7-O-Acetyl-GD₃ in Human T-lymphocytes Is Detected by a Specific T-cell-activating Monoclonal Antibody*

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The monoclonal antibody U5, which is a potent inducer of proliferation in human T-cells, was found to bind to an alkali-sensitive derivative of ganglioside GD₃. Using immunochromatographic and spectroscopic methods, the structure of the U5 antigen was determined as 7-O-acetyl-GD₃. The antibody U5 did not react with 9-O-acetyl-GD₃ and bound severalfold more strongly to 7-O-acetyl-GD₃ than to GD₃. U5 is the first antibody known to detect preferentially 7-O-acetyl-GD₃. Flow cytometric analysis showed that each major class of human leukocytes contained a significant fraction of cells binding the U5 antibody.

Gangliosides are sialic acid-containing glycosphingolipids (GSLs) consisting of an oligosaccharide chain attached to a lipid core structure. They are plasma membrane constituents of all mammalian cells. Recently, we showed that normal human leukocytes contain disialogangliosides with an 9-O-acetyl group on their terminal sialic acid (1). Not only was there a very restricted surface expression of this GSL on human blood cells (2), but it was also found to be the first surface marker for helper cells within the CD8 positive T-cell population (3). These findings suggested that slight modifications of cell surface molecules, such as O-acetylation, might suffice to define new functional subpopulations of leukocytes. This hypothesis is in accordance with observations that the pattern of glycolipids expressed on human hematopoietic cells is cell type-specific (4-6). Our studies also indicated that O-acetylated disialogangliosides other than the 9-O-acetylated forms were present on human cells (1). During the Fifth Workshop and Conference on Human Leukocyte Differentiation Antigens (Boston, 1993), we presented evidence that a monoclonal antibody (mAb), U5, bound strongly to an alkali labile form of GD₃, which was different from 9-O-acetyl-GD₃ and, furthermore, that antibodies specific for 9-O-acetyl-GD₃ failed to bind to this labile GD₃ derivative. This, taken together with the observation that binding of mAb U5 to human CD4⁺ and CD8⁺ cells is induced a strong T-cell proliferation, which was accompanied by up-regulation of antigen expression (7), stimulated our interest in characterizing the structure of the U5 antigen. In this report, we describe the purification of the U5 antigen and identify it as the ganglioside 7-O-acetyl-GD₃. In addition, the distribution of the U5 antigen on human blood cells is analyzed.

EXPERIMENTAL PROCEDURES

Antibodies—mAb U5, R24, and E11 were prepared as described previously (7). mAb UM4D4 (CDw60) (8) was donated by Dr. D. A. Fox, University of Michigan, Ann Arbor, MI. The mAb M-T32 (CDw60) (9) was a kind gift of Dr. E.P. Rieber, University of Dresden, F.R.G.

