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CDw60 Glycolipid Antigens of Human Leukocytes: Structural Characterization and Cellular Distribution

By Bernhard Kniep, Willy A. Flegel, Hinnak Northoff, and Ernst Peter Rieber

Monoclonal CDw60 antibodies recognize glycolipid antigens with restricted surface expression on human leukocytes. They allow us to define new functional subpopulations of T lymphocytes and are able to induce costimulatory signals. In this report, we describe the molecular composition of CDw60 glycolipid antigens derived from different human leukocyte subpopulations. The glycolipids were isolated and their structures were identified by immunochemical methods. All molecules containing the CDw60 determinant were found in the disialoganglioside fraction. They were O-acetylated derivatives of the gangliosides II³ (Neu5Ac)₂-LacCer (G_{D3}), IV³ (Neu5Ac)₂-nLc₄Cer

(DSPG), and VI³ (Neu5Ac)₂-nLc₆Cer (DSnHC), respectively. The most common CDw60 glycolipid antigen in human leukocytes was 9-O-acetyl G_{D3}. In a comparison of various cell types, the highest concentration of 9-O-acetyl G_{D3} on a per cell basis was determined in granulocytes and in blood T lymphocytes, whereas B lymphocytes, thymus cells, and monocytes contained considerably smaller amounts of this molecule. Polar CDw60 antigens such as 9-O-acetyl DSPG and 9-O-acetyl DSnHC were only detected in granulocytes.

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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. The pattern of gangliosides expressed on human hematopoietic cells is cell-type-specific¹⁻³ and is presumed to play an important role in membrane-mediated processes such as cell signaling and recognition events involved in cell differentiation,⁴ hormone receptor modulation,^{5,6} and cell-cell interaction.⁷⁻⁹ The function of GSLs in cellular recognition is substantiated by recent reports showing that carbohydrate sequences on both GSLs and glycoproteins are specific ligands of a group of mammalian lectins called selectins.¹⁰⁻¹² Furthermore, GSLs, particularly gangliosides, and monoclonal antibodies (MoAbs), which recognize gangliosides, have been reported to control growth and function of lymphoid cells^{13,14} and phagocytes.¹⁵

Since the structure and composition of the carbohydrate moieties of glycoconjugates change during ontogeny and functional differentiation of cells,³ the glycoconjugate pat-

terns on the cell surface might serve as functional markers of leukocyte subpopulations. To address this question, a large number of leukocyte-specific MoAbs were screened for reactivity with glycoconjugates using complex glycolipid mixtures prepared from leukocytes. These investigations led to the definition of new clusters of MoAbs that were specific for carbohydrate differentiation antigens on the surface of leukocytes such as the myeloid cell-restricted CDw65 MoAbs,^{16,17} the B-cell-specific CD77^{18,19} and CD76²⁰ mAbs, and the T-cell-restricted CDw60 MoAbs.^{21,22} Previously, we have found that CDw60 mAbs define new functional subpopulations of both the CD4⁺ and CD8⁺ T-cell subsets^{22,23} and that they bind to several different disialogangliosides.²¹ Recently, we identified one of the target structures of CDw60 MoAbs as II³ (Neu5, 9Ac₂-Neu5Ac)-LacCer (9-O-acetyl G_{D3}), which had been isolated from bovine buttermilk.²⁴ To date, this structure has mainly been reported as a melanoma-associated antigen in humans.²⁵⁻²⁷ In this report, an analysis of the structures and distribution of CDw60 glycolipid antigens in various human hematopoietic cell types is presented. We show that the main CDw60 glycolipid of