Preparation of Monoclonal Antibodies against
a Glycolipid Asialo GM1

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Abstract Five IgM monoclonal antibodies (MAbs), MW-1, MW-2, MW-3, MW-4, and MW-5, against a glycolipid asialo GM1 were prepared from hybridoma clones obtained by the fusion of mouse NS-1 myeloma cells with spleen cells from a mouse immunized with asialo GM1 adsorbed to naked Salmonella. All the MAbs reacted only with asialo GM1 when their reactivities were examined by enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatography (TLC)-immuno-staining using structurally related glycolipids. The MAbs showed a complement-dependent lysis of mouse natural killer (NK) cells, but the lytic activities were weaker than that of a rabbit polyclonal anti-asialo GM1 antibody. When they were mixed, the anti-NK activity was increased to a level almost comparable to that of the polyclonal antibody. These results suggest that all the MAbs obtained are specific for asialo GM1 and that they may be different in fine specificity for the glycolipid. Significance of the MAbs in immunological and neurochemical studies is discussed.

Asialo GM1, gangliotetraosylceramide, has been characterized as a specific surface marker or a differentiation-related antigen of NK cells (7, 14, 20), macrophages (1, 12), and thymocytes (4, 12) in rats and mice. These were revealed mainly by immunological methods with a conventional antibody against asialo GM1 raised in rabbits (13). The antibody was polyclonal and had the possibility to contain a variety of specificity, and a production of MAbs specific for asialo GM1 has long been desired.

However, in spite of the wide propagation of MAb production technique, attempts made by many investigators to obtain MAbs specific for asialo GM1 have ended in failure because of a low immunogenicity of the glycolipid in rats and mice. Therefore, there has been no report as to anti-asialo GM1 MAbs.

In the present paper, we report a successful production of five MAbs specific for asialo GM1.

MATERIALS AND METHODS

Glycolipids. Glycolipids used are listed in Table 1. Asialo GM1, GM1, GD1a, GD1b, asialo GM2, GM2, GM3, CDH, and CMH were prepared from
bovine brain as described by Arita et al (2). Human erythrocyte CTH, globoside, and paragloboside were purchased from Dia-Iatron Company, Ltd. (Tokyo). The purity of these glycolipids was confirmed by TLC (2).

Animals. Female New Zealand white (NZW) mice and female CD-1 nude (nu/nu) mice were purchased from Clea Japan, Inc. (Tokyo) and Charles River Japan, Inc. (Atsugi, Kanagawa, Japan), respectively. Female mice of other strains used and male NZW rabbits were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan). The mice were bred under specific-pathogen-free conditions at a constant temperature and humidity and used at 6–7 weeks of age.

Rabbit polyclonal anti-asialo GM1 antibody. Polyclonal anti-asialo GM1 antibody was produced in our laboratory by immunizing NZW rabbits with an emulsion containing asialo GM1, methylated bovine serum albumin (Sigma Chemical Com-

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Table 1. Glycolipids used

<table>
<thead>
<tr>
<th>Glycolipids</th>
<th>Structures&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMH</td>
<td>Glcβ1→1Cer</td>
</tr>
<tr>
<td>CDH</td>
<td>Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>CTH</td>
<td>Galα1→4Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>Globoside</td>
<td>GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>Paragloboside</td>
<td>Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer</td>
</tr>
<tr>
<td>Asialo GM2</td>
<td>GalNAcβ1→4Galβ1→4Glcβ1→1Cer</td>
</tr>
</tbody>
</table>
| Asialo GM1  | Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Cer | 3
| GM3         | Galβ1→4Glcβ1→1Cer       |
| GM2         | GalNAcβ1→4Galβ1→4Glcβ1→1Cer | 3
| GM1         | Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Cer | 3
| GD1a        | Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Cer | 3
| GD1b        | Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1Cer | 3

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<sup>a</sup> Gal = galactose; Glc = glucose; GalNAc = N-acetylgalactosamine; GlcNAc = N-acetylglucosamine; NeuAc = N-acetyleneuraminic acid; Cer = ceramide.
MONOClonal ANTI-ASIALO GM1 ANTIBODIES

pany, St. Louis, Mo., U.S.A.), and Freund’s complete adjuvant as described previously (15). IgG fraction was obtained from antisera by ammonium sulfate precipitation (33% saturation).