Purification of the U5 Antigen, of 9-O-Acetyl-GD₃, and of GD₃ from Bovine Buttermilk—1460 g of buttermilk powder, obtained by freeze drying 7.5 liters of buttermilk, was suspended in 7.5 liters of chloroform/methanol/water (30:60:8) (v/v/v) and stirred for 1 h at ambient temperature. The suspension was filtered under reduced pressure through a Buchner funnel. The residue was reextracted twice as above. The extracts were combined, and the solvent was evaporated in vacuo at a maximum of 25 °C. The evaporated lipid extract was dialyzed for 3 days at 4 °C against several changes of water. The desalted extract was lyophilized and dissolved in chloroform/methanol/water (30:60:8) (v/v/v). After removal of insoluble material, the extract was pumped onto a 3.5 × 20-cm column filled with DEAE-Sepharose (acetate form) in three separate runs. Elution was performed with 1 liter of chloroform/methanol/water (30:60:8), 1 liter of methanol, 1 liter of 20 mM, 2 liters of 50 mM, and finally with 1 liter of 150 mM ammonium acetate in methanol, respectively. The 50 mM ammonium acetate eluates contained all of the U5 antigen, the 9-O-acetyl-GD₃, and the GD₃, as shown by immunostaining of thin-layer chromatograms using the mAbs U5, M-T32, and R24, respectively (see below). The 50 mM ammonium acetate eluates were pooled, concentrated by evaporation, dialyzed, lyophilized, dissolved in chloroform/methanol/water (85:15) (v/v) and pumped onto a HPLC column (16 × 500 mm) filled with LiChrosorb Si 60 5-μm particles (Merck). Elution was performed using a linear gradient from chloroform/methanol/water (v/v/v) (82:6.16:4.1) to (40:50:10) in 400 min at a flow rate of 2 ml/min. Fractions were collected every 2 min. Fractions 112–128 contained 9-O-acetyl-GD₃. Fractions 129–134 contained the U5 antigen, fractions 135–140 contained a mixture of the U5 antigen and GD₃, and fractions 141–159 contained GD₃. Fractions 129–134 were pooled and further purified on an analytical 4 × 250 mm Partisil 5-μm silica HPLC column (Whatman Ltd., Maidstone, United Kingdom) using a gradient from chloroform/methanol/water (v/v/v) (82:6.16:4.1) to (40:50:10) in 200 min at 1 ml/min. Fractions were collected every minute. Fractions 36–40 contained the U5 antigen together with an impurity that showed a somewhat higher chromatographic mobility than the U5 antigen. After a final HPLC separation using the Partisil column and a gradient from chloroform/methanol/water (v/v/v) (82:6.16:4.1) to (50:45:5) in 200 min at 1 ml/min (200 fractions), apparently pure U5 antigen (about 87 μg) was found in the fractions 57–65. Fractions 54–56 and 66–75 also contained the U5 antigen.

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†† The abbreviations used are: GSL, ganglioside; mAb, monoclonal antibody; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; GD₃, Neu5Acα₂→8Neu5Acα₂→3Galβ1→4Glcφ1→1′-ceramide; 7-O-acetyl-GD₃, Neu5,7Acα₂→8Neu5Acα₂→3Galβ1→4Glcφ1→1′-ceramide; 9-O-acetyl-GD₃, Neu5,9Acα₂→8Neu5Acα₂→3Galβ1→4Glcφ1→1′-ceramide; HPTLC, high performance thin layer chromatography; DIG, digoxigenin-succinyl-e-amino-caproyl acid hydrazide; PBS, phosphate-buffered saline; Neu5,7Acα₂, 5-N-acetyl, 7-O-acetyleneuraminic acid; Neu5,9Acα₂, 5-N-acetyl, 9-O-acetyleneuraminic acid; 9-O-acetyl-DSPG, Neu5,9Acα₂→8Neu5Acα₂→3Galβ1→4GlcNAcα1→3Galβ1→4Glcφ1→1′-ceramide; Gb1₃₄, 1′(NeuAc)₃ NeuAc-GGosCer; Gb1₃₃, 1′(NeuAc)₂ NeuAc-GGosCer; Gb1₃₂, 1′(NeuAc)₁ NeuAc-GGosCer.
contained the US antigen as major component but of less purity. The antigen containing fractions were dried and stored at −70 °C.

Preparation of the Disialoganglioside Fraction from Unseparated Human Leukocytes—The disialogangliosides were prepared exactly as has been described previously (1).

Thin Layer Chromatography (TLC)—TLC analysis was carried out on TLC (HPTLC) silica gel 60 (Merck). The running solvent was chloroform/methanol/water (50:40:10) (v/v/v) on high performance TLC (HPTLC) silica gel 60 plates (Merck). The has been described previously (1).

Quantitation—For quantitation of TLC-separated gangliosides, the HPTLC plates were sprayed with resorcinol/HCl, and then covered with a glass plate and heated at 95 °C for 30 min. Densitometric measurements were made in transmission mode at 580 nm (10) using a Shimadzu dual wavelength TLC Scanner CS9501 (PC, Shimadzu, Düsseldorf, FRG). 0.5–3.0 μg of G1a was used for calibration.

TLC Immunostaining—Ganglioside antigens separated on HPTLC plates were detected by immunostaining using the method of Betheke et al. (11) with modifications (12).

