ABSTRACT

Binding of tritium-labeled monosialoganglioside (GM₁) by intact thymocytes of young AKR/J mice was studied in vitro. The binding was dependent on time, temperature, concentration of cells, and ganglioside, suggesting that the cells possess a finite number of GM₁-binding sites. Binding, which could be observed as early as 15 sec, was very rapid, reaching a plateau in about 5 min, followed by a slow and steady increment up to 4 hr of incubation. Cellular binding of GM₁ appeared to be tight with very little exchange with exogenous gangliosides. GM₁ was almost entirely recovered intact 30 min after it was bound by thymus cells. The structural components of GM₁ (galactose, glucose, N-acetylneuraminic acid, mixed ceramides, and lactosylceramide) did not compete with GM₁ for cellular binding. The relative specificity of the GM₁-binding site was revealed by the lack of competition by lactosylceramide and very weak competition by disialoganglioside and trisialoganglioside that appeared to be due to a GM₁ contamination in these preparations. Further characterization of the GM₁-thymocyte interaction indicated that it was a Ca²⁺-dependent process, since chelating agents ethyleneglycolbis(β-aminoethyl ether)-N,N'-tetraacetic acid and ethylenediaminetetraacetic acid inhibited the binding up to 75 to 80%, and the inhibition by ethyleneglycolbis(β-aminoethyl ether)-N,N'-tetraacetic acid was reversed by the addition of excess of Ca²⁺. Also, there appeared to be no overlap of the GM₁-binding site with the binding site for concanavalin A. Finally, a comparison of the amount of GM₁ bound by leukemic and nonleukemic thymus cells revealed that the tumor cells had a higher capacity to bind GM₁ molecules than do normal thymus cells on a per cell basis under several experimental conditions.

INTRODUCTION

GM₁³ is a plasma membrane component of cells (7). It is believed to be the receptor for cholera toxin (5, 36) and has been shown to have affinity for binding to other microbial products such as tetanus toxin, to interferon (1, 37), and to mammalian glycoprotein hormones such as bovine thyrotropin and human chorionic gonadotropin (17, 24). Very little is known about the biological functions of gangliosides except a possible role in cellular recognition [by virtue of their presence on the cell surface (9, 10)] or suppression of cell growth and division in vitro (13, 18). In recent years, similar patterns of changes in ganglioside composition of virally transformed cells and chemically induced tumors have been reported (8). Three recent observations from this laboratory and others have implicated gangliosides in the immunosuppression observed in tumor-bearing animals: (a) Kloppel et al. (16) have reported an increased level of glycolipid-bound sialic acid in serum of humans and mice bearing mammary carcinoma. We have extended this observation to show an increase in the total ganglioside content of thymic lymphoma from AKR/J mice (19); (b) an elevated serum ganglioside level in such tumor bearers (19); and (c) the inhibition of Con A-induced lymphocyte transformation in the presence of exogenous gangliosides in vivo (18, 20). We have proposed that gangliosides shed by leukemic thymus or by normal cells in response to the presence of neoplastic cells may produce immunosuppressive effects in these animals. A direct interaction of gangliosides with lymphoid cells is implicit in this hypothesis. Glycolipids have been implicated as surface markers on T- and B-cells (6, 25, 32) as well as immunoregulatory molecules in their functions (20, 22, 28). Fishman et al. (8) and Calissi et al. (2) have reported the uptake and metabolism of exogenously added gangliosides in transformed mouse fibroblasts. However, no information is available on the binding of gangliosides to lymphocytes. In order to define the cellular basis of immunosuppressive action of gangliosides, it is necessary to demonstrate a direct interaction of gangliosides with lymphoid cells. The present studies deal with the binding characteristics of radiolabeled GM₁ to normal mouse thymocytes in vitro. Also, a comparison of the binding of [³H]GM₁ to leukemic and nonleukemic mouse thymus cells is presented.