Other materials. Horseradish peroxidase-conjugated antibodies against rabbit and mouse immunoglobulins were purchased from Dako Corporation (Santa Barbara, Calif., U.S.A.). Mouse immunoglobulin class determination kits, which include rabbit antibodies against light and heavy chains of mouse immunoglobulin isotypes, horseradish peroxidase-conjugated goat antibody against rabbit immunoglobulins, and the substrate solution, were purchased from Zymed Laboratories, Inc. (South San Francisco, Calif., U.S.A.). Kits for the determination of immunoglobulin concentration by the single radial immunodiffusion method (10) were obtained from Tago, Inc. (Burlingame, Calif., U.S.A.). Bovine serum albumin (BSA) and Tween 20 (polyoxyethylene sorbitan monolaurate) were from Sigma. Pristane (2,6,10,14-tetramethylpentadecane) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, Wis., U.S.A.). Polyethylene glycol (4,000 molecular weight) was obtained from E. Merck A.G. (Darmstadt, West Germany). Polystyrene latex beads (0.4 μm in diameter) were from Japan Synthetic Rubber Inc. (Tokyo).

Preparation of hybridomas. Various strains (A/J, C57BL/6, BALB/c, DBA/2, C3H/He, and NZW) of mice were immunized with asialo GM1 adsorbed to naked Salmonella minnesota (3) (weight ratio of glycolipid to bacteria, 1:4) as described by Young et al (19) with some modifications. The mice were injected intravenously with the glycolipid-bacterial complex: 5 μg of asialo GM1 on day 0, 10 μg on day 4, 15 μg on day 7, and 20 μg on days 12 and 20. At appropriate intervals, a small volume of blood was taken from the mice by retro-orbital plexus puncture, and serum was prepared and assayed for anti-asialo GM1 antibody ELISA titer and anti-mouse NK activity as described below. The booster injections with 20 μg of asialo GM1 were repeated every two weeks until a positive antibody titer appeared. Animals with a high level of the titer were selected for the following experiment.

Four days after the last booster injection, spleen cells were fused with HAT (hypoxanthine-aminopterine-thymidine)-sensitive mouse NS-1 myeloma cells at a cell number ratio of 10:1 in a 50% (w/v) solution of polyethylene glycol. The cells were seeded in flat-bottomed, 96-well Costar microculture plates (Data Packaging Corporation, Cambridge, Mass., U.S.A.) in a final volume of 0.2 ml of Dulbecco’s modified Eagle medium (Gibco Laboratories, Grand Island, N.Y., U.S.A.) supplemented with 10% fetal bovine serum (Gibco), 1 × 10^{-4} M hypoxanthine, 4 × 10^{-7} M aminopterine, 1.6 × 10^{-5} M thymidine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Ten to fourteen days later, wells containing growing hybridomas were screened for anti-asialo GM1 antibody titer by ELISA. Hybridomas positive for the titer were cloned and recloned by limiting dilution.

