[CANCER RESEARCH 44, 5262–5265, November 1984]

Differential Expression of Ganglioside G_{D3} by Human Leukocytes and Leukemia Cells[1]

Bader Siddiqui, Joanne Buehler, Michael W. DeGregorio,[2] and Bruce A. Macher[3]

Gastrointestinal Research Laboratory, Veterans Administration Hospital, San Francisco 94143 [B. S.]; Cancer Research Institute [J. B., B. A. M.] and Department of Pharmaceutical Chemistry [B. A. M.], University of California, San Francisco 94143; and Children's Cancer Research Institute, Pacific Medical Center, San Francisco, California 94115 [M. W. D.]

ABSTRACT

Gangliosides from normal leukocytes and the cells of 25 patients with acute and chronic leukemia were tested for the presence of the disialoganglioside II^3-α-*N*-acetylneuraminosyl-α2→8-*N*-acetylneuraminosyllactosylceramide (G_{D3}). G_{D3} was detected by immunostaining thin-layer chromatographs with an anti-G_{D3} monoclonal antibody (AbR_{24}). Among the myeloid cells tested, acute leukemia cells were positive for G_{D3}, whereas chronic leukemia cells and normal neutrophils did not have detectable G_{D3}. A range of G_{D3} reactivity was apparent within the acute myeloid leukemia cells; gangliosides from pure myeloid leukemia cells stained more intensely than those from leukemia cells with monocytic characteristics. All lymphocytic leukemia cells (chronic and acute) contained G_{D3}, but this ganglioside could not be detected in extracts from normal lymphocytes. A ganglioside extract from the cells of a patient with hairy cell leukemia was also positive for G_{D3} immunostaining. These results demonstrate that normal leukocytes and chronic myelogenous leukemia cells are distinguished from other lymphoid and nonlymphoid leukemia cells on the basis of G_{D3} ganglioside expression.

INTRODUCTION

Our analyses of the glycosphingolipids of normal human leukocytes (7) and leukemia cells (4, 13, 14) have shown that these cells contain several types of monosialogangliosides but only one disialoganglioside, G_{D3}.[4] Of 8 patients with acute leukemia previously studied, only one contained G_{D3}. This ganglioside was also detected in the cells of a CLL patient but not in the cells of 2 hairy cell leukemia patients or in neutrophils from normal donors. These analyses were done by classical methods which require μg quantities of gangliosides. Recently, a sensitive immunostaining technique (8) has been developed which allows detection of ng quantities of glycosphingolipids. We have used this method and a hybridoma-derived, monoclonal anti-G_{D3} antibody (AbR_{24}) (2, 10) to determine the distribution of G_{D3} among normal leukocytes and leukemia cells. Our results show a differentiation and cell lineage-related distribution of G_{D3} in human leukocytes.

MATERIALS AND METHODS

Human Leukocytes and Leukemia Cells. Cells from 25 newly diagnosed and untreated patients with leukemia were available for analysis. The leukemic cells were classified according to the French-American-British classification scheme (1). Lymphoblasts were considered to be of T-cell type if they formed rosettes with sheep RBC. Normal leukocytes were obtained from blood products prepared at Irwin Memorial Blood Bank, San Francisco, CA. Procedures for purifying normal neutrophils, lymphocytes, and leukemic cells have been described previously (7, 14).

