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Detection of GD_3 Ganglioside in Childhood Acute Lymphoblastic Leukemia With Monoclonal Antibody to GD_3: Restriction to Immunophenotypically Defined T-Cell Disease

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We have recently reported that the disialoganglioside GD_3 is found in cellular lipid extracts of T-cell acute lymphoblastic malignancies (T-ALL) but is not detectable by resorcinol staining in extracts of non-T acute lymphoblastic leukemia blasts (non-T-ALL). We have now extended this study to assess the detectability of GD_3 in T-ALL vs non-T-ALL utilizing an anti-GD_3 antibody, R_{24}. Gangliosides isolated from T-ALL and non-T-ALL blasts by two different methods were separated by thin-layer chromatography and stained with anti-GD_3 and a control antibody specific for GM_3 and sialosylparagloboside (SPG). Anti-GD_3 reactivity was observed in extracts from T-ALL cells in all cases, whereas GD_3 was not detected in any of the non-T-ALL samples. The anti-GM_3/SPG antibody stained GM_3 in all of the leukemic samples analyzed as well as SPG in the non-T-ALL samples. Indirect immunofluorescence was used to assess the expression of GD_3 at the surface of leukemic blasts. Fluorescence-activated cell sorting analysis with R_{24} showed that whereas T-ALL blasts were highly reactive with this antibody, non-T-ALL blasts were totally unreactive. In an analysis of a larger number of leukemia patients by fluorescence microscopy, 20 out of 28 samples with the T-ALL phenotype were positive for R_{24}, whereas zero out of 11 non-T-ALL samples were reactive. These results confirm our earlier finding of the specificity of GD_3 to the T-ALL subclass of childhood leukemias and furthermore suggest the potential value of anti-GD_3 as an immunological tool for the diagnosis and therapy of T-cell ALL.

Abbreviations used: Gangliosides GD_3 and GD_2 are abbreviated according to the nomenclature of Svennerholm [45]; ALL, acute lymphoblastic leukemia; SPG, sialosylparagloboside, IV^3-α-N-acetylneuraminosylneolactotetraosylceramide; HPTLC, high-performance thin-layer chromatography; CALLA, common acute lymphoblastic leukemia antigen; FACS, fluorescence-activated cell sorting; IL-2, interleukin 2.

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Studies of glycolipids of various human tumors have shown that specific gangliosides (sialic-acid-containing glycosphingolipids) accumulate in certain tumors, which has led to the proposal that specific gangliosides may be antigenic markers for these tumors [see for review 1–3]. With the aid of hybridoma techniques, a large number of monoclonal antibodies with carbohydrate specificity have been raised against human tumors [4]. While incompatible blood-group-related antigens may be expressed in tumors of epithelial origin on either glycolipids or glycoproteins [5–7], accumulations of gangliosides of the ganglio series appear to be specific for glycolipid alterations in malignancy. Examples include increased GD_2[2] ganglioside in neuroblastoma [8], increased GD_3 and GD_2 in melanomas [9,10], and increased GD_3 in certain acute and chronic leukemias [11–14]. In the case of melanoma, monoclonal antibodies specific for GD_3 of both the IgM [15] and the IgG [16,17] subclasses have been derived in different laboratories. The IgG3 anti-GD_3 monoclonal antibody (R_{24}) developed at Sloan-Kettering Intitute is of particular interest in that this antibody inhibits melanoma cell growth in vitro [18], mediates complement-mediated cytolysis and antibody-dependent cytotoxicity with human effector cells [19,20], and also has been shown in phase I trials to have potential clinical antitumor value against melanoma [21].

We have recently described ganglioside alterations in childhood lymphoblastic malignancies, and have shown the accumulation of GD_3 in cells of seven T-cell lymphoblastic malignancies relative to normal peripheral lymphoid tissue, while non-T-ALL cells contained no detectible GD_3 [14]. In the current study we have utilized the R_{24} antibody to further assess the specificity of the GD_3 alteration in childhood leukemias. With R_{24}, T-cell ALL and non-T-ALL blasts were compared with respect to the presence of GD_3 by (1) the HPTLC immunostaining technique, which detects this ganglioside at the ng level and (2) indirect immunofluorescence microscopy and FACS analysis. The results show the clear absence of GD_3 in non-T-ALL and that this ganglioside distinguishes a major subclass of T-cell ALL.