Anti-asialo GM1 MAb. The anti-asialo GM1 MAb were prepared as ascites fluids obtained by injection of the established hybridoma cells to pristane-primed CD-1 nude mice and used without further purification. The class and the concentration of the MAb were determined by use of commercially available kits described above.
ELISA. Wells of flat-bottomed, 96-well Falcon polystyrene microtiter plates (Becton Dickinson and Company, Oxnard, Calif., U.S.A.) were seeded with 0.5 μg of asialo GM1 in ethanol, and the solvent was evaporated. The wells were then seeded with 0.2 ml of phosphate-buffered saline (PBS) containing 0.5% BSA (solution A). After incubation at 37°C for 1 hr, the plates were washed with PBS containing 0.05% Tween 20. Then, 0.1 ml of anti-asialo GM1 antibody diluted serially with solution A was seeded to each well, and the plates were incubated at 37°C for 1 hr. After washing with solution A, 0.1 ml of peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in dilution of 1:200 with PBS containing 2% BSA and 0.05% Tween 20 was added to each well. The plates were incubated at 37°C for 1 hr and seeded with o-phenylenediamine/H2O2 substrate solution. The peroxidase reaction was stopped by addition of 8 N H2SO4 (0.05 ml/well) 30 min later. Anti-asialo GM1 titer was expressed as a reciprocal of the highest antibody dilution showing positive reaction, or as an optical density at 490 nm read with a double wavelength microtiter spectrophotometer (ImmunoReader NJ; InterMed, Roskilde, Denmark).

Absorption of anti-asialo GM1 antiserum. Anti-asialo GM1 antiserum was mixed with packed polystyrene latex beads coated with asialo GM1 as described by Taki et al (17). The mixture was held at 4°C for 1 hr, and the antiserum was separated by centrifugation. The absorption was repeated three times. Antiserum treated with asialo GM1-uncoated beads was used as an unabsorbed control.

TLC. Glycolipids (5 μg) were chromatographed on a plastic TLC plate (Polygram Sil G; Macherey-Nagel, West Germany) in chloroform–methanol–0.25% aqueous KCl (50:40:10, v/v/v). The glycolipids were detected by the orcinol reaction.

TLC-immunostaining. The procedure described by Higashi et al (6) was used with some modifications. After the glycolipids were chromatographed as above, the TLC plate was soaked in PBS containing 2% BSA (solution B) for 2 hr and then incubated with anti-asialo GM1 antibody (1:1,000 dilution) at room temperature (the same as below) for 1 hr. The plate was washed with solution B and incubated with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins (1:1,000 dilution) for 1 hr. The plate was washed with solution B and soaked in 4-chloro-1-naphthol/H2O2 substrate solution for 10–15 min. The reaction was stopped by washing with water, and the plate was air-dried.

NK assay. The procedure was described previously (15). Briefly, 5 x 10^3 to 1 x 10^4 51Cr-labeled YAC-1 cells were incubated at 37°C in wells of U-bottomed, 96-well Costar microculture plates with C57BL/6 mouse spleen cells at an effector-to-target (E:T) ratio of 50:1 in a final volume of 0.2 ml. After a 4-hr incubation period, 51Cr radioactivity released in the supernatant was counted, and the specific cytotoxicity was calculated as described previously (15).

Treatment of spleen cells with anti-asialo GM1 plus complement. Spleen cells (3 x 10^6) from C57BL/6 mice were suspended in Hanks' balanced salt solution (HBSS) containing serially diluted anti-asialo GM1 antibody and held at 4°C for 30 min. The cells were washed with HBSS and incubated with an appropriate concentration
of guinea pig complement at 37 C for 40 min. After being washed twice with HBSS, the cells were assayed for NK activity as described above.

RESULTS

Antibody Response to Asialo GM1

Three to four mice of various strains were immunized with asialo GM1 according to the schedule described in "MATERIALS AND METHODS." Only one mouse of NZW strain elicited a high anti-asialo GM1 titer (12,800) in serum after 10 injections with asialo GM1. The other mice elicited no or very low titer. The NZW mouse antiserum showed a marked anti-mouse NK activity in the presence of guinea pig complement (Table 2). The anti-NK activity of the antiserum was

Table 2. Complement-dependent anti-mouse NK activity of the NZW anti-asialo GM1 antiserum