MATERIALS AND METHODS

Thymus Cells. Suspensions of normal thymus cells were prepared from young AKR/J mice, 3 to 5 months old, as described previously (14). Leukemic thymocytes were obtained from 6- to 9-month-old mice of the same strain. The suspension and culture medium was HMEM-TBH, without antibiotics, glutamine, or serum (14). All operations involving the cells were carried out at room temperature except for the incubation, which was maintained at 37°C.

Hexoses and hexose derivatives were from Applied Science Laboratories, Inc. (State College, Pa.) a-MM and NANA were obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.) and Sigma Chemical Co., (St. Louis, Mo.), respectively. Ceramide, GM₁, G₂a, G₁₁, and GM₇ were purchased from Supelco, Inc. (Belleville, Pa.).

Labeling of Gangliosides. GM₁ was radiolabeled by combining the galactose oxidase: sodium borohydride method of labeling the terminal galactose of ganglioside described by Suzuki and Suzuki (34) and the method of Schwarzmann (30) in which potassium borohydride is used to introduce label into the ceramide portion of GM₁. In the presence of palladium as catalyst. Briefly, 2 mg of GM₁ were oxidized with bacterial

1 Supported by USPHS Grant CA 21631 from the National Cancer Institute.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: GM₁, monosialoganglioside; Con A, concanavalin A; HMEM-TBH, Hanks' minimal essential medium with tryptophol, methylglutamate, methyl-2-aminoethanesulfonic acid, and N-, N-bis(2-hydroxyethyl)glycine; NANA, N-acetylneuraminic acid; a-MM, methyl-2-aminoethanesulfonic acid, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffers, pH 7.4; a-MM, methyl-2-aminoethanesulfonic acid, N-acetylneuraminic acid; TCA, trichloracetic acid; G₃₁, trisialoganglioside; G₂a, disialoganglioside; EGTA, ethyleneglycolbis(β-aminoethyl ether)-N,N'-tetraacetic acid.

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2808 CANCER RESEARCH VOL. 40

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galactose oxidase (EC 1.1.3.9) in 10 mM phosphate buffer, pH 7.0, at room temperature for 24 hr. After sodium azide (0.2 mM) was added to inhibit the enzyme (4), the reaction mixture was adjusted to an alkaline pH by the addition of 1 M NaOH. The terminal galactose of GM₁ was reduced with 2 mCi of KB₃H₄ in presence of the catalyst palladium chloride (2.5 mg/ml) for 4 hr. Palladium and the enzyme were precipitated by methanol and removed by centrifugation. Labile tritium in the supernatant was removed by repeated addition of water and evaporation to dryness under N₂. The residue was dissolved in water and passed through a 2-ml Dowex 50-H⁺ column to remove Na⁺ and K⁺. The filtrate was extracted with toluene and finally with methanol repeatedly to remove boric acid.

The reaction product was purified by chromatography on a precoated preparative Silica Gel G thin-layer plate (Brinkmann Instruments, Inc.) using CHCl₃:CH₃OH:H₂O (60:35:8, v/v) containing 0.02% CaCl₂ for 2.5 hr. Ganglioside regions on the plate were detected by spraying the plate with resorcinol reagent (38). Silica gel was scraped from the region corresponding to standard GM₁, and the glycolipid was eluted by repeated washings with methanol and chloroform:methanol (2:1). The eluates were pooled, dried, and tested for radiopurity by analytical TLC using the same solvent system. A single peak of radioactivity corresponding to standard GM₁ was noted under conditions that would have detected a 5% contaminant. The specific activity (9.21 and 42.3 Ci/mol for 2 different preparations) of [³H]GM₁ was calculated by determining NANA content by a micromodification of the thiobarbituric acid method (39) and determining the radioactivity by scintillation spectrometry.