MATERIALS AND METHODS

Cell Preparation and Characterization

Lymphoblasts were obtained before therapy from whole blood or from bone marrow from patients with ALL. Blasts were isolated by Ficoll-Hypaque density gradient centrifugation, as previously described [22]. Thymic tissue was obtained from children undergoing cardiac surgery, and a single cell suspension of thymocytes was prepared. Lymphoblasts were evaluated for T-cell, B-cell, and leukemia-associated surface antigens by indirect immunofluorescence techniques with monoclonal antibodies [23]. Specifically, lymphoblasts expressing either CD2 or CD7 antigens antigens were considered as T-cell ALL while lymphoblasts expressing BA-1(CD24), B4(CD19), HLA-DR, and/or CALLA were considered as non-T ALL. In all cases, lymphoblasts were positive for terminal deoxytidyl transferase [24] and negative for surface immunoglobulin.

Isolation and Purification of Gangliosides

Gangliosides were isolated from lymphoblasts and thymocytes either by the method of Ledeen et al. [25], as previously described [14], or, for smaller samples, by lipid

extraction by the methods of Svennerholm [26,27] followed by ganglioside purification by the di-isopropyl ether-butanol extraction method of Ladisch and Gillard [28]. For the latter method, samples were homogenized with a Dounce homogenizer in a small volume of water, and methanol and chloroform were added sequentially to yield a final ratio of water:methanol:chloroform of 3:8:4 (v/v/v). After extraction for 3 h, the samples were centrifuged, and the pellets were rehomogenized in water and reextracted as above. After centrifugation, the supernatants were combined and evaporated. The total lipid fraction was suspended in di-isopropyl ether:1-butanol (60:40) (DIPE/butanol) by alternating pulses of vortexing and sonication; 0.5 vol of either 0.1% or 0.3% NaCl was added; and the suspension was resonified and vortexed to ensure thorough mixing of the phases. The phases were separated by centrifugation; the upper organic phase was removed and discarded, and the lower phase was mixed with the same volume of DIPE/butanol as above. After centrifugation, the upper phase was removed, and the lower phase was lyophillized. This material was then dissolved in chloroform:methanol:water, 60:30:4.5 (v/v/v) [26], and insoluble material was pelleted. The supernatant was removed to another tube and evaporated to dryness. This lipid fraction was desalted by using a Sep-Pak-C_{18} cartridge [29]. Specifically, the material was dissolved in 1–3 ml of 0.1 M KCl, and this solution was passed four times through an extensively prewashed cartridge. After elution of salts from the column with 30 ml of water, gangliosides were eluted with 20 ml of chloroform:methanol (2:1, v/v). The samples were dried under N_2 gas and stored at $-20°C$ for further analysis.

HPTLC Analysis of Gangliosides by Immunostaining With Antiganglioside Monoclonal Antibodies

Isolated gangliosides from T-ALL, non-T-ALL, and thymocytes were spotted on 10 × 10 cm aluminum-backed HPTLC plates (E. Merck) together with standards GD_3 and GM_3 (Bachem Inc., Torrance, CA) and the total ganglioside fraction prepared from human erythrocyte membranes [30]. The plates were developed in the solvent system, chloroform:methanol:0.25% $CaCl_2$ (60:40:9, v/v/v). Gangliosides GD_3, GM_3, and SPG were detected by the HPTLC immunostaining overlay method with anti-GD_3 and anti-GM_3/SPG monoclonal antibodies by the method of Magnani et al. [31]. Anti-GD_3 antibody was an IgG3 (R_{24}) [16], a gift from Dr. Kenneth Lloyd, Sloan-Kettering Institute, New York. Anti-GM_3 was an IgM (M2590) [32], a gift from Meiji Seika Kaisha, Ltd, Tokyo, and Dr. M. Taniguchi, Chiba University, Japan. This antibody has equal reactivity with sialosylparagloboside (SPG), the major human red blood cell ganglioside, and GM_3 [33]. Plates were exposed to either anti-GD_3 or anti-GM_3/SPG at 5–7 μg/ml, washed, and then exposed to murine ^{125}I-anti-IgG and/or ^{125}I-anti-IgM ($2–3 \times 10^6$ cpm/ml antibody solution), gifts from Dr. John Magnani, NIH, Bethesda, Maryland. Plates were washed and exposed to Kodak X-Omat film for 18 h.