Nonspecific Immunochromochromic Detection of Gangliosides on HPTLC Plates—by Digoxigenin-succinyl-ε-aminoacryloic Acid Hydrazide (DIG) Labeling—5-Bromo-4-chloro-indolyl-3-phosphate, p-toluidine salt, was obtained from Boimol, Hamburg, FRG. All other reagents were purchased from Boehringer Mannheim. DIG labeling was performed essentially as described previously (13). In order to obtain improved sensitivity, a 1:333 dilution of the DIG-ε-aminoacryloic acid hydrazide solution and incubation with the phosphatase-conjugated antidigoxigenin antibody (1:2000 dilution) was prolonged to 48 h at 30 °C. Total gangliosides were detected after O-deacetylation as described below.

Isomerization of the US Antigen to 9-O-Acetyl-GD3—Dried US antigen in a glass test tube was incubated in 20 μl aqueous ammonia for 30 min at ambient temperature (14) followed by lyophilization. US antigen on HPTLC plates immobilized with polyisobutylmethacrylate (13) was isomerized by incubation of the plate in 0.1 n glycine-NaOH buffer, pH 10.0 for 2 h at 37 °C (1). The plates were then washed 3 times for 5 min with phosphate-buffered saline (PBS), pH 7.3. Isomerized US antigen was visualized by immunostaining using mAb U5/800 as described above.

O-Deacetylation of Gangliosides—Alkaline hydrolysis of gangliosides after separation on HPTLC plates was performed by incubating the plates for 17 h at ambient temperature in a chamber with an atmosphere saturated with 13.3% aqueous ammonia. The ammonia-treated plates were dried for at least 3 h in vacuo in the presence of P2O5. In vitro O-deacetylation was done by treating the dried sample for 1 h at 37 °C with 1.0 or 13.3% aqueous ammonia followed by evaporation of the ammonia in vacuo at 30 °C.

Release and Characterization of O-Acetylated Sialic Acids—Sialic acids were released enzymatically from gangliosides, purified, and analyzed by HPLC as described previously (15, 14). To confirm the identity of 7-O-acetyl-5-N-acetylneuraminic acid, which was not easily separable from 7-acetylneuraminic acid, an aliquot of the released acid was treated with 20 μl aqueous ammonia as described previously (14). The expected rearrangement product 9-O-acetyl-5-N-acetylneuraminic acid was identified using an authentic standard, which was a kind gift from Dr. R. Schafer, Kiel, FRG.

Electrospray Mass Spectrometry—A Finnigan MAT TSQ 700 triple quadrupole mass spectrometer equipped with a Finnigan electrospray ion source (Finnigan MAT Corp., San Jose, CA) was used. The O-acetylated Gd3 derivative was dissolved in methanol and injected at a flow rate of 2 μl/min into the electrospray chamber. A voltage of 4.5 kV was applied to the electrospray needle. For collision-induced decomposition experiments, the doubly charged parent ions were selectively transmitted by the first mass analyzer and directed into the collision sphere saturated with 13.3N aqueous ammonia. The ammonia-treated ions were then transmitted by the first mass analyzer and directed into the collision sphere saturated with 13.3N aqueous ammonia. The ammonia-treated ions were then transmitted by the first mass analyzer and directed into the collision sphere saturated with 13.3N aqueous ammonia.

Phosphate buffer (Sigma) in the glycine-NaOH buffer. Absorbance was read at 405 nm in an SLT Spectra III ELISA reader (SLT, Crailsheim, FRG) after 1 h.

Isolation of Leukocyte Cell Populations—CD4+ and CD8+ T-cells were prepared by a combination of “panning” technique and complement-mediated lysis. Flow cytometric analysis showed that CD4+ and CD8+ T-cells comprised 90% of the respective preparations, with not more than 5% CD16+ NK cells and less than 2% CD8+CD4+ T-cells, B-cells, and monocytes.

CD3+ T-cells were obtained by depletion of monocytes and B-cells followed by complement-mediated lysis of CD16+ NK cells. CD16+ NK cells were isolated immunomagnetically using a magnetic cell sorting system (Miltenyi, Bergisch Gladbach, FRG). The final cell suspension contained 85–90% CD16+ with approximatively 6% CD3+ cells.