Isolation and Purification of Gangliosides. Cells were extracted with 20 volumes of each of the following chloroform:methanol mixtures (v/v): 2:1, 1:1, and 1:2. The total lipid extract was dried, dissolved in chloroform:methanol:water, 30:60:8 (v/v), and chromatographed on a column (1 × 20 cm) of DEAE-Sephadex. Neutral glycosphingolipids were eluted with 2 column volumes of the starting solvent and 1 column volume of methanol. Gangliosides were displaced from the column with 4 column volumes of 0.2 M sodium acetate in methanol. The gangliosides were dialyzed, lyophilized, and Folch (3) partitioned to remove contaminating phospholipids. In some cases (Fig. 4), gangliosides were separated by HPLC to obtain fractions enriched in G_{D3}. This was done by chromatographing the Folch partition upper phase on a column (2.2 mm × 25 cm) of Iatrobeads (6RS-8010; Iatron Chemical Products, Tokyo, Japan) using a linear gradient of isopropyl alcohol:hexane:water, 55:35:10 to 55:30:15, at 2 ml/min for 150 min. Lipid-bound sialic acid was determined by the resorcinol method (12). Sample amounts are expressed as μg of gangliosides assuming a molelcular weight of 1800. Gangliosides were separated by thin-layer chromatography on Silica Gel 60 HPTLC plates, using chloroform:methanol:0.2% $CaCl_2$, 55:50:9 (v/v), and visualized with resorcinol reagent or immunostained. For immunostaining, the plates were dipped (20 sec) in a solution of 0.05% polyisobutylmethylacrylate (Polyscience Inc., Warrington, PA) in hexane, air dried, and soaked in PBS:BSA for 10 to 15 min. The plate was placed in a solution of AbR_{24} (1:500 dilution in PBS:BSA) for 2.5 hr, rinsed with 0.9% NaCl solution (saline), and soaked in a solution of ^{125}I-Protein A (6) (10^6 cpm/ml in PBS:BSA) for 2 to 3 hr. All incubations were done at room temperature. The plate was washed 5 times with saline and dried, and an autoradiograph was prepared (Kodak X-O-mat RXR, 24 to 48 hr at −70°).

RESULTS

TLC Profiles of Human Leukocyte and Leukemia Cell Gangliosides. Gangliosides were obtained from 3 preparations of normal human neutrophils and one preparation of normal human lymphocytes. Leukocytes from 4 normal donors were combined to obtain a mixed-leukocyte ganglioside preparation. Gangliosides were also isolated from the cells of 25 patients with leukemia. Each ganglioside preparation was quantitated (see

[1] This study was supported in part by Grant 1462 from the Council for Tobacco Research-USA, Inc.

[2] Recipient of Louis R. Lurie Foundation funds.

[3] Recipient of National Cancer Institute Grant CA 32826. To whom requests for reprints should be addressed.

[4] The abbreviations used are: G_{D3}, II^3-α-*N*-acetylneuraminosyl-α2→8-*N*-acetylneuraminosyllactosylceramide; DSPG, disialoparagloboside or IV^3-α-*N*-acetylneuraminosyl-α2→8-*N*-acetylneuraminosylneolactotetraosylceramide; G_{M3}, II^3-α-*N*-acetylneuraminosyllactosylceramide; G_{T1a}, II^3-α-*N*-acetylneuraminosyl-α2→8-*N*-acetylneuraminosyl-IV^3-α-*N*-acetylneuraminosylgangliotetraosylceramide; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; ANLL, acute nonlymphocytic leukemia; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia; AMoL, acute monocytic leukemia; AMMoL, acute myelomonocytic leukemia; TLC, thin-layer chromatography; BSA, 1% solution of bovine serum albumin; PBS, phosphate-buffered saline (150 mM NaCl:10 mM phosphate, pH 7.4); HPLC, high-performance liquid chromatography.

Received May 8, 1984; accepted August 3, 1984.

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Refs. 4, 7, 13, and 14 for data on the concentration of ganglioside per cell) by the method of Svennerholm (12) and examined by TLC (Fig. 1). Resorcinol-positive bands (which chromatographed with G_{M3}) were apparent in each sample. All samples also contained more polar ganglioside bands, some of which had relative mobilities similar to that of G_{D3} (see especially Fig. 1*B*, *Lanes 2*, *3*, *6*, and *7*; and Fig. 1*C*, *Lane 7*). CML cells contained bands which chromatograph near G_{D3} (Fig. 1A, *Lanes 4* to *8*); however, we have recently shown that this compound is VI^3NeuAc-nLc-Ose_6Cer (14).