Binding Assay. Cells were incubated at 37° in a total volume of 0.25 ml of HMEM-TBH buffer containing [³H]GM₁ (about 55,000 to 60,000 dpm) in polypropylene microfuge tubes. At the end of incubation, the tubes were chilled and centrifuged at 12,000 x g for 30 sec. The cell pellet was resuspended in 1 ml of ice-cold phosphate-buffered saline, (NaCl, 8 g/liter; KCl, 0.2 g/liter; Na₂HPO₄, 1.15 g/liter; KH₂PO₄, 0.2 g/liter, pH 7.4), and immediately centrifuged for 1 min at the same speed. This process was repeated once more, and pellets were taken up in 1.2 ml of Scintiverse (Fisher Scientific Co., Chicago, Ill.) for radioactivity determination. In each experiment, "nonspecific binding" was determined by parallel assay tubes that contained an excess of unlabeled GM₁ (30 to 50 μg) but no [³H]GM₁ during incubation with cells. [³H]GM₁ was added immediately prior to centrifugation and washing steps. Specific binding is defined as the difference between the total binding and nonspecific binding. The difference between duplicate assays was less than 4%. The average molecular weight of 1544.7 for GM₁, as given by Supelco was used for calculation. Where necessary, the modified Student's t test was used to assess statistical significance of data.

RESULTS

Characteristics of Cellular Binding of [³H]GM₁

Chart 1 demonstrates that normal mouse thymus cells are capable of binding [³H]GM₁ in vitro. The amount of GM₁ bound was proportional to the number of cells in the incubation medium in the range of 10⁶ to over 10⁷ cells/incubation. The binding per cell decreased above 10⁶ cells probably due to clumping and settling. A density of 10⁶ cells/incubation in subsequent experiments was used to facilitate washing and to give data with reasonably high cpm. Chart 2 shows the kinetics of binding process at 10⁶ cells/incubation; the initial binding reaction was rapid with the time point of 15 sec showing significant binding. Whereas a plateau was reached (representing 25% of maximal binding) by 5 min and continued for about 30 min, a second but slow increment in binding was observed that appeared to reach maximal binding at about 4 hr. Furthermore, the amount of GM₁ bound to the cells was proportional to the amount of GM₁ present in the medium. Thus, saturation kinetics could be demonstrated whether the binding was followed for 15 min or 3 hr (Chart 3). Double reciprocal plots of the same data (see Chart 3, inset) revealed no significant differences between the affinities of binding (9.5 μM) in both the early and the late phases of the binding. The binding appeared to be temperature dependent (see Chart 6); although only 60% was bound at 0° as compared to 37°, binding was still significant at low temperatures.
Effect of Structural Components of Gangliosides on [3H]GM1 Binding

In order to understand whether the hydrophobic or hydrophilic portion of the GM1 molecule was involved in the attachment of the ganglioside to its binding site on the thymocyte surface, an excess (40 to 100 times) of D-galactose, D-glucose, D-galactosamine, NANA, lactosylceramide, and mixed cerebrosides were added to the cells 10 min before the addition of [3H]GM1. None of the above compounds were competitive with GM1 for binding, suggesting that the configuration of the entire molecule was involved in the binding process (Table 1). However, other glycolipids preparations such as G018 and GD18 showed some limited ability to compete with [3H]GM1 binding. In order to check whether the GM1-binding site was distinct from that of the lectin-binding site for Con A, the latter was added at mitogenic doses before the addition of [3H]GM1. The lectin did not interfere with GM1 binding. Furthermore, α-MM, a compound known to specifically occupy Con A receptor on lymphocytes, did not significantly alter the binding of GM1 to thymocytes (Table 1). Data presented in Chart 4 show the relative specificity of the GM1-binding site toward other glycolipids tested at different doses. Whereas partial competition at higher doses was noted with sialic acid-containing glycolipids G018 and GD18, lactosylceramide showed no competition with GM1 binding. A contamination of G018 that was about 10% GM1 would have given inhibition that was solely due to GM1. TLC analysis showed the presence of 8% GM1 in the sample of G018, which was similarly impure. It is thus likely that the binding is specific for GM1, with little or no competition by GD18 and G018.