B-cells were also obtained by the panning technique. The adherent cell fraction contained 80% CD20+ cells, 4–8% monocytes, 4% CD16+, and 2–5% CD3+ cells. The above methods have been described in detail elsewhere.

Monocytes were purified to >90% by adherence to tissue culture dishes (Greiner, Solingen, FRG) for 60 min at 37 °C.

Granulocytes were prepared from the erythrocyte layer obtained by Ficoll-Hypaque centrifugation of peripheral blood mononuclear cells. Cells were suspended in 20 ml of PBS, pH 7.2, and 5 ml of dextran 250 solution (5% w/v) in physiological saline) were added. After 20 min of incubation at room temperature, the supernatant was removed and washed in 40 ml of PBS (150 × g, 7 min, without brake). Remaining erythrocytes were lysed using a solution containing 0.82% NH4Cl, 0.1% KHCO3, 0.1 ml EDTA, pH 7.27. The preparations consisted of 90% granulocytes as shown by flow cytometry.

Immunoprecipitation—Purified CD3+ T-cells (1 × 107) were washed three times with 50 ml Tris-HCl, pH 8.0, containing 0.15 M NaCl and 5 mM EDTA and solubilized in 1.4 ml of this buffer containing, in addition, 0.5% Nonidet P-40, 1% phenylmethylsulfonyl fluoride, and 10.5% aprotinin. The suspension was sonicated for 2 min and incubated 15 min on ice. After centrifugation (4000 × g, 4 °C, 15 min) the supernatant was prefiltered by stirring for 30 min at 4 °C with 100 μl of Pansorbin pellet (Pharma- cia Biotech Inc.), followed by incubation with 20 μg of mAb US800 of supernatant for 1 h under the same conditions.

500 μl of this cell lysate/antibody mixture were incubated under rotation with 50 μl of protein A-Sepharose (Phar macia) for 3 h or overnight at 4 °C. The protein A-Sepharose-antibody-antigen complex was washed three times with 50 ml Tris-HCl, pH 8.0, containing 0.15 M NaCl and 5 mM EDTA buffer by successive centrifugation (1250 × g, 3 min) and resuspension, and then incubated with 30 μl of lysis buffer (62.5 mM Tris, 2% SDS, 10% glycerol, pH 6.8) for 30 min in an ultrasonic bath. The suspension was then centrifuged (4000 × g, 4 °C, 15 min), and the resulting supernatant was shock frozen in liquid nitrogen.

Analysis of the Lipid Constituents of the Immunoprecipitates—The immunoprecipitates were analyzed as described previously (16).

Flow Cytometric Analysis of Cell Surface Antigen For immunofluorescence microslides, 2 × 105 purified cells were incubated with 5 μl of the purified anti-Gd3 antibodies or the control antibody H-141–30 (mouse IgG3 anti-H-2D, a kind gift from Dr. G. Hämmerling, Heidelberg, FRG) followed by 50 μl of goat anti-mouse IgG-fluorescein isothiocyanate (1:200) (Coulter Electronics, Krefeld, FRG) as secondary antibody. The conjugated antibody MAbGG-fluorescein isothiocyanate was purchased from Dianova (Hamburg, FRG). Cells were analyzed with a fluores- cence-activated cell sorter (FACSscan, Becton Dickinson, Heidelberg, FRG).

Proliferation Assays—Separated T-cells were incubated in triplicate in microwell culture plates (Nunclon 1–67008, Nunc, Roskilde, Denmark) in the presence or absence of the anti-Gd3 antibodies. The antibodies were used at final concentrations from 100 to 1.56 μg/ml. Phy- tohaemagglutinin (HA17, Wellcome Diagnostics, Dartford, UK) was applied at 0.5 μg/ml. The cultures were pulsed with 0.5 μCi/ml [3H]thymidine (Dupont NEN) for 18 h on day 5 of incubation, and incorporated radioactivity was measured in a Betaplate Counter (Pharmacia).