Immunofluorescence Analysis With R_{24} Antibody

For analysis of surface expression of GD_3 on a large number of leukemia samples of both the T-cell and non-T-cell subclasses, blasts were assayed by indirect immunofluorescence microscopy with the R_{24} antibody, at a concentration of 50 μg/ml, utilizing standard techniques [34]. The second antibody was fluorescein-conjugated goat antimouse IgG (Cooper Biomedical, Malvern, PA).

For flow cytometric determinations, cells obtained from leukemic patients were resuspended in phosphate-buffered saline containing 1% bovine serum albumin and

stained with 40 μg/ml of R_{24} monoclonal antibody and a fluorescein-conjugated goat $F(ab)_2$ antimouse IgG antibody (Cooper Biomedical) according to standard procedures [35]. Cells were fixed in 1% formaldehyde and analyzed in a Bectin-Dickenson FACS IV flow cytometer; 10,000 cells gated to exclude nonviable cells were accumulated for each histogram. The percentage of GD_3-positive cells was determined against a background of nonspecific labelling with MOPC-21 (a nonspecific IgG obtained from mouse myeloma cells) (Litton Bionetics). Logarithms of fluorescence intensity in individual histograms were distributed over 256 channels.

RESULTS

HPTLC Immunostaining With Monoclonal Antiganglioside Antibodies

Gangliosides from four T-cell and four non-T-ALL blasts were isolated and individual gangliosides were separated on HPTLC aluminum-backed plates for immunostaining with R_{24} anti-GD_3 monoclonal antibody. Between 10 and 50 ng ganglioside was spotted for each leukemia sample. As seen in Figure 1A, GD_3 was detected in all four T-cell leukemias analyzed, whereas R_{24} staining was absent in the non-T-ALL samples. The results demonstrate that at the ng level of sensitivity, GD_3 is not found in non-T-ALL blasts but is a major ganglioside of T-cell ALL.

In order to assess the presence of GD_3 relative to other gangliosides in leukemic lymphoblasts with the immunostaining technique, the separated gangliosides were reacted with an anti-GM_3 preparation which cross-reacts with SPG. For this determination, gangliosides previously isolated from blasts from two patients with T-ALL and two with non-T-ALL by the method of Ledeen et al. [25] were spotted alongside gangliosides which were freshly isolated from $1–2 \times 10^8$ cells from two patients with T-ALL, two with non-T-ALL, and a thymocyte preparation. After development, the plate was reacted with a mixture of anti-GD_3 and anti-GM_3/SPG antibodies, followed by a mixture of ^{125}I-anti-IgG and ^{125}I-anti-IgM. As seen in Figure 1B, both GM_3 and GD_3 were detected in the four T-cell ALL samples which were studied, and the ratio of the two gangliosides varied among the samples. On the other hand, the non-T-ALL blasts contained both GM_3 and SPG, and no GD_3 was detected. The thymocyte preparation contained not only GM_3 but also detectable levels of GD_3.

Reactivity of T-ALL and Non-T-ALL Blasts With R_{24} by Immunofluorescence Techniques

In order to assess the surface expression of GD_3 on ALL lymphoblasts, blasts from T-ALL and non-T-ALL patients were reacted with R_{24} anti-GD_3 antibody, and the cells were processed for FACS analysis. The results show that GD_3-positive cells were found in three out of three T-ALL patient blasts tested (32–60% positive cells). A representative experiment is shown in Figure 2. Analysis of the fluorescence intensity distribution of the GD_3-positive cell populations revealed an heterogeneous distribution, indicating that different concentrations of GD_3 are present on the cell surface of different cells (Fig. 2). On the other hand, no GD_3-positive cells were observed in any of the three non-T-ALL patients tested.

