies using ELISA. Initially we have tested the specificity of the ELISA absorption assay using erythrocytes known to express \( G_D \) or devoid of \( G_D \), on their cell surface. Sera were tested for both IgG and IgM antibodies after absorption with erythrocytes. As shown in Fig. 6, sera preabsorbed with ox erythrocytes showed an undiminished dosimetric response to \( G_D \), with the ELISA varying dilutions. The reactivity of the sera preabsorbed with pig and ox erythrocytes, respectively, were tested for both IgG and IgM antibodies after absorption with erythrocytes. This preliminary test indicates the validity of the assay for demonstration of anti-\( G_D \) antibody binding to cell surface \( G_D \). In the M25 melanoma cell absorption, all of the sera were preabsorbed initially with ox erythrocytes. This is particularly important because melanoma cell lines grown in a medium containing fetal bovine serum can express gangliosides containing \( N \)-glycolylsialic acids (24). In order to avoid cross-reactivity of the antibodies with \( N \)-glycolylsialic acid containing gangliosides on melanoma cell surface, the serum was preabsorbed to ox erythrocytes which predominantly express gangliosides with \( N \)-glycolylsialic acids (21).

As shown in Table 3, both IgM and IgG antibodies showed remarkable reduction in their reactivity to \( G_D \) after absorption to M25 melanoma cell, confirming the ability of the antibodies to bind to the tumor cells directly.

**DISCUSSION**

In this investigation we tested sera from melanoma patients who received MCV immunotherapy for the presence of anti-\( O-\)
AcG\textsubscript{D3} and anti-G\textsubscript{D3} antibodies and observed that antibodies reacting to G\textsubscript{D3} occurred only in the sera containing anti-O-AcG\textsubscript{D3} antibodies. MCV contained 7- to 12-fold higher G\textsubscript{D3} than O-AcG\textsubscript{D3}. These findings led to the inference that G\textsubscript{D3} might not have elicited an immune response. This suggestion gains support from the works of Livingston (9) who was not able to induce anti-G\textsubscript{D3} antibodies in melanoma patients using a purified G\textsubscript{D3} vaccine. The unresponsiveness against G\textsubscript{D3} does not appear to be due to the purified form of the immunogen because the same purified G\textsubscript{D3} vaccine was found to be immunogenic when used to immunize mice. The same group has also observed that purified G\textsubscript{M2} vaccine was immunogenic in melanoma patients (25). It is also highly unlikely that the immunization methodology was inappropriate, in the light of these investigators' previous success in inducing anti-G\textsubscript{M2} and anti-G\textsubscript{D3} antibodies in melanoma patients using the same administration techniques (25). Another indication that G\textsubscript{D3} might not have elicited the observed immune response is its occurrence in large quantity in normal tissues. G\textsubscript{D3} accounts for over 20% of the total gangliosides in several normal tissues as shown in Table 4. Table 4 indicates that G\textsubscript{D3} is a normal component in humans and thus humans may have acquired immunological tolerance against G\textsubscript{D3}. Finally, if G\textsubscript{D3} were immunogenic in humans, a melanoma tumor would not have escaped from complement-mediated antibody cytotoxicity and antibody-dependent cellular cytotoxicity (36) because G\textsubscript{D3} is so prominently expressed on the melanoma cell surface.

The evidence that the anti-G\textsubscript{D3} antibodies found in MCV-treated patients may have been induced by the antigenic stimulation of O-AcG\textsubscript{D3} contained in the MCV is: (a) antibodies were not found in the preimmune sera of immunized patients; (b) there is a paucity of evidence demonstrating the presence of O-AcG\textsubscript{D3} in normal human tissues; (c) previous reports demonstrate the nonimmunogenic nature of G\textsubscript{D3} in humans; (d) there are no anti-G\textsubscript{D3} antibodies alone in any of the immunized sera analyzed in spite of the higher amount of G\textsubscript{D3} than O-AcG\textsubscript{D3}; and (e) the anti-G\textsubscript{D3} antibody activity was abolished by absorption not only with G\textsubscript{D3} but also with O-acetylsialic acid-containing cells suggesting that the antibody detects a common epitope between G\textsubscript{D3} and O-AcG\textsubscript{D3}. Moreover a recent study by Hirabayashi et al. (24) indicates that chemical alterations in the sialic acid residues of gangliosides can render the molecule immunogenic. It was demonstrated in this study that the gangliosides substituted with the N-glycolyl group in the position of N-acetyl group elicited an antibody response against those gangliosides, which are normally nonimmunogenic (24). If such a modification can render immunogenicity to a nonimmunogenic molecule on the cell surface, then such alterations could be of immense value in the immunotherapy of cancer, particularly for those tumors that express large quantities of cell surface molecules which by themselves are not foreign to the host.