RESULTS

Different Proliferative Responses of Human T Cells Induced by Binding of the mAbs R24, U5, and E11—Previous reports had suggested that mAb R24 could induce T-cell proliferation (17). When we compared two different anti-Gd3 antibodies, R24 and E11, with the putative anti-Gd3 mAb U5 in an effort to corroborate and extend those findings, we found that there were extreme variations in the capacities of these mAbs to
induce T-cell growth as assayed by thymidine incorporation (Fig. 1). The mAbs R24 and U5 stimulated CD4+ and CD8+ T-cell growth without addition of exogenous cytokines, whereas E11 did not. Antibody U5 always induced higher levels of proliferation at significantly lower antibody concentrations than R24. This finding correlated with the antibody reactivity measured by flow cytometry. mAb U5 stained 30–70% of the T-cells, separated as described previously (7), was measured after stimulation with mAb U5 or R24 at final concentrations between 100 and 1.56 μg/ml. The S.D. ranged within 15%. The values of phytohaemagglutinin stimulation (0.5 μg/ml), mAb E11 (100 μg/ml), and medium control for CD4+ T-cells were 152,930, 189, and 286 cpm, respectively; for CD8+ T-cells 90,411, 49, and 68 cpm, respectively.

Detection of T-cell Antigen 7-O-Acetyl-Gd3

The overall concentration of disialogangliosides in human leukocytes is very low (about 122 μg of lipid-bound sialic acid in 10^12 unseparated leukocytes (1)), making this a poor source for the purification of the U5 antigen. We therefore searched for an alternative source of the antigen and detected it in bovine milk, which has been reported to contain several disialogangliosides of the Gd3 type (19). The purification of the U5 antigen from bovine buttermilk was achieved by ion-exchange chromatography and three consecutive fractionations on HPLC silica columns as described under “Experimental Procedures.” Two different methods were used to identify the U5 antigen in the course of the purification. The first was direct TLC immunostaining using the mAb U5 as already shown in Fig. 2. However, because of the cross-reactivity of this antibody with the non-O-acetylated ganglioside Gd3 (Fig. 2, lane B) and because the upper Gd3 band and the U5 antigen migrated very close together, it was, especially in the presence of the large amounts of Gd3 found in bovine buttermilk, often difficult to distinguish between these two GSLs using mAb U5. For this reason, we developed a second method to identify the U5 antigen, which took advantage of the alkali-induced (pH 10) rearrangement of the U5 antigen to 9-O-acetyl-Gd3, which could be detected with mAb UM4D4 (Fig. 3). In the left half of Fig. 3 the characteristic binding patterns of the 9-O-acetylated disialogangliosides from bovine buttermilk (lane a) and from unseparated human leukocytes (lane b) are shown. After treatment of the plate at pH 10, one new major band appeared in both lanes (Fig. 3, panel, large arrows). As shown below, this major band originated from the U5 antigen. The band could clearly be distinguished from 9-O-acetyl-Gd3 because of its different chromatographic mobility. Using both methods, the U5 antigen could be distinguished with certainty from both 9-O-acetyl-Gd3 (by U5 staining) and Gd3 (by staining with 9-O-acetyl-Gd3-specific antibodies after the alkali-induced rearrangement).

The changes in the U5 antigen upon mild and strong alkali treatment in vitro are shown in Fig. 4. Reaction products were separated by TLC and analyzed by immunostaining with different antibodies and by the nonspecific detection of all GSL antigens using DIG staining (13). U5 antigen (Fig. 4A, lane 1) purified from bovine buttermilk was not detectable by the strictly 9-O-acetyl-Gd3-specific mAb (15) UM4D4 (Fig. 4B,
lanewas another band (lane 1) of minor intensity that had the same mobility as reference ganglioside GD3 (Fig. 4C, lane 3). Treatment of the U5 antigen with strong alkali (13.3 M ammonia, 30 min, 22 °C) resulted in a single product with the mobility of GD3 (Fig. 4C, lane 3).