Immunostaining of Leukocyte Gangliosides with AbR_{24}. Preliminary experiments with gangliosides isolated from cells of 2 leukemia patients which we had previously characterized (4, 13) as containing G_{D3} demonstrated that the amount of ganglioside extract needed to obtain a positive immunostain varied by more than 50-fold. Therefore, ganglioside samples from new patients were analyzed at various concentrations ranging from 0.1 to 15 μg. Pukel *et al.* (10) previously determined that the immunostaining assay with AbR_{24} could detect 25 ng of G_{D3}, a result which we have confirmed. Therefore, a negative response with 15 μg of ganglioside would indicate that G_{D3} would represent less than 0.2% of the ganglioside being tested.

Anti-G_{D3} Binding to Human Myeloid Cell Gangliosides. Ganglioside from 3 groups of myeloid cells were tested by immunostaining for the presence of G_{D3}: normal human neutrophils; CML cells; and ANLL. Normal neutrophils (3 donors) and CML cells (5 patients) were negative for G_{D3} when tested (15 μg of gangliosides for neutrophils and 12 μg for CML cells). Gangliosides from the cells of patients with 4 forms of ANLL were tested by immunostaining for the presence of G_{D3}. Fig. 2 shows an autoradiograph of a TLC plate on which 3 μg of ganglioside were chromatographed from the cells of 12 ANLL patients. All of the ganglioside preparations contained a component which bound AbR_{24}, but the amounts of this component differed dramatically. The greatest amount of G_{D3} was detected in ganglioside preparations from the cells of 2 ANLL patients with AML (Fig. 2, *Lanes 1* and *2*). Gangliosides from 4 other AML patients (Fig. 2, *Lanes 3* to *6*) contained less, but detectable, amounts of G_{D3}. In addition to the G_{D3} bands, 2 other bands are visible in *Lanes 1* and *2* (Fig. 2*A*). In *Lane 1*, bands running in front of G_{D3} are seen which may represent an additional ganglioside component which is recognized by AbR_{24} but which may also be due to smearing of the G_{D3} in this sample. Bands more polar than G_{D3} are seen in *Lane 2*. These bands chromatographed near standard DSPG (data not shown). When a TLC of DSPG and G_{D3} was immunostained with AbR_{24}, both compounds were found to bind the antibody (Fig. 3). Therefore, the bands running below G_{D3} in *Lane 2* (Fig. 2) are most probably due to DSPG. This compound was not found in gangliosides from any of the other cells tested in this study.

Gangliosides from 2 other sets of ANLL cells were tested for AbR_{24} binding. *Lanes 7* and *8* (Fig. 2) show that gangliosides from AmoL patients contain small but detectable quantities of G_{D3}. Greater quantities of G_{D3} were found in the gangliosides of 4 AMMoL patients (Fig. 2, *Lanes 9* to *12*). A variation in the amount of G_{D3} was also found among the AMMoL patients tested. In summary, all of the immature myeloid cells (ANLL) tested contained G_{D3}, whereas mature myeloid cells (normal neutrophils and CML cells) did not contain G_{D3}.

Anti-G_{D3} Binding to Human Lymphoid Cell Gangliosides. Gangliosides were obtained from 2 preparations of normal lymphocytes, 4 preparations of ALL cells, and 3 preparations of CLL cells and were immunostained with AbR_{24}. G_{D3} was detected in all samples except those of normal lymphocytes (15 μg of ganglioside tested). Fig. 4 shows an autoradiograph obtained from an immunostained TLC plate which contained 6 μg of gangliosides from each ALL sample and 3 μg of gangliosides from each CLL sample. This experiment demonstrates that CLL cells contain greater quantities of G_{D3} than do ALL cells; quantities which were similar to those of AML cells. Therefore, all of the lymphocytic leukemia cells tested contain G_{D3}.

Gangliosides were also prepared from the cells of 2 patients with hairy cell leukemia and tested with AbR_{24}. Fig. 4, *Lane 8*, shows that G_{D3} was detectable when 6 μg of hairy cell gangliosides were tested (the immunostaining pattern for the second patient was similar).