With a larger patient population, immunofluorescence microscopy was also utilized to determine the ubiquity of GD_3 expression in T-ALL and whether the absence of GD_3 is a general phenomenon of non-T ALL. We have observed that cells from 20 of 28 T-ALL patients (anti-$CD7^+$) were positive for R_{24} ($\geq$ 10% of cells positive). In ten of the

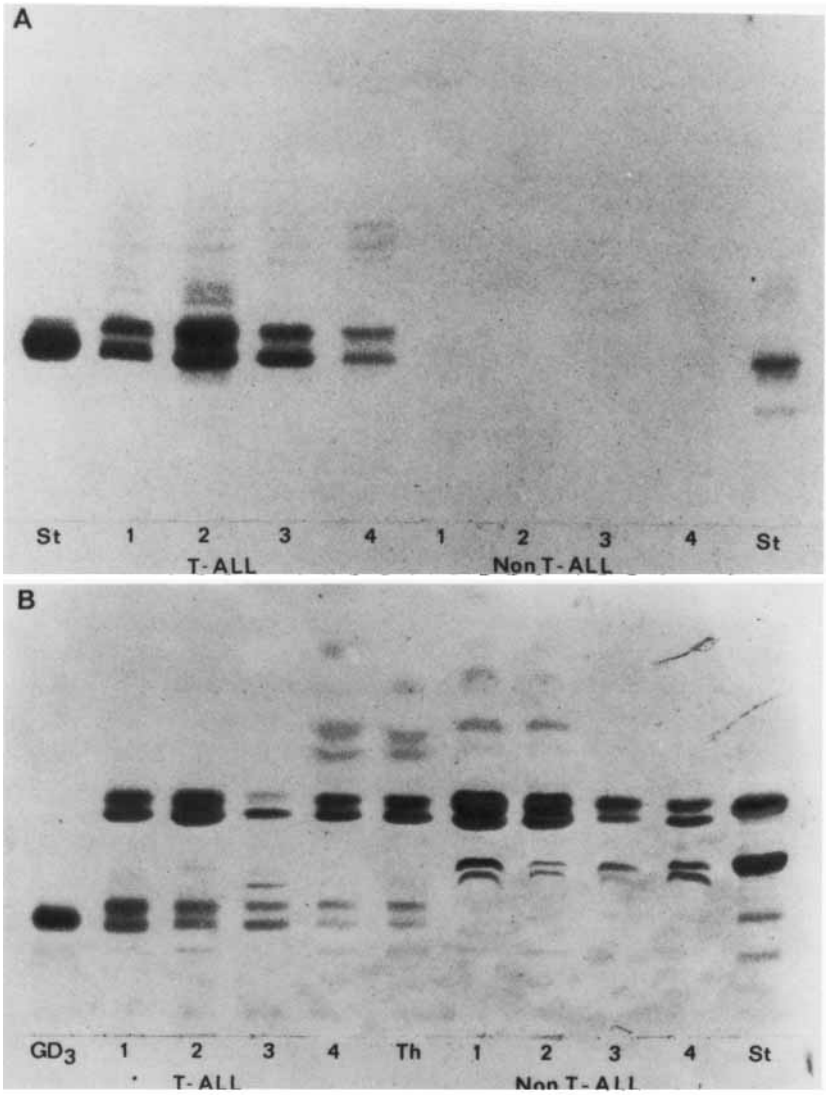

Fig. 1. HPTLC immunostaining of T-ALL and non-T-ALL gangliosides with antiganglioside monoclonal antibodies. **A:** Anti-GD_3 immunostaining. Gangliosides extracted from four T-ALL patient blasts (T-ALL, 1–4) and four non-T-ALL patient blasts (Non T-ALL, 1–4) were developed along with standards (ST, left side: GM_3 and GD_3; right side, total human erythrocyte membrane gangliosides, 25 ng ganglioside/standard; 10–50 ng ganglioside of each leukemia sample was spotted. Anti-GD_3 was utilized at 5 μg/ml. Anti-GD_3 detects the GD_3 standard and GD_3 in each T-ALL extract but not GM_3 or erythrocyte SPG. Small quantities of GD_3 and another more polar ganglioside, probably disialoparagloboside, which also reacts with R_{24} [13], are also visualized in the erythrocyte standard with anti-GD_3. **B:** Anti-GD_3 plus anti-GM_3/SPG immunostaining. Gangliosides from four T-ALL extracts (T-ALL, 1–4) and four non-T-ALL extracts (Non-T-ALL, 1–4) and a thymocyte sample were developed along with standards GD_3 (left side) and GM_3 plus total erythrocyte membrane gangliosides (20 ng of each standard). Gangliosides from 1–2 × 10^8 cells were spotted from each sample. Samples 1 and 2 for T-ALL and samples 3 and 4 for non-T-ALL were those spotted in the same lanes in A, while the other samples in each case were from different leukemias. The antibody mixture detected any GM_3, GD_3, or SPG in each sample, as well as low levels of several contaminating lipids in certain samples.

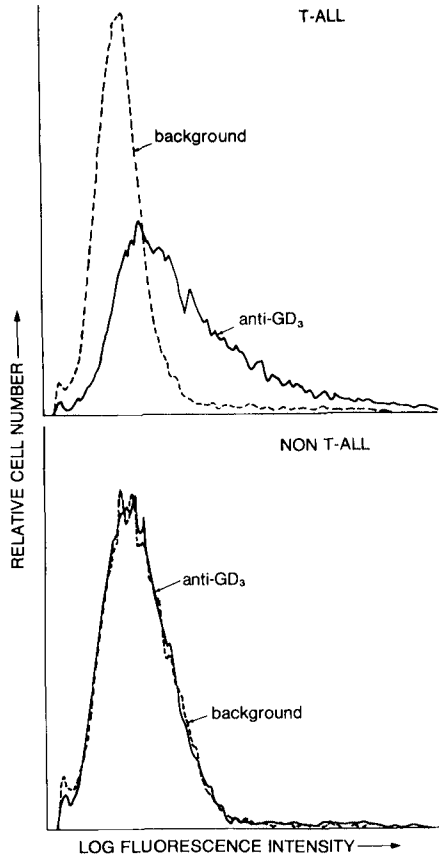

Fig. 2. GD_3 antigen expression on T-ALL and non-T-ALL. Cells obtained from T-cell ALL and non-T-ALL patients were stained with anti-GD_3 R_{24} monoclonal antibody and analyzed by flow cytometry as discussed in Materials and Methods. In this experiment, 51.5% of the T-ALL cells and 1.8% of the non-T-ALL cells stained positive with the anti-GD_3 monoclonal antibody.