From these experiments it was concluded that the U5 antigen was an O-acetylated derivative of ganglioside GD3 different from 9-O-acetyl-GD3. Because the conditions of the in vitro mild alkali treatment were the same as those used by Diaz et al. (14) to achieve an intramolecular migration of O-acetyl groups from the 7- to the 9-position of the O-acetylated sialic acid, we predicted that the U5 antigen should be identical with or closely related to 7-O-acetyl-GD3. HPLC analysis of sialic acids released by Arthrobacter ureafaciens sialidase treatment of the purified U5 antigen showed the presence of a small peak as a shoulder eluting before the 5-N-acetylated product (Fig. 5B). This shoulder (R₁⁹5Ac = 0.95) has been reported to be 7-O-acetyl-5-N-acetyleneuraminic acid (20). Although the 7-O-acetyl-5-N-acetyleneuraminic acid (Neu5,7Ac₂) derivative could only be partially separated from the 5-N-acetyleneuraminic acid originating from the penultimate sialic acid residue (elution times in this system were 57 min for Neu5Ac and 70 min for 7-O-acetylated and unsubstituted 5-N-acetyleneuraminic acid, respectively, Fig. 5B, arrow), its disappearance concomitant with the appearance of 9-O-acetyleneuraminic acid (standard in Fig. 5A) after treatment with 20 M aqueous ammonia was a further indication of the identity of the shoulder at 57 min as the 7-O-acetylated product (Fig. 5C). The fact that the peak areas of Neu5Ac (from the penultimate sialic acid residue) and of Neu5,9Ac₂ were not equal is most likely the result of some overall de-O-acetylation occurring during the induction of acetyl group migration.

Another indication of the 7 position of the O-acetyl group came from periodate oxidation experiments combined with DIG staining. This latter method is dependent on periodate oxidation of cis diol groups for the formation of the digoxigenin hydrazones, which are then manifested immunologically. Periodate attack of the non-O-acetylated ganglioside GD3 can only take place in the exocyclic glycerol-like side chain of the terminal sialic acid residue (21). The presence of an O-acetyl substitution in this side chain in either the 9 or 8 position would prevent an attack by mild periodate and subsequent DIG staining. For 7-O-acetyl-GD3, a cleavage between the terminal (C-9) and the subterminal (C-8) carbon atoms in the sialic acid side chain could be expected with attendant detectability by DIG.
staining. An in situ periodate oxidation followed by DIG labeling with unsubstituted GD3 (lane 1), with the U5 antigen (lane 2), and with 9-O-acetylated GD3 (lane 3) is shown in Fig. 6. In panel A, GD3 and the U5 antigen but not the 9-O-acetylated GD3 were oxidized by periodate as shown by DIG staining. In panels B, C, and D, control stains with the mAbs US, UM4D4 after alkali-induced rearrangement, and UM4D4 without alkali-induced rearrangement, respectively, are shown. Thus, the detectability of the U5 antigen by DIG staining also suggested that the O-acetyl group was located in the 7 position.

Mass Spectrometric Analysis of the U5 Antigen—Structural analysis of the U5 reactive ganglioside from bovine buttermilk was also performed using negative ion electrospray mass spectrometry (Fig. 7). A cluster of six intense doubly charged molecular ions was detected at m/z 770.6, 777.4, 783.9, 791.1, 798.0, and 805.1, whereas the corresponding singly charged species were detectable but of rather low abundance (Fig. 7A). These molecular ions suggest the presence of a series of gangliosides incorporating a homogeneous carbohydrate moieties, i.e., a monooxacylated tetrasaccharide of the composition Ac-NeuAc-NeuAc-Hex, linked to a heterogeneous ceramide portion with C19-C24 fatty acids bound to a C18 sphingosine. These assumptions were confirmed by tandem mass spectrometric experiments. After collision-induced decomposition of the doubly charged parent ion at m/z 791, the spectrum depicted in Fig. 7B was obtained. The location of the O-acetyl group in the terminal sialic acid moiety was unequivocally demonstrated by the detection of a weak daughter ion at m/z 350 (NeuNAcOAc) accompanied by more intense fragments at m/z 332 (NeuNAc-O-Ac-H2O), m/z 290 (NeuNAcOAc-CH2COOH), and m/z 272 (NeuNAcOAc-H2O-CH2COOH). A signal at m/z 641 characteristic of the mono-O-acetylated disialosyl moiety was not observed, but an intense fragment at m/z 623 (NeuNAcOAcNeuNAc-H2O) and weaker signals at m/z 581, 563, and 535, which can be explained by loss of CH2COOH, H2O+CH2COOH, and HCOOH+CH2COOH, respectively, were detected.