Identification of G_{D3} in Polar Ganglioside Fractions of Normal Human Leukocytes. When total ganglioside extracts from human neutrophils and lymphocytes were tested with AbR_{24}, G_{D3} could not be detected even when 15 μg of gangliosides were analyzed. To determine if normal human leukocytes did indeed lack G_{D3}, total gangliosides from these cells were separated by HPLC to obtain polar ganglioside fractions. Gangliosides from the cells of one of the ANLL patients (see Fig. 1*B*, *Lane 6*, and Fig. 2, *Lane 5*) were separated by HPLC to serve as a positive control. Immunostaining of TLC plates from these HPLC runs are shown in Fig. 5*A*. G_{D3} was detected in the second major ganglioside fraction from the ANLL cells (Fig. 5*B*). This fraction was devoid of G_{M3} and represented less than 20% of the starting material (0.5 μg of ganglioside was spotted from each fraction). The HPLC procedure resulted in an approximate 5-fold enrichment of G_{D3}.

Immunostaining of the HPLC fractions of normal leukocyte gangliosides gave a faint band in Fraction 2. In this case, 5 μg of each fraction were spotted. Assuming that the HPLC procedure consistently resulted in a 5-fold enrichment of G_{D3}, this ganglioside represents less than 0.05% of the total leukocyte gangliosides. Such a minor amount may represent a contaminant from serum (9, 11); therefore, normal leukocyte may be devoid of G_{D3}.

DISCUSSION

Our results show that there is a direct relationship between the expression of G_{D3} by human myeloid leukemia cells and their degree of differentiation, with only immature cells expressing the ganglioside. Among human lymphoid cells, G_{D3} is expressed by both immature and mature cell forms of leukemic leukocytes. G_{D3} was undetectable in lipid extracts from normal neutrophils and lymphocytes but could be detected in purified preparation of human leukocyte gangliosides following enrichment of the polar ganglioside fraction by HPLC. Therefore, G_{D3} expression in human leukocytes and leukemia cells apparently is regulated by one or more of the following: cell lineage; degree of cellular differentiation; and/or leukemogenesis. Further studies detailing the presence or absence of G_{D3} in ganglioside extracts from normal, immature leukocytes are required to determine if the expression of G_{D3} occurs only with leukemogenesis or is related to normal differentiation.

The antibody used in our study was thought to react only with G_{D3}, but we have demonstrated that AbR_{24} also immunostains DSPG. Recently, Hansson *et al.* (5) have reported that an anti-

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melanoma antibody (4.2 antibody) which binds to G_{D3} also immunostains DSPG. These 2 gangliosides share the following nonreducing terminal sequence NeuAcα2→8NeuAcα2→3Gal. Another ganglioside (*e.g.*, G_{T1a}), which has the same terminal trisaccharide, was not recognized by AbR_{24} (10). Therefore, NeuAcα2→8NeuAcα2→3Gal is required for binding, but other structural features must also affect binding. In our analysis of leukemia cell gangliosides, only one sample contained a component that was more polar than G_{D3} and which reacted with AbR_{24}. Although the AbR_{24}-reactive compound had chromatographic properties similar to those of DSPG, further analyses are required to determine its exact structure.

ACKNOWLEDGMENTS

We would like to thank Dr. Lloyd J. Old, Memorial Sloan-Kettering Cancer Center, for providing the antibody AbR_{24}; Dr. Samar Kundu for the gift of human erythrocyte DSPG; and Jim Harris for typing this manuscript. Michael W. DeGregorio would like to thank Dr. Jordan R. Wilbur for his support.