20 positive samples, greater than 50% of the cells were positive for R_{24}. On the other hand, zero out of 11 non-T-ALL samples studied reacted with this antibody. The results of these immunofluorescence studies indicate that GD_3 is expressed at the cell surface of a large population of T-ALL blasts but is not found in non-T-ALL blasts.

DISCUSSION

This report confirms and extends our previous findings, which indicated that T-cell ALL and non-T-ALL can be distinguished on the basis of GD_3 ganglioside content [14]. Our earlier work was limited to resorcinol detection of gangliosides which were isolated from blasts from seven T-cell lymphoblastic malignancies and from three non-T-ALL patients. The current study shows that at the nanogram level of detection, utilizing the technique of HPTLC immunostaining with an anti-GD_3 antibody, GD_3 was not found in ganglioside extracts of cells from all six non-T ALL patients examined, whereas this antibody detected significant amounts of GD_3 in extracts of cells from six T-ALL patients.

Furthermore, indirect immunofluorescence determination with R_{24} revealed positive staining in blasts from a large majority of patients with T-ALL, while staining was not observed in cells from patients with non-T-ALL either by fluorescence microscopy or by the more sensitive technique of FACS analysis.

Interestingly, we have not observed GD_3 in lymphoblasts of all of the T-ALL patients tested: eight out of 28 of the patients had less than 10% blasts positive for R_{24}, and FACS analysis and fluorescence microscopy analysis to date have shown that the population of GD_3-positive T-ALL cells is highly heterogeneous with respect to extent of surface expression. Since resorcinol [14] and immunostaining patterns (Fig. 1) of T-ALL also show variation in the relative amount of GD_3, heterogeneity of R_{24} staining by immunofluorescence is probably not due to variation in cryptogenicity of the ganglioside at the cell surface but rather due to true variation of GD_3 content among and within the lymphoblast populations.