<table>
<thead>
<tr>
<th>Treatment of spleen cells</th>
<th>Dilution of antiserum</th>
<th>% Lysis of YAC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Antiserum + C'</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td>38.0 ± 1.6b</td>
</tr>
<tr>
<td>Normal NZW serum</td>
<td>1:100</td>
<td>39.6 ± 0.8</td>
</tr>
<tr>
<td>NZW anti-asialo GM1</td>
<td>1:10,000</td>
<td>36.0 ± 0.8</td>
</tr>
<tr>
<td>antiserum + C'</td>
<td>1:3,000</td>
<td>20.3 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>1:1,000</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>1:300</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.1 ± 0.7</td>
</tr>
<tr>
<td>Rabbit anti-asialo GM1</td>
<td>1:80</td>
<td>1.6 ± 0.6</td>
</tr>
</tbody>
</table>

a) C57BL/6 mouse spleen cells were treated with the serially diluted NZW mouse anti-asialo GM1 antiserum and further treated or untreated with guinea pig complement (C'). The cells were tested for NK activity against YAC-1 cells by a 4-hr 51Cr release assay at an E:T ratio of 50:1.
b) Mean ± S.D.
c) Not done.

Table 3. Effect of absorption of the NZW anti-asialo GM1 antiserum with asialo GM1 on the ELISA titer and the anti-NK activity

<table>
<thead>
<tr>
<th>Antiserum or antibody</th>
<th>Absorption</th>
<th>ELISA titer</th>
<th>% Lysis of YAC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZW anti-asialo</td>
<td>-</td>
<td>12,800</td>
<td>35.4 ± 1.6</td>
</tr>
<tr>
<td>GM1 antiserumb)</td>
<td>+</td>
<td>&lt;32</td>
<td>0.2 ± 0.9</td>
</tr>
<tr>
<td>Rabbit anti-asialo</td>
<td>-</td>
<td>64,000</td>
<td>34.0 ± 1.4</td>
</tr>
<tr>
<td>GM1 antibodyc)</td>
<td>+</td>
<td>&lt;32</td>
<td>2.0 ± 0.5</td>
</tr>
</tbody>
</table>

a) The NZW mouse anti-asialo GM1 antiserum and the rabbit anti-asialo GM1 antibody were absorbed or unabsorbed with asialo GM1 and assayed for the anti-asialo GM1 ELISA titer and the complement-dependent anti-mouse NK activity.
b) Dilution used for the anti-NK assay was 1:300.
c) Dilution used for the anti-NK assay was 1:80.
stronger than that of the rabbit polyclonal anti-asialo GM1 antibody (ELISA titer 64,000): The NZW mouse antiserum abolished the mouse NK activity completely even in dilution of 1:1,000. Both the ELISA titer and the complement-dependent anti-NK activity were completely diminished by absorption with asialo GM1 (Table 3).

On the basis of these results, the NZW mouse received the last booster injection and was used for the hybridization experiment four days later.

Anti-Asialo GM1 MAbs

Five single clones of hybridomas secreting anti-asialo GM1 MAb were established from one fusion. All the MAbs (designated MW-1, MW-2, MW-3, MW-4, and MW-5) were of the IgM class of immunoglobulin with kappa light chains. ELISA titers of the ascites fluids were 256,000 to 1,024,000, and the antibody concentrations were 1.0 to 1.9 mg/ml.

Specificity of the Anti-Asialo GM1 MAbs by ELISA

All the five MAbs reacted only with asialo GM1 when their reactivities were tested with 12 structurally-related glycolipids by ELISA (Fig. 1).

Specificity of the Anti-Asialo GM1 MAbs by TLC-Immunostaining

The reactivities of the five MAbs were examined by TLC-immunostaining. All the MAbs reacted only with a band of asialo GM1 (Fig. 2). Ascites fluids of the five MAbs obtained from various nude mouse individuals consistently showed the same reactivity, while the rabbit polyclonal antibody sometimes cross-reacted with GM1 and/or paragloboside.