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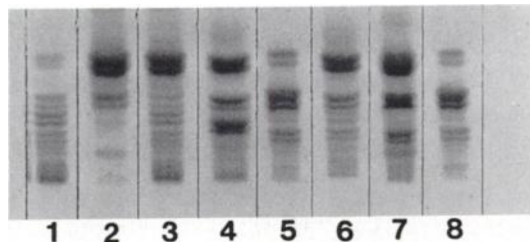
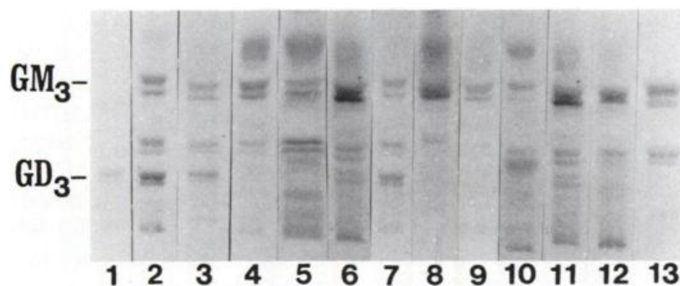


1A



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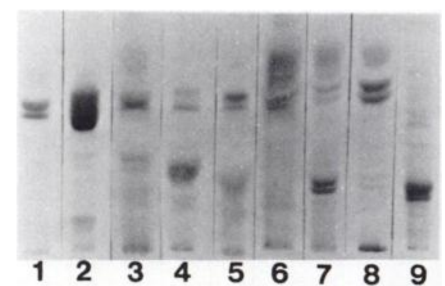
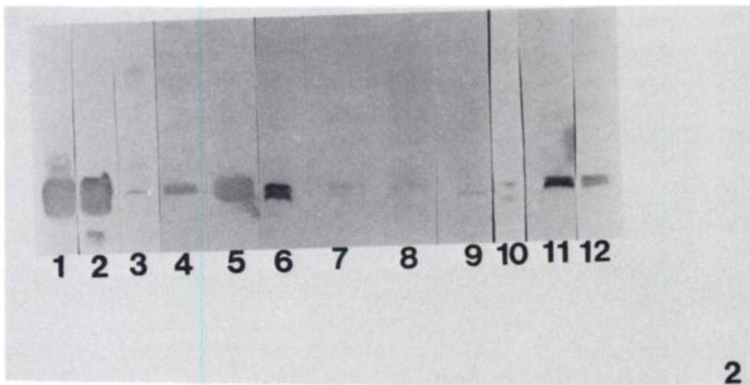
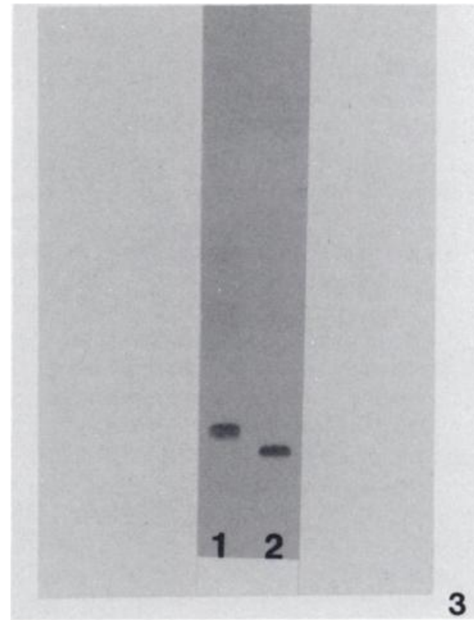
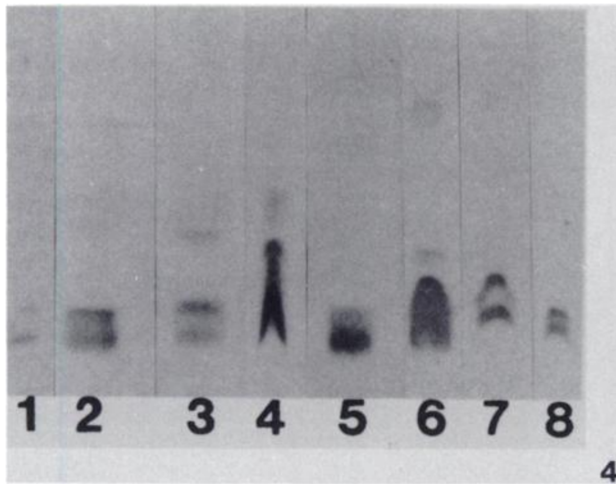


