Cell Surface Accessibility of Individual Gangliosides in Malignant Melanoma Cells to Antibodies Is Influenced by the Total Ganglioside Composition of the Cells

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ABSTRACT

The reactivity of a panel of anti-ganglioside monoclonal antibodies with a number of melanoma cell lines having different ganglioside composition profiles was studied. One cell line synthesized only GM3, one produced both GM3 and GD2, 2 had GM3 and GD3 as their major gangliosides, and 2 others synthesized approximately equal amounts of GM3, GM2, GD3, and GD2 gangliosides. Antibody reactivity with viable cells was analyzed by: (a) flow cytometry on suspension cells; and (b) mixed hemagglutination assays or immune adherence assays on monolayer cells in culture. GM3 was efficiently detected only in the cell line having GM3 as its sole ganglioside. In the other cell lines, GM3 was difficult to detect even in cells in which it made up a high proportion (up to 50%) of the total ganglioside content. GM2 was easily detectable only in JB-RH melanoma cells (which contain only GM3 and GM2). GD3 was the most reactive ganglioside in 2 cell lines and GD2 in 2 other lines. In general, the most complex ganglioside present in a cell was the one most accessible to antibody. The differential exposure at the cell surface of specific gangliosides may have implications for antibody-directed tumor detection and therapy and for cell-protein or cell-cell interactions that involve glycolipids.

INTRODUCTION

Melanoma cells, as well as other cell types of neuroectodermal origin, are comparatively rich in gangliosides (1). In human melanoma tissue and cell lines, the predominant gangliosides are GM3 and GD3, although in some instances GM2 and GD2 can also be expressed in substantial amounts (2). mAbs, both mouse and human, have been used extensively in the analysis of ganglioside expression in melanoma (1, 3). Moreover, mAbs to certain gangliosides have found use in the diagnosis and therapy of tumors. Specifically, anti-GD3 mAbs have been studied for the therapy of malignant melanoma (4), and anti-GD2 has been applied to both the therapy and diagnosis of neuroblastoma (5).

Although the reactivity of anti-ganglioside mAbs with specific cells and tissue is, in general, related to ganglioside composition of the cells, there are indications that other factors also play a role in this process. For example, although GM3 ganglioside is widely distributed in a variety of cell types, antibodies to GM3 react selectively with certain melanoma cell lines (6–8). This phenomenon has been partially explained by the high density of GM3 found on melanoma cells (7). Another important parameter, which has not been as well studied, may be the influence of other components of the cell surface, i.e., glycoproteins and other glycolipids, on antibody reactivity.

In this study, we have compared the total ganglioside composition of a panel of melanoma cell lines with the ability of a number of anti-ganglioside mAbs (anti-GM3, -GM2, -GD3, and -GD2) to react with these cell lines. The results show that the reactivity of a given mAb with a cell line is dependent not only on the presence or absence of its cognate ganglioside antigen but also on the expression of other gangliosides by the target cell.

MATERIALS AND METHODS

Cell Lines. Human melanoma cell lines SK-MEL-28, -31, -37, and MeWo have been described (9). B78 is a subclone of the mouse melanoma cell line B16 (10) and was kindly provided by Dr. A. Albino, Sloan-Kettering Institute. JB-RH is also a mouse melanoma cell line, and it was derived by Dr. Jane Berkelhammer, University of Missouri, Columbia, MO, and subsequently subcloned for high GM2 expression (11). The cell lines were cultured in minimal essential medium supplemented with fetal bovine serum (10%), l-glutamine (2 mM), nonessential amino acids (1%), and penicillin-streptomycin (100 μg/ml) as described previously (9).

Antibodies. mAb M2590 [anti-GM3; IgM (6)], DH2 [anti-GM3; IgG3 (12)], and GMR6 [anti-GM3; IgM (13)] were kindly provided by Drs. T. Itoh and M. Taniguchi, by Dr. S. Hakomori, and by Dr. T. Tai, respectively. mAb 3F8 (anti-GD2; IgG3) was a gift from Dr. N-K. Cheung (5). mAb R24 (anti-GD3; IgG3) and 5-3 (anti-GM2; IgM) have been described previously (11, 14).