Calcium Requirement for GM1 Binding

Since Ca2+ has been reported to bind with GM1 (23, 31), it was of interest to check whether the cellular binding of GM1 is a Ca2+-dependent phenomenon. Increasing concentrations of EGTA (a specific Ca2+-chelating agent) in the medium did result in progressively lesser amounts of GM1 bound to cells (Chart 5). A similar degree of inhibition was noted with EDTA at the same range of concentrations. At 0.5 mM EGTA, binding was reduced by 55%, and this inhibition was indeed relieved by addition of increasing doses of CaCl2, thereby suggesting the importance of Ca2+ in the ganglioside-cell interaction.

Metabolism of GM1 after Binding to Cells

To determine whether the GM1 once bound to the cells remained unmetabolized or was degraded due to the presence of ganglioside-degrading enzymes, chloroform:methanol (2:1) extracts of cell pellets that were previously incubated with [3H]GM1, for 30 min were analyzed by TLC. A boiled cell pellet was used as a control to correct for the efficiency of the extraction and analytical procedures. The amount of radioactivity that cochromatographed with authentic GM1 was essentially the same in both cases, suggesting very little catabolism of cell-bound [3H]GM1.

The question of reversibility of the binding reaction was also examined by first exposing the cells to [3H]GM1, for 10 min at 37°, washing the cell suspension as previously described, and then dividing the suspension into 3 equal portions (A, B, and C).

Table 1

<table>
<thead>
<tr>
<th>Additions</th>
<th>Quantity (μg)</th>
<th>[3H]GM1 bound (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>(100)</td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td>25</td>
<td>111</td>
</tr>
<tr>
<td>NANA</td>
<td>30</td>
<td>101</td>
</tr>
<tr>
<td>Mixed cerebrosides</td>
<td>25</td>
<td>107</td>
</tr>
<tr>
<td>Above 3 compounds</td>
<td>25, 30, 25</td>
<td>93</td>
</tr>
<tr>
<td>D-Galactosamine</td>
<td>250</td>
<td>116</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>250</td>
<td>101</td>
</tr>
<tr>
<td>Lactosylceramide</td>
<td>30</td>
<td>120</td>
</tr>
<tr>
<td>G11</td>
<td>30</td>
<td>50</td>
</tr>
<tr>
<td>GD18</td>
<td>30</td>
<td>72</td>
</tr>
<tr>
<td>GM1</td>
<td>30</td>
<td>2</td>
</tr>
<tr>
<td>Con A</td>
<td>0.5</td>
<td>95</td>
</tr>
<tr>
<td>α-MM</td>
<td>1870</td>
<td>87</td>
</tr>
</tbody>
</table>

* Glycolipids added simultaneously with [3H]GM1, and mixed, and binding with cells was determined for 15 min.

* Unpublished observations.
C). Each aliquot was incubated for further periods of 10, 20, and 30 min with an excess (25 μg) of unlabeled GM1 in 2 volumes of HMEM-TBH at 37°. At the end of the second incubation, cells were centrifuged and washed once more, and all the supernatants with washings were collected separately. At each point, the wash solution contained approximately 11 to 13% of the total radioactivity. These results suggest that most of the GM1 was tightly bound and that only a small fraction exchanged with unlabeled exogenous GM1 under these experimental conditions.

Comparison of [3H]GM1 Binding by Leukemic and Nonleukemic Thymocytes

Virally transformed cells exhibit altered cell surface characteristics (9). Since the thymic leukemia of AKR/J mice is known to be of viral origin, the amount of GM1 bound to leukemic and nonleukemic thymocytes in vitro was determined. Charts 6 and 7 depict these results. Equal numbers of cells were incubated with [3H]GM1 for varying time periods, different temperatures, and increasing cell and ganglioside concentrations. The results showed that the leukemic cells have a higher capacity to bind GM1 molecules than do the normal thymocytes at all the above experimental conditions.