1C

Fig. 1. Thin-layer chromatograms of gangliosides from normal human leukocytes and leukemia cells. The separation was on a plate of Silica Gel 60 (HPTLC) using the solvent system $CHCl_3$:methanol:0.2% $CaCl_2$, 55:40:9. *A, Lane 1,* normal neutrophils; *Lane 2,* normal lymphocytes; *Lane 3,* normal leukocytes; *Lanes 4* to *8,* CML cells from 5 patients (each lane contains 8 to 10 μg lipid-bound sialic acid). *B, Lane 1,* standard G_{D3}; *Lanes 2* to *7,* AML cells from 6 patients; *Lanes 8* and *9,* AMoL cells from 2 patients; *Lanes 10* to *13,* AMMoL cells from 4 patients (each lane contains 4 to 6 μg lipid-bound sialic acid). *C, Lanes 1* to *4,* ALL cells from 4 patients; *Lanes 5* to *7,* CLL cells from 3 patients; *Lane 8,* hairy cell leukemia cells; *Lane 9,* G_{D3} standard (each lane contains 4 to 6 μg lipid-bound sialic acid).







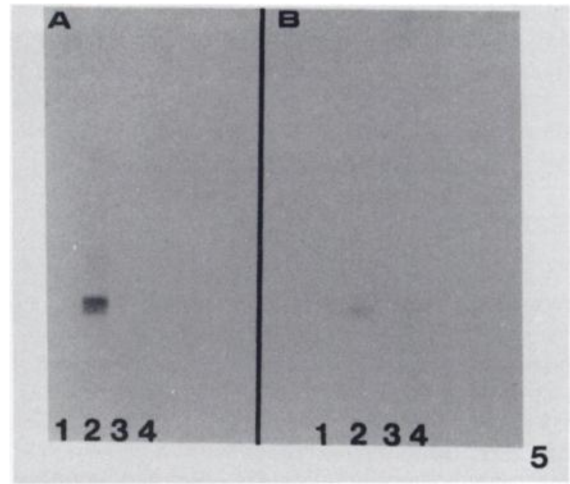


Fig. 2. Autoradiographs of AbR_{24} (anti-G_{D3}) immunostains of gangliosides from human myeloid leukemia cells. *Lanes 1* to *6*, AML cells (see Fig. 1*B*, *Lanes 2* to *7*); *Lanes 7* and *8*, AMoL cells (see Fig. 1*B*, *Lanes 8* and *9*); *Lanes 9* to *12*, AMMoL cells (see Fig. 1*B*, *Lanes 10* to *13*).

Fig. 3. Autoradiograph of AbR_{24} immunostain of G_{D3} and DSPG gangliosides. *Lane 1*, AbR_{24} binding to G_{D3}; *Lane 2*, AbR_{24} binding to DSPG.

Fig. 4. Autoradiograph of AbR_{24} (anti-G_{D3}) immunostains of gangliosides from human lymphoid and hairy cell leukemia cells. *Lanes 1* to *4*, ALL cells (see Fig. 1*C*, *Lanes 1* to *4*); *Lanes 5* to *7*, CLL cells (see Fig. 1*C*, *Lanes 5* to *7*); *Lane 8*, cells from a patient with hairy cell leukemia (see Fig. 1*C*, *lane 8*).

Fig. 5. Autoradiograph of AbR_{24} immunostains of leukocyte gangliosides separated by HPLC. *A*, *Lanes 1* to *4*, HPLC fractions from AML patient gangliosides (see Fig. 1*B*, *Lane 6*, and Fig. 2*A*, *Lane 5*); *B*, *Lanes 1* to *4*, HPLC fractions from normal human leukocyte gangliosides (see Fig. 1*A*, *Lane 3*).

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