![Fig. 1. ELISA specificity of the anti-asialo GM1 MAb (MW-3). The five anti-asialo GM1 MAbs MW-1, MW-2, MW-3, ME-4, and MW-5 were examined for the reactivity with various glycolipids (0.5 μg) by ELISA as described in "MATERIALS AND METHODS." All the MAbs showed the same reactivity pattern, and the result obtained with the MW-3 MAb is representatively shown here.](image-url)
Fig. 2. Specificity of the anti-asialo GM1 MAb (MW-3) by TLC-immunostaining. Various glycolipids (5 µg), after having been chromatographed simultaneously in triplicate on a TLC plate, were visualized with orcinol reagent (panel 1) and stained by the immunostaining method (see under "MATERIALS AND METHODS") with the rabbit polyclonal anti-asialo GM1 antibody (panel 2) or with the five anti-asialo GM1 MAbs. All the MAbs showed the same reactivity pattern, and the result with the MW-3 MAb is representatively shown here (panel 3).

Table 4. Anti-mouse NK activities of the anti-asialo GM1 MAbs in the presence of guinea pig complement

<table>
<thead>
<tr>
<th>Treatment of spleen cells</th>
<th>Dilution of antibody</th>
<th>% Lysis of YAC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td>41.2±1.4</td>
</tr>
<tr>
<td>C' alone</td>
<td></td>
<td>40.9±1.9</td>
</tr>
<tr>
<td>MW-1 MAb+C'</td>
<td>1: 10</td>
<td>32.4±1.1</td>
</tr>
<tr>
<td>MW-2 MAb+C'</td>
<td>1: 3</td>
<td>24.5±0.5</td>
</tr>
<tr>
<td>MW-3 MAb+C'</td>
<td>1: 10</td>
<td>31.4±1.3</td>
</tr>
<tr>
<td>MW-4 MAb+C'</td>
<td>1: 3</td>
<td>20.0±1.8</td>
</tr>
<tr>
<td>MW-5 MAb+C'</td>
<td>1: 10</td>
<td>36.3±1.2</td>
</tr>
<tr>
<td>MW-6 MAb+C'</td>
<td>1: 3</td>
<td>31.2±1.9</td>
</tr>
<tr>
<td>Control MAb(9)+C'</td>
<td>1: 10</td>
<td>22.7±0.9</td>
</tr>
<tr>
<td>Rabbit anti-asialo GM1</td>
<td>1: 3</td>
<td>10.8±0.6</td>
</tr>
<tr>
<td>antibody+C'</td>
<td></td>
<td>24.1±1.4</td>
</tr>
<tr>
<td>Rabit anti-asialo GM1</td>
<td>1: 80</td>
<td>20.4±0.7</td>
</tr>
<tr>
<td>antibody+C'</td>
<td></td>
<td>41.6±1.0</td>
</tr>
</tbody>
</table>

a) C57BL/6 mouse spleen cells were treated with the anti-asialo GM1 MAbs and treated further with guinea pig complement (C'). The cells were assayed for the NK activity.

b) The relative NK cell activities are shown in parentheses.

c) An IgM MAb (ascites fluid) unrelated to asialo GM1.
The five MAbs more or less reduced C57BL/6 mouse spleen NK activity in the presence of complement. However, the anti-NK activities of the MAbs were weaker than that of the rabbit polyclonal antibody. They reduced the NK activity only partially even in dilution of 1:3, while the rabbit antibody completely abolished the NK activity in dilution of 1:80 (Table 4). When equal volumes of the five MAbs were mixed, the anti-NK activity elevated to a level almost comparable to that of the rabbit polyclonal antibody: Approximately 80% of the NK cell activity was lost by the MAb mixture in dilutions of 1:10 to 1:3 (Table 5).

**DISCUSSION**

In the present study, five MAbs against asialo GM1 were obtained. The glycolipid is known to be present on the surface of various rat and mouse cells including NK cells (7, 14, 20). According to the hybridoma technique developed by Köhler and Milstein (8), MAbs are derived from single clones of hybridomas produced by fusing myeloma cells with antibody-producing lymphocytes. At present, the myeloma cells available usually are those from rats and mice. Therefore, it is almost impossible to obtain MAbs against specific substances unless they are immunogenic in these animals. Asialo GM1, as stated above, is a “self-antigen” in them and shows no or low immunogenicity. Therefore, attempts made by many investigators to obtain anti-asialo GM1 MAbs have not been successful.