Fragment ions incorporating the ceramide portion were detected at m/z 659 (Cer-Hex3NeuAc-CO-COO)2−, at m/z 958 (Cer-Hex3)3−, together with weak signals at m/z 796 (Cer-Hex)−, accompanied by peaks at m/z 778 and 760 generated by the loss of one or two molecules of H2O and at m/z 634 (Cer+). Only the latter signals shifted by the expected mass increment when a different parent ion was decomposed, confirming the assignments. These results confirmed the structure of the U5 antigen as a terminally O-acetylated derivative of ganglioside GD3.

Quantitative Binding of mAbs U5, R24, and E11 to GD3 and 7-O-acetylated-GD3—As shown above, mAb U5 did not bind to 9-O-acetylated-GD3 (Fig. 2, lane B). However, the antibody bound to some extent to GD3. We therefore compared quantitatively binding of the mAbs U5, R24, and E11 to 7-O-acetylated-GD3 and GD3 in an ELISA assay (Fig. 8). The affinity of mAb U5 for 7-O-acetylated-GD3 was severalfold higher than that of the other two mAbs for this antigen, whereas all three mAbs had relatively low affinities for GD3. The high binding affinity and specificity of mAb U5 for 7-O-acetylated- versus nonacetylated GD3 classifies this mAb as the first with a preferential specificity for 7-O-acetylated-GD3.

Detection of 7-O-Acetyl-GD3 in Human T-Cells—Direct evidence for the presence of 7-O-acetylated-GD3 in human T-cells was obtained by analysis of the lipids extracted from mAb U5 immunoprecipitates of purified human T-cells (Fig. 9). Lipid extracts from total leukocytes (A) or T-cell immunoprecipitates (B) were separated on TLC plates and detected by immunostaining with mAb UM4D4 before and after alkali treatment. The band indicated by the large arrow originated from 7-O-acetylated-GD3 as inferred from the facts that it could only be detected after pH 10 treatment of the lipid extract and that it migrated between the positions of 9-O-acetylated-GD3 and 9-O-acetyl-DSPG.

Expression of U5 Positive Gangliosides by Different Human Leukocyte Populations—The detection of the U5 antigen in different leukocyte populations as determined by flow cytometry is shown in Table I. Although our findings suggest that this antigen serves as a receptor for the functional activation of T-cells, it is not a specific marker for them, as surface expression of U5 antigen was also found in a significant fraction of the cells in all other classes of leukocytes analyzed.

DISCUSSION

In this study, we have identified the target antigen of the mAb U5 as 7-O-acetylated-GD3 and have shown that this GSL is present in the disialoganglioside fraction of human leukocytes, where it was hitherto unknown. 7-O-Acetyl-GD3 has recently been identified in bovine buttermilk and in melanoma cells of hamsters and humans (19, 22, 23). Its occurrence in normal human leukocytes may have been overlooked for two reasons. First, this antigen shows a migration on TLC very similar to that of unsubstituted GD3; second, the classical method for the detection of alkali-labile GSL, a characteristic decrease in their TLC mobility upon ammonia treatment (24), failed in this case since there is essentially no difference in the mobilities of GD3 and 7-O-acetylated-GD3.