**Time Course.** The amount of GM1 bound by leukemic thymocytes was higher than that of nonleukemic cells. The greatest part of that increment was associated with the fast phase (up to 5 min) of the binding process. There was an increment in the binding of GM1 with increasing time of incubation in both leukemic and nonleukemic thymocytes (Chart 6a).

**Effect of Temperature.** Increased binding of GM1 was observed with increase in temperature. Furthermore, the magnitude of difference in binding between leukemic and nonleukemic cells was greater at 37° than at 0° (Chart 6b).

**Effect of Substrate and Cell Concentrations.** At a fixed cell concentration, the pmoles of GM1 bound increased progressively with increasing amounts of GM1 present in the medium up to a point of saturation (Chart 7). Although the leukemic cells did not seem to attain a plateau level of binding at high concentration of GM1, with 5 × 10^6 cells, saturation of the binding could be demonstrated at a lower cell number (1 × 10^6 cells).

**DISCUSSION**

Loss of general immunocompetence is a common observation in tumor-bearing animals and patients. Immunosuppressive factors present in the sera of such tumor bearers are capable of inhibiting some of the common tests of immunocompetence of normal homologous lymphocytes (12, 33, 40). Kim et al. (15) have demonstrated that tumor cells can shed tumor antigens and membrane components into their environment. Vitetta et al. (38) observed that Thy-1 antigen is shed from thymocytes in the form of aggregates composed of plasma membrane lipids and proteins. Among the lipids, prior exposure of murine lymphocytes to lecithin liposomes was found to suppress the Con A response (27), and treatment of spleen cells with GM1 liposomes resulted in temporary inhibition of the number of plaque-forming cells (22). Elevation of total plasma gangliosides in leukemic AKR/J mice as well as an inhibitory action of gangliosides at similar doses of the Con A-induced mitogenic response to mouse thymocytes in vitro have recently been reported from this laboratory (18–20). Implicit in this scheme
of immunosuppression by gangliosides is the interaction of gangliosides with the lymphocytes, and results presented here demonstrate binding of GM₁ to thymus cells.

The cellular binding of GM₁ was observed to be dependent on time, temperature, concentration of GM₁, and the number of cells available for binding (Charts 1 to 6). An extremely rapid initial binding was observed. This observation is in good agreement with the reports describing cholera toxin and prostaglandin binding to mouse and rat thymocytes in which 80% of cholera toxin molecules were found to bind the cells within 1 min, and it took 40 to 50 sec only to achieve half-maximal uptake of prostaglandin E₁ (11, 29). Similarity in the saturation kinetics and absence of any difference in the affinity of GM₁ to its binding sites both at early rapid phase (15 min) and at the late time point (3 hr) perhaps indicate the existence of additional GM₁-binding sites on thymocytes that are generated or exposed with progression of time (Chart 2). A rearrangement of membrane components is possible and could be due to initial binding of GM₁ molecules resulting in further exposure of more sites. Because it is inhibited by sodium azide, it would appear to be an energy-dependent process. Reduced binding observed at 0°C could be due to factors such as: (a) decreased rate of "collision" between free GM₁ molecules and cellular binding sites; (b) decreased fluidity of the membrane at low temperatures; and (c) lower rate of production of energy, assuming that the slow phase of GM₁ binding is an energy-dependent process. In fact, when 5 to 10 mM sodium azide was present during incubation, only 50% binding was observed. The proportionality of the amount of GM₁ bound to the number of cells suggests the presence of a finite number of GM₁-binding sites on the murine thymocytes. This does not necessarily imply specific structural sites, inasmuch as steric or electrostatic interactions could also limit the number of molecules of GM₁ that could bind per given membrane area. On the other hand, saturable binding with a single high-affinity binding constant argues for a defined "binding site."