We used various strains of mice to immunize with asialo GM1. Only one mouse of NZW strain elicited a high antibody titer in serum as determined by ELISA and complement-dependent lysis of mouse NK cells. The mouse strain is known to possess a genetic background for autoimmunity although they do not
develop autoimmune diseases (18). However, it is not clear whether it was by mere chance or whether it was due to the immunopathological property that the NZW mouse elicited a high anti-asialo GM1 titer in serum after repeated immunizations with asialo GM1: The data of the present investigation are insufficient for making any conclusion.

The MAbs obtained were shown to be specific for asialo GM1 by ELISA and TLC-immunostaining. The epitope structure recognized by the MAbs is estimated to be a carbohydrate chain of Galβ1-3GalNAcβ1-4Galβ1-4Glc (Gal=galactose, GalNAc = N-acetylgalactosamine, and Glc = glucose).

Like many other MAbs directed to carbohydrate chains in glycolipids and glycoproteins, the five anti-asialo GM1 MAbs were all of the IgM class of immunoglobulin.

Each of the MAbs lysed mouse NK cells in the presence of complement, but their lytic activities were weaker than that of the rabbit polyclonal antibody. This can be explained by a general difference between MAbs and polyclonal antibodies that epitope structures recognized by the former are more restricted than those recognized by the latter. It was shown in the present study that the anti-NK activity was increased when the MAbs were mixed. It was also shown that the anti-NK activity of the NZW mouse antiserum was stronger than that of the rabbit polyclonal antibody. These results may support the above explanation. Further, the results raised a possibility that the five MAbs might be divided into two or more groups in terms of fine specificity for the steric portions within the carbohydrate moiety of asialo GM1. This line of investigation is now under way, and the results will be reported elsewhere.

In addition, the weak anti-NK activities of the anti-asialo GM1 MAbs are not due to a possibility that the concentrations of the MAbs in ascites fluids might be too low: all the ascite fluids contained IgM at sufficiently high concentrations of 1-2 mg/ml, and their anti-asialo GM1 ELISA titers were higher than that of the rabbit polyclonal antibody.

To our knowledge, there has been no paper as to a preparation of MAbs specific for asialo GM1. MAbs reactive with asialo GM1 have been reported. Miller et al (11) reported a MAb which is specific for phenyl-β-galactoside and cross-reactive with the non-reducing terminal diasaccharide structure of asialo GM1. Solomon and Higgins (16) also described a MAb which is specific for the terminal β-linked galactosyl residue of asialo GM1 and cross-reactive with GM1 and CDH (asialo GM3). Thus, our present paper is probably the first to report a successful production of asialo GM1-specific MAbs.

Recent studies have shown that cellular development, differentiation, and oncogenesis are associated with changes in composition and metabolism of glycolipids and glycoproteins (5). The changes have been well characterized by MAbs with desired epitope specificity. Asialo GM1 is reported to exist in mouse brain tissue (9) as well as in NK cells and macrophages. Therefore, the anti-asialo GM1 MAbs produced in the present investigation will serve as useful probes in immunological and neurochemical studies.
REFERENCES


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Note added in proof. After the acceptance of the present one, a paper by Watarai et al (1987. J. Biochem. 102: 59–67) appeared describing a production of 6 anti-asialo GM1 IgM MAbs. These authors used, unlike us, culture supernatant of the hybridoma clones to examine the specificities of the MAbs and showed that they are all specific for asialo GM1 in liposome immune lysis assay and TLC-immunostaining. Further, they showed that the MAbs are incapable of removing mouse NK cell activity in vitro. Thus, there seems to be a certain similarity between their MAbs and ours in the glycolipid reactivity. However, at present, it is impossible to evaluate how they can be compared in more detail because they have not been studied simultaneously in one experiment.