In human leukocytes, O-acetylated sialic acid residues are ubiquitous components of disialogangliosides. We found in previous work that a majority of the disialogangliosides from human leukocytes were O-acetylated and identified the major component as 9-O-acetylated GD3, and two minor components as 9-O-acetylated GD3 analogs containing in addition one and two lactosamine disaccharide units (1). We also showed that treatment of the disialogangliosides from unseparated leukocytes with mild alkali caused a considerable increase in the amount of 9-O-acetylated gangliosides (1). This suggested the presence of unknown O-acetylated forms of the gangliosides that had rearranged to the 9-O-acetates during mild alkali treatment, a supposition that we have now confirmed with the identification of the U5 antigen as 7-O-acetyl-GD3.

Theoretically, the O-acetyl group of the U5 antigen could also be located at the 8 position. However, HPLC separation of enzymatically released sialic acid showed the characteristic shoulder of the 7-O-acetylated derivative (the position of the 8-O-acetylated N-acetylaceamic acid is not known in this HPLC system because of the extreme lability of this molecule).
In addition, the U5 antigen was susceptible to mild periodate, which could only be expected for the 7-O-acetyl derivative. The presence of unsubstituted GD3 in our purified antigen could be excluded by mass spectrometry. It was not possible to quantitate the proportion of 7-O-acetylated, 9-O-acetylated, and non-acetylated forms of disialogangliosides originally present in human leukocytes or in purified T-cells since it could not be excluded that the O-acetylated gangliosides were partially deacetylated during purification. Indeed, it is conceivable that the non-O-acetylated disialogangliosides originate entirely through deacetylation during purification.

Investigations into the existence and the properties of 7-O-acetyl-GD3 have been conducted primarily in two laboratories (19, 22, 23). However, their results concerning the general properties of this molecule differed in several points. The first matter of controversy is the stability of the antigen. Manzi et al. (23) found that 7-O-acetyl-GD3 was an extremely labile compound with a strong tendency to rearrange to the 9-O-isomer, which is in agreement with our present results. In contrast, Ren et al. (22) reported that the 7-O-isomer could be purified

**Fig. 7.** Electrospray mass spectrometry of U5 antigen. A, negative ion electrospray mass spectrometry showing mainly six doubly charged molecule ions. B, collision induced decomposition of the doubly charged parent ion at m/z 791.

**Fig. 8.** Binding of mAbs U5, R24 and E11 to 7-O-acetyl-GD3 and GD3 tested by ELISA. The indicated amounts of each antigen were assayed with the three mAbs as described under "Experimental Procedures."
concentrations of GM3.

An argument in favor of a surface expression of the U5 antigen comes through inference, from the evidence that it is involved in mediating T-cell activation. Our interest in the characterization of the U5 antigen originated from the observation (Fig. 1) that the T-cell stimulatory capacity of mAb U5 was severalfold higher than that of mAb R24, although, as noted above, both bound to ganglioside GD3 with comparable affinities. This suggested that the primary antigen recognized by U5 and responsible for T-cell activation was different from GD3. The U5 antigen as well as non-O-acetylated GD3 have also been implicated in earlier studies as activation molecules on T-cells (7). In contrast, eight different monoclonal antibodies specific for 9-O-acetylated derivatives of GD3, tested under the auspices of the Fifth Workshop and Conference on Human Leukocyte Differentiation Antigens (26), were found to not induce T-cell proliferation (data not shown). Further detailed functional studies with a panel of related antibodies will be necessary to clarify and confirm the roles that these different disialogangliosides may or may not play in T-cell activation.

Whether or not gangliosides are directly involved in signal transduction from the cell surface is still largely unknown. An involvement of gangliosides in signaling through direct binding of GD1b, GD2b, GM2a, and GM2 to calmodulin and the calmodulin-dependent enzyme cyclic nucleotide phosphodiesterase has been demonstrated (27, 28). Moreover, Hanunn (29) and Yuan et al. (30) have suggested roles for the GSL metabolites ceramide and sphingosine 1-phosphate in the regulation of cell growth, differentiation, and apoposis (29, 30). Recently, a tight and specific association of the signal transducing GPI-linked surface molecule CD59 with the ganglioside GM3 was described (16). Thus, as a working hypothesis for future investigations, it might be speculated that 7-O-acetyl-GD3 operates in a similar manner by forming a close association in a membrane microdomain with a T-cell-activating molecule such as CD2 or CD3.

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