Flow Cytometry. For flow-cytometric experiments, confluent cell cultures were disrupted with 10 mM EDTA-PBS, trypsin (0.1%)-EDTA (0.02%), or Pronase (1 μg/ml in PBS; Calbiochem, San Diego, CA) to obtain single cell suspensions. Cells (1.0 x 10^6/ml) were incubated with saturating concentrations of antibody as follows: M2590 (20 μg/ml), DH2 (culture supernatant), GMR6 (culture supernatant), 5-3 (ascites fluid, 1:100), R24 (20 μg/ml), and 3F8 (20 μg/ml). After incubation at 0°C for 45 min, the cells were washed 3 times with PBS-1% bovine serum albumin. They were then incubated for 30 min at 4°C with anti-mouse IgM-fluorescein conjugate (Sigma Chemical Co., St. Louis, MO) for mAbs M2590, GMR6, and 5-3 and anti-mouse Ig (H- and L-chain-specific)-fluorescein conjugate (Sigma) for mAbs DH2, R24, and 3F8. After washing as described above, the cells were suspended in PBS-1% bovine serum albumin at 1.0 x 10^6 cells/ml and analyzed in an Epics Profile II fluorocytometer.

Mixed Hemagglutination and Immune Adherence Assays. MHA and IA assays for cell surface reactivities on adherent cells were carried out as described previously (9, 15). The MHA method with protein A-conjugated red blood cells was used for the IgG3 mAbs (DH2, R24, and 3F8), whereas the IA method was used for the IgM mAbs (M2590 and 5-3).

Glycolipids. Gangliosides were isolated from cells as described previously (16). Briefly, cell pellets were extracted with chloroform-methanol (2:1; 1:1, and 1:2, sequentially) and the glycolipid fraction was isolated by Florisil chromatography of the acetylated derivatives (17). Neutral glycolipids were separated from gangliosides by DEAE-Sephadex ion exchange chromatography (18). The ganglioside fraction was finally isolated by chromatography on a Sep-Pak C18 (Waters-Millipore, Milford, MA) column (19).

The sialic acid content of the ganglioside fractions was determined by hydrolysis in 0.1 N HCl at 80°C for 1 h and separation of the released...
sugars was done with high performance anion exchange chromatography using a pulsed amperometric detector (Dionex Corp., Sunnyvale, CA) according to a modification (20) of the method of Hardy et al. (21).

Fatty acid composition was determined after methanolysis in 1 N HCl in methanol at 80°C for 6 h, and analysis of the released fatty acid esters by gas chromatography-mass spectrometry using a Delsi-Nermag (CA) according to a modification (20) of the method of Hardy et al. (21).

Thin Layer Chromatography. Thin layer chromatography of the isolated gangliosides was carried out in chloroform-methanol-0.2% CaCl2 (60:40:9) on high performance silica gel G TLC plates (E. Merck), and the gangliosides were visualized with resorcinol-HCl. Plates were then scanned with a Shimadzu TLC scanner, and the percentage of individual gangliosides determined by comparison with known amounts of gangliosides. TLC immunostaining was performed using aluminum-backed silica gel G plates (E. Merck) as described (16), except that the reactivity was visualized by using rabbit anti-mouse IgG-horseradish peroxidase as the second reagent.

Radiolabeling of Cell Surface Components Containing Sialic Acid. Melanoma cells were specifically labeled in cell surface sialic acid residues using a slight modification of the method of Gahmberg and Andersson (22). A monolayer culture of melanoma (grown in a T-75 flask) was mixed with PBS and after cooling the flask to 4°C, 3.0 ml of 2 mM sodium periodate in PBS were added. After incubating at 4°C for 30 min, 3 ml of 100 mM glycerol-PBS were added. After 5 min at room temperature, the cell layer was rinsed 4 times with PBS. Sodium [3H]-borohydride (5 mg/ml, 1.0 GBq/mmol; New England Nuclear-Dupont, Boston, MA) in PBS (3 ml) was then added and allowed to react for 30 min at room temperature. Unlabeled sodium borohydride (5 mg/ml, 1.0 GBq/mmol) was then added. After 5 min, the cell layer was rinsed gently with 4 portions of PBS. The cells were then detached from the flask by scraping, and acidic glycolipids were extracted from the cell pellet as described above and analyzed by TLC and fluorography (16).