An attempt was made to understand the nature of interaction of GM₁ with its cellular binding site. Mixed ceramides or lactosylceramide did not compete with intact GM₁, suggesting that the nonpolar portion of GM₁ does not play a major role in the binding process (Table 1). Besançon et al. (1) reported a similar finding in a study of interferon-ganglioside interaction. Furthermore, carbohydrate and NANA moieties alone were observed not to be inhibitory in our system (Table 1). Requirement for Ca²⁺ for the binding process (Chart 5) could have important biochemical implications on the mechanistic aspects of ganglioside actions on the cells in general. The calcium:ganglioside complex could be the actual structure being bound by the cells. In fact, formation of Ca²⁺-ganglioside complexes have been demonstrated recently (23, 31).

Lymphocytes possess a number of receptors for antigens, bacterial products, hormones, lectins, etc. Whereas GM₁ itself is believed to be a receptor for cholera toxin (36), it remains to be explored whether GM₁ has a defined structure with which it interacts at the cellular binding site. Our results suggest that there is no overlapping of the Con A-binding site and the GM₁-binding site on the thymocytes in view of the following evidence: (a) gangliosides do not prevent Con A binding as indicated by direct binding experiments using [acetyl-³H]Con A (20); (b) conversely, Con A did not interfere with [³H]GM₁ binding (Table 1); and (c) α-MM, which specifically occupies the lectin-binding site, did not show significant inhibition of [³H]GM₁ binding (Table 1).

Results of our studies and those of others strongly suggest that GM₁ is inserted into mammalian cell membranes (2, 8, 26). Whether or not the exogenous gangliosides inserts into the cell membrane in the same manner as the endogenous ganglioside is, however, not clear. Since the exchange with unlabeled GM₁ is poor, it is possible that once inserted either the off reaction is extremely slow or the ganglioside becomes inaccessible for replacement. Our results showed that GM₁ was almost entirely recovered as an intact molecule 30 min after incubation with the cells, thus excluding metabolism of bound GM₁. This finding agrees well with the report of O'Keefe and Cuatrecasas (26) that exogenous GM₁ remains as an intrinsic part of the membrane of 3T3 mouse fibroblasts and is not degraded. Also, the cell-bound GM₁ was observed to be functionally equivalent to endogenous ganglioside with respect to responsiveness to cholera gen.

Further studies on the postbinding biochemical events of GM₁-thymocyte interaction should be interesting. Stimulation by exogenous gangliosides of microsomal ATPase activities has been reported recently (3). It will be interesting to determine if, due to insertion of GM₁ into the thymocyte membrane, the activity of plasma membrane-bound adenylate cyclase or phosphodiesterase may be kept at a relatively high-low level, respectively, resulting in an extended period of high intracellular levels of cyclic-adenosine 3',5'-monophosphate. These possibilities are being investigated currently.

A calculation of the number of GM₁ molecules bound per cell under optimal conditions shows that about 40 and 100 x 10⁶ molecules were bound by each nonleukemic and leukemic cell, respectively. It may be pointed out here that the thymocytes from the above animals consist of cell populations differing in the state of differentiation, cell size (and hence surface area), and cell surface properties. Assuming the GM₁ was bound to small, medium, and large lymphocytes with equal affinity, re-calculation of the above data to arrive at the average density of GM₁-binding sites per unit surface area of nonleukemic and leukemic thymocytes gave values of 2.4 and 3.75 x 10⁶ (GM₁ molecules bound per sq μm), respectively. Thus, our studies suggest modest differences in the number of GM₁-binding sites between leukemic and nonleukemic thymocytes when compared on a per cell basis.

REFERENCES


5 Values reported by Metcalf (21) on the relative distribution of small, medium, and large lymphocytes in normal thymus (89, 10, and 1%) and thymus lymphoma (19, 56, and 25%) of AKR mice were used for these calculations. The surface area of small, medium, and large lymphocytes was 154, 254, and 380 sq μm based on the mean diameter of 7, 9, and 11 μm, respectively. Thus, the mean surface areas of given populations of leukemic and nonleukemic thymocytes were calculated to be 28,689 and 16,631 sq μm/100 cells, respectively.
Binding of Monosialoganglioside by Murine Thymus Cells in Vitro

Rajabather Krishnaraj, Yeheskel A. Saat and Robert G. Kemp


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