A significant, albeit low level of HPTLC immunostaining of thymocyte gangliosides with R_{24} anti-GD_3 was observed. Thus, a clear difference exists in the amount of GD_3 found in thymocytes and in the amount found in non-T-ALL. With preliminary FACS analysis, we have also observed R_{24} staining in thymocytes at a level intermediate between that of non-T-ALL and T-cell ALL. Recently, Hersey et al. [36] have shown that while unseparated peripheral blood lymphocytes express a very low but detectable level of GD_3, as measured by binding of R_{24}, cells stimulated by lectin bound significantly higher amounts of anti-GD_3. These studies, together with our observation of the heterogeneous expression of GD_3 within a patient population of T-ALL, suggest that the expression of GD_3 may be a function of the state of blastogenesis in T cells, and that leukemogenesis may involve the immortalization of a particular differentiative state of the thymocyte.

The function of GD_3 in T-ALL is unknown; however, lack of GD_3 in certain cases of T-ALL suggests that this particular ganglioside is not absolutely required for maintenance of the transformed state. It has been recently shown that anti-GD_3 antibodies can enhance the proliferation of lectin-stimulated and interleukin 2 (IL-2)-driven lymphocytes [36]. Thus, GD_3 in T-ALL may be a potent selection molecule expressed at the cell surface which mediates the proliferation of these cells. Although GD_3 may be the most potent functional ganglioside for T-cell growth, other gangliosides may take the place of GD_3 in certain rare cases of T-ALL. SPG may function similarly in non-T-ALL.

A second possible mechanism for selective expression of GD_3 at the surface of T-ALL relates to the potential immunosuppressive activity of this ganglioside. Ladisch has shown that ganglioside GD_2 which accumulates in neuroblastomas is found in high concentrations in the serum of neuroblastoma patients [8,37]. Gangliosides shed from murine lymphoma cells [38] as well as those isolated from the lymphoblasts of patients with chronic or acute nonlymphocytic leukemias [39] are immunosuppressive, and both macrophage function [40] and IL-2-driven proliferation [41] are inhibited by gangliosides. The immunosuppressive activity of gangliosides in vivo has been recently reported by Ladisch et al. [42]. Although the presence of increased ganglioside GD_3 in T-ALL patient serum and ganglioside shedding from T-ALL cells have not been shown, such studies are in progress in our laboratory to examine this hypothesis.

Our findings that GD_3 is expressed at the cell surface of a large subpopulation of T-ALL and that this ganglioside is detectable in these cells with a well-described anti-GD_3 antibody suggest a possible clinical utility for anti-GD_3 antibodies in the diagnosis

and therapy of T-ALL. The R_{24} antibody is reactive to leukemic T-lymphoblasts at a concentration similar to that which is reactive to melanoma cells (40–50 μg/ml) [21]. This antibody is cytotoxic to melanoma cells in vitro, in both complement-mediated and cell-mediated cytotoxicity assays [19,20], and we have recently demonstrated in vitro cytotoxicity of R_{24} to T-cell leukemic lymphoblasts exhibiting GD_3 [43]. R_{24}, currently in use in phase 1 trials for melanoma [21], or other anti-GD_3 antibodies, may be useful immunotherapeutic agents for T-cell ALL either alone or in the form of an immunocytotoxic conjugate. Those patients which express > 50% R_{24}-positive cells would be particularly good candidates for this form of therapy. Furthermore, anti-GD_3 antibody may be a useful purging agent in autologous bone marrow transplantation therapy in T-ALL. Monoclonal antibodies raised against normal human granulocytes and specific for blood group fucooligosaccharides expressed on both glycolipids and glycoproteins are now being used in autologous bone marrow purging regimens for treatment of acute myelogenous leukemia [44]. The encouraging results of this study suggest that specific antiganglioside monoclonal antibodies such as anti-GD_3 may be similarly useful agents for this type of therapy in T-cell ALL.

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