RESULTS

Ganglioside Profiles of Melanoma Cell Panel. The total ganglioside profile of 6 melanoma cell lines was determined by TLC and visualization with resorcinol-HCl reagent (Fig. 1A). The TLC plates were also scanned and the percentage of individual gangliosides determined (Table 1). The identity of the individual gangliosides was confirmed by antibody immunostaining (Fig. 1B). The cell lines were chosen for their range of ganglioside composition. Thus, mouse B78 cell line has GM3 as its only detectable ganglioside; mouse JB-RH has both GM3 and GM2 in approximately equal amounts. SK-MEL-28 and MeWo have GM3 and GD3 as their major ganglioside components (this pattern is typical of the majority of human melanoma cell lines and tumor tissue samples (1–3)). Even though GD2 could not be detected in these 2 cell lines on TLC by resorcinol-HCl reagent, small amounts of GD2 could be detected by immunostaining with anti-GD2 mAb (Fig. 1B); the level of GD2 detected was estimated to represent less than 1% of the total cell ganglioside. SK-MEL-31 and -37 are human melanoma cell lines showing more complex ganglioside patterns with GM3, GM2, GD3, and GD2 all being expressed in substantial amounts.

The total ganglioside content of the different cell lines was determined by assaying the amount of sialic acid present in the extracted ganglioside fractions. The lipid-bound sialic acid contents of the cell lines were: 0.42 µg/107 cells in B78 cells, 5.07 µg/107 cells in JB-RH; 4.20 µg/107 cells in SK-MEL-28; 2.72 µg/107 cells in MeWo; 5.00 µg/107 cells in SK-MEL-37; and 1.08 µg/107 cells in SK-MEL-31.

The fatty acid composition of the gangliosides (GM3 and GD3) isolated from SK-MEL-28 cells was also determined. The major fatty acids in GM3 were C16:0 (11.9%), C18:1 (14.1%), C18:0 (8.3%), C22:1 (62.5%), C24:1 (1.3%), and C24:0 (1.9%), and in GD3 were C16:0 (17.6%), C18:1 (7.9%), C18:0 (15.9%), C22:1 (33.7%), C24:1 (15.7%), and C24:0 (9.7%). The data on the fatty acid composition of GD3 differ from those presented by Nudelman et al. (23), who found a predominance of C24 fatty acids. The reason for this discrepancy is unclear, but it could be due to differences in culture conditions or isolation procedures.

Reactivity of Antiganglioside Antibodies with Melanoma Cells as Analyzed on Suspension Cells by Flow Cytometry. The reactivity of the panel of antiganglioside mAbs with cell suspensions of the 6 melanoma cell lines described in the previous section was analyzed by flow cytometry. Typical patterns are shown in Fig. 2, and quantitative data are given in Table 2. Several interesting aspects of the reactivity are evident. For most of the cell lines, only one mAb reacted with the majority of the cells in the culture with strong uniform staining. For SK-MEL-28 and MeWo, this was mAb R24, reacting with GD3; for SK-MEL-31, mAb 3F8 (identifying GD2) reacted maximally; and for JB-RH only mAb 5–3 (identifying GM2) reacted strongly. Only SK-ME-37 was an exception, showing quite strong immunoreactivity with both mAb R24 (anti-GD3) and 3F8 (anti-GD2). Mouse melanoma cell line B78 reacted only with anti-GM3 mAbs (mAbs M2590, GMR6, and DH2). The degree of reactivity depended on the antibody used: with mAb DH2, 58.2% of the cells were positive and the intensity of fluorescence was 7.3; with mAb M2590, 55% of the cells were positive with a mean fluorescence intensity of 5.4; and mAb

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![TLC immunostaining of gangliosides from melanoma cell lines.](image-url)