The immunostaining results of G\textsubscript{D3} degraded by endoglycosidase and neuraminidase have provided evidence that the sialic acid residues of G\textsubscript{D3} and O-AcG\textsubscript{D3} are the important components of the epitope recognized by these antibodies. Although the disialyl residues occur in G\textsubscript{D3}, the erythrocyte absorption studies reveal that the antibodies recognizing G\textsubscript{D2} are not absorbed by erythrocytes expressing G\textsubscript{D3} and hence the epitope requirement of G\textsubscript{D3} and O-AcG\textsubscript{D3} antibodies are differ-

### Table 3 ELISA immunostaining of antibodies reacting to G\textsubscript{D3} before and after absorption with M25 human melanoma cells

For this assay the wells were coated with 4 µg of bovine brain G\textsubscript{D3} diluted in absolute ethanol. Aliquots (50 µl) were added to each well and evaporated to dryness in vacuum. Peroxidase-conjugated anti-human IgG or IgM were used as second antibodies. Sera were preabsorbed to 1 ml of packed ox erythrocytes. For absorption with M25 cells, sera were initially preabsorbed with 0.1 ml of packed ox erythrocytes followed by 0.2 ml of packed melanoma cells. melanoma cells were removed from culture flasks after trypsinization. Values are expressed as means of triplicate analyses.

<table>
<thead>
<tr>
<th>Sera</th>
<th>Treatments (presorption)</th>
<th>1:800</th>
<th>1:1600</th>
<th>1:4000</th>
<th>1:800</th>
<th>1:1600</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-8-BR</td>
<td>Ox RBC</td>
<td>0.940</td>
<td>0.850</td>
<td>0.700</td>
<td>0.620</td>
<td>0.415</td>
</tr>
<tr>
<td></td>
<td>M25 cells</td>
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<td>0.230</td>
<td>0.085</td>
<td>0.080</td>
<td>0.070</td>
</tr>
<tr>
<td>B-2-HO</td>
<td>Ox RBC</td>
<td>0.700</td>
<td>0.580</td>
<td>0.420</td>
<td>0.160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M25 cells</td>
<td>0.640</td>
<td>0.640</td>
<td></td>
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<tr>
<td>D-8-SE</td>
<td>Ox RBC</td>
<td>1.160</td>
<td>1.100</td>
<td></td>
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<tr>
<td></td>
<td>M25 cells</td>
<td>0.640</td>
<td>0.640</td>
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</tbody>
</table>
ent from those which bind to \( \text{GD}_2 \). Antibodies failed to bind to \( \text{GD}_3 \) after periodate oxidation but reacted to periodate-treated \( \text{O-AcGD}_3 \), which is resistant to periodate oxidation. This finding demonstrates that the antibodies recognize both substituted (with an \( \text{O}-\text{acyl} \) group) and unsubstituted glycerol side chain of sialic acids on the \( \text{GD}_3 \)s and do not bind to the gangliosides if the side chain is destroyed by periodate oxidation.

One question that arises from this investigation is the consequence of inducing an immune response to a nonimmunogenic molecule commonly found on normal cells. This is an interesting point, especially in light of the clinical trial with a murine monoclonal anti-\( \text{GD}_3 \) antibody (R-24) which did not demonstrate direct cytotoxic effects to normal tissues containing \( \text{GD}_3 \). Why normal cells expressing \( \text{GD}_3 \) are not affected by this antibody is still unknown, but it has been hypothesized that the antigens on the nonreactive cell surface are immunologically cryptic. If this is the case, \( \text{O-AcGD}_3 \) might be an effective means for inducing cross-reactive antibody since it is found in a majority of biopsied melanomas (83%, \( n = 52 \)) (4). Since \( \text{O-AcGD}_3 \) is a minor component on melanoma cells (<5% of the total gangliosides), it may be insufficient to induce a strong immunity in melanoma patients without a deliberate immunization. However, if \( \text{O-AcGD}_3 \) can induce antibodies that react to melanoma-associated \( \text{GD}_3 \), the immunization of patients with \( \text{O-AcGD}_3 \) may be a useful approach to manipulating melanoma cell growth in vivo. In addition, the accumulation of \( \text{GD}_3 \) has been reported for other human malignancies, such as astrocytoma (37), glioma (38), meningioma (28, 29), neurofibrosarcoma (31), and acute lymphoblastic (39) and chronic lymphocytic leukemia (40). Therefore the model developed for the melanoma system may apply to other human cancers.

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REFERENCES


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