**Table 1** Ganglioside composition of panel of melanoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>GM3 (%)</th>
<th>GM2 (%)</th>
<th>GD3 (%)</th>
<th>GD2 (%)</th>
<th>Other (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B78</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>JB-RH</td>
<td>49</td>
<td>47</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>SK-MEL-28</td>
<td>51</td>
<td>0†</td>
<td>32</td>
<td>0†</td>
<td>17</td>
</tr>
<tr>
<td>MeWo</td>
<td>46</td>
<td>0†</td>
<td>36</td>
<td>0†</td>
<td>18</td>
</tr>
<tr>
<td>SK-MEL-37</td>
<td>19</td>
<td>20</td>
<td>15</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>SK-MEL-31</td>
<td>24</td>
<td>15</td>
<td>19</td>
<td>36</td>
<td>3</td>
</tr>
</tbody>
</table>

* Determined by TLC and detection of gangliosides by resorcinol-HCl spray as described in "Materials and Methods."
† Unidentified gangliosides and nonspecifically stained components.
‡ Percentage of total ganglioside (average of 2 experiments).
§ Small amounts of GD2 could be detected by TLC immunostaining with mAb 3F8.
GMR6 was more reactive with, with 90% of the cells being positive with an intensity of 10.4. It should be noted that mAb GMR6 shows some cross-reactivity with GD1α and GM1b, but since these gangliosides are not present in melanoma cells, this is not a complication of the analysis.

Anti-GM3 mAbs DH2, M2590, and GMR6 did not react significantly with any of the other cell lines tested. mAb M2590 reacted with 35% of SK-MEL-37 and GMR6 reacted with 76% of MeWo cells, but the mean intensities were only 2.4 and 2.9, respectively. The low reactivity of anti-GM3 with all the cell lines, except B78, was quite surprising, considering that the GM3 ganglioside content was quite high, ranging from about 50% of total ganglioside content for SK-MEL-28 and JB-RH to 19% for SK-MEL-37. A similar phenomenon was noted with some of the other antibodies. Thus, even though SK-MEL-31 had substantial amounts of GD3 (20% of total gangliosides), anti-GD3 mAb (R24) reacted only weakly with this cell line — 71% of the cells were positive but the average intensity was only 4.1. Likewise, SK-MEL-37 had 19% of its ganglioside content as GM2, but anti-GM2 (mAb 5–3) reacted only weakly (19.7% positive cells with a mean intensity of 6.8). As expected, anti-GD2 reacted very strongly with SK-MEL-31 and SK-MEL-37, which contain large amounts of GD2. More surprisingly, a high proportion of SK-MEL-28 and MeWo cells, which have extremely low GD2 levels, reacted with anti-GD2, although the intensity of fluorescence was rather low. From these data, it appears that the reactivity of an antiganglioside mAb with a melanoma cell is strongly influenced by the nature of the other gangliosides expressed by the cell. There was a tendency for a mAb to react most strongly with the most complex ganglioside in the cell when more than one ganglioside was present.

These data were derived from cell suspensions prepared using either an EDTA-containing or trypsin-EDTA buffer. Essentially the same results were obtained using either procedure. The effect of more vigorous protease treatment was examined by treating the cell suspensions with Pronase (using a maximum of 1 μg enzyme/ml enzyme, inasmuch as higher concentrations resulted in cell lysis). Under these conditions, the reactivity of the 2 cell lines tested (B78 and SK-MEL-28) with anti-GM3 was slightly enhanced, but reactivity with anti-GD3 was unchanged (data not shown).

Reactivity of Antiganglioside Antibodies as Analyzed on Melanoma Monolayer Cells by Mixed Hemagglutination and Immune Adherence Assays. In order to determine whether the accessibility of cell surface gangliosides to antibodies was the same on monolayer cells as with cell suspensions, the reactivity with mAbs was studied on melanoma cells growing in the wells of microtiter plates using two red blood cell rosetting assays: either the MHA or the IA. The former was used for the IgG3 antibodies using protein A-coupled erythrocytes and the latter assay was used for IgM antibodies. In general, the results (Table 3) were very similar to those obtained with flow cytometry. Thus, anti-GM3 mAbs were strongly reactive only with B78 cells; JB-RH cells reacted only with anti-GM2 and not with anti-GM3 antibodies, and SK-MEL-31 was reactive only with anti-GD2 mAb despite having substantial amounts of the other 3 gangliosides.

Accessibility of Gangliosides to Cell Surface Labeling by Periodate-[3H]Borohydride. Since GM3 was poorly recognized by antibodies in a number of cell lines, the accessibility of this ganglioside to chemical reagents was analyzed. Specifically, the radiolabeling of gangliosides by treatment with sodium periodate and sodium [3H]borohydride was analyzed in 3 cell lines (B78, JB-RH, and SK-MEL-28). This procedure has been demonstrated to specifically label cell surface components containing sialic acid, at least in erythrocytes (22). The results showed that GM3 was efficiently labeled in all 3 cell lines (Fig. 3). This was despite the fact that GM3 was poorly accessible to antibody in JB-RH and SK-MEL-28 cells. GM2 was efficiently labeled in JB-RH cells and GD3 was radiolabeled, although not strongly, in SK-MEL-28 cells. These data confirm that these gangliosides are cell surface components in the cell lines examined.

DISCUSSION

The phenomenon of the "crypticity" of some glycolipids in the plasma membrane of mammalian cells has been known for some time. The classic example is the inaccessibility of globoide, the major neutral glycolipid of adult human erythrocytes, to antigloboside antibodies (24). In this instance, treatment of the cells with trypsin or sialidase enhances globoide

<table>
<thead>
<tr>
<th>Table 2 Reactivity of antiganglioside antibodies with melanoma cells as assayed by fluorescence staining and flow cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-GM3</strong></td>
</tr>
<tr>
<td><strong>Cell line</strong></td>
</tr>
<tr>
<td>B78</td>
</tr>
<tr>
<td>JB-RH</td>
</tr>
<tr>
<td>SK-MEL-28</td>
</tr>
<tr>
<td>MeWo</td>
</tr>
<tr>
<td>SK-MEL-37</td>
</tr>
<tr>
<td>SK-MEL-31</td>
</tr>
</tbody>
</table>

<sup>a</sup> % Pos, percentage of cell showing positive fluorescence in comparison to staining with fluoresceinated anti-Ig alone; Int, mean fluorescence intensity.

<sup>b</sup> Average of 2 separate experiments.

4950
Fig. 3. Fluorogram of TLC plate showing cell surface radiolabeling of gangliosides with sodium periodate-[3H]borohydride method. Lane 1, B78; Lane 2, JB-RH; Lane 3, SK-MEL-28.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>M2590a</th>
<th>DH2b</th>
<th>Anti-GM2, 5-3a</th>
<th>Anti-GD3, R24b</th>
<th>Anti-GD2, 3F8b</th>
</tr>
</thead>
<tbody>
<tr>
<td>B78</td>
<td>64 2+</td>
<td>128 3+</td>
<td>0</td>
<td>1024 3+</td>
<td>1024 3+</td>
</tr>
<tr>
<td>JB/RH</td>
<td>0</td>
<td>0</td>
<td>128 2+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SK-MEL-28</td>
<td>0</td>
<td>4 (+)</td>
<td>0</td>
<td>128 3+</td>
<td>512 3+</td>
</tr>
<tr>
<td>MeWo</td>
<td>32 (+)</td>
<td>8 (+)</td>
<td>0</td>
<td>128 3+</td>
<td>32 (+)</td>
</tr>
<tr>
<td>SK-MEL-37</td>
<td>16 (+)</td>
<td>32 (+)</td>
<td>0</td>
<td>&gt;1024 3+</td>
<td>&gt;1024 3+</td>
</tr>
<tr>
<td>SK-MEL-31</td>
<td>0</td>
<td>2 (±)</td>
<td>4 (+)</td>
<td>2 (±)</td>
<td>1024 3+</td>
</tr>
</tbody>
</table>

a Assayed on monolayer cells with IA method.
b Assayed on monolayer cells with protein A-red blood cell MHA method.
c Last dilution showing positive cells.
d Estimate of strength of reaction: 3+, very strong; 2+, strong; +, weak; ±, very weak.

*K. O. Lloyd, unpublished observations.
Fig. 4. Summary of ganglioside content and immunoreactivity (flow cytometry) of melanoma cells. A, ganglioside composition (percent of total ganglioside in cell line). 1, GM3; 2, GM2; 3, GD3; 4, GD2. B, flow-cytometric analysis with antibodies (percent of cells positive compared to negative control). 1, DH2 (anti-GM3); 2, 5–3 (anti-GM2); 3, R24 (anti-GD3); 4, 3F8 (anti-GD2). * intensity of reactivity with antibodies is low (2–3 intensity units).

could be physically blocking the smaller by virtue of its size alone. Indeed, molecular modeling studies have indicated that the oligosaccharide moiety of GD3 is not appreciably longer than that of GM3, inasmuch as the conformation of GD3 oligosaccharide was calculated to be hook-shaped with the terminal NeuAc being folded back onto the GM3 sequence. A possible explanation of this phenomenon lies in an understanding of the nature of glycolipid-glycolipid interactions in the cell membrane and of interactions of the oligosaccharide chain with the lipid surface of the membrane. These are both very poorly understood processes. Pertinent to this topic are the recent studies of Strömberg et al. (31) who, using molecular modeling techniques, showed that the orientation of the saccharide chain of globo-series glycolipids plays an important role in determining their binding to Escherichia coli. Specifically, they suggested that the sugar chain of globotriosyl-ceramide (GbOse3) is oriented parallel to the membrane surface, whereas the sugar moieties of longer glycolipids (GbOse4 and GbOse5) have more vertical orientations. In the context of the present study, it could be suggested that the presence of longer glycolipids in a cell causes the shorter glycolipids to assume a more horizontal, and therefore less accessible, orientation at the cell surface. Biophysical studies, such as those that have been carried out on model membrane systems, may help in understanding this phenomenon (32–34). The tendency of certain glycolipids to coalesce in clusters or patches, as demonstrated in these studies, could influence their reactivity with antibodies.

A number of studies have suggested that subtle differences in ceramide structure, such as fatty acyl chain length, can influence the antigenicity of glycolipids (29). In the present study, we compared the fatty acid composition of GM3 and GD3 isolated from SK-MEL-28 melanoma cells. The overall fatty acid composition of the 2 gangliosides was found to be quite similar. Although the GD3 sample had a slightly greater proportion of C24 fatty acids, this difference does not seem to be large enough to result in greater exposure of the majority of the GD3 molecules to antibody.

Although the function of glycolipids is still poorly understood, recent work has shown that they can play important roles in cell-cell interactions by virtue of glycolipid-glycolipid (35) and glycolipid-protein (selectin) recognition (36). They also serve as important targets for the reactions of cells with antibodies. Specifically, since tumors often express altered glycolipid patterns, antibodies to glycolipids provide important reagents for the analysis, diagnosis, and therapy of tumors. Obviously, the degree of exposure of individual glycolipids at the cell surface will be an important parameter in both cell-cell and cell-antibody interactions. Specifically, the presence of a certain glycolipid in the cell membrane does not necessarily mean that the glycolipid is able to participate in these interactions. A specific example of this could be the selectively shown by anti-GM3 monoclonal antibodies for a few melanoma cell lines even though GM3 is widely distributed in many cell types (6, 8, 12). Nores et al. (7) have explained this phenomenon on the need to exceed a certain antigen density before an antibody can react effectively with a target cell. While this could be one important parameter, the data presented here indicate that the total ganglioside composition of the cells is another contributing factor. The question of accessibility to antibody could also be important in selecting antibodies for use in tumor diagnosis and therapy. Thus, when a cell type has more than one major glycolipid, then the use of an antibody to the more complex glycolipid in the target cell could be most effective. To some extent this has already happened in that anti-GD3 is the antibody of choice for studying malignant melanoma cells (4, 14), even though GM3 is found in approximately equal amounts in the melanoma cells (14, 37). Also anti-GD2 is widely used in studies on neuroblastoma (5, 38), even though GM2 is an equally prominent ganglioside in this cell type (39). Nevertheless, attention to the question of antigen accessibility could help in the choice of antibodies or combination of antibodies for use in tumor diagnosis and therapy in the future.

Acknowledgments

We thank Marvin Olsen (Sloan-Kettering Institute) for assistance with the mass spectrometric analysis of fatty acids and Ken Class for the flow cytometric data.

References

ACCESSIBILITY OF GANGLIOSIDES IN TUMOR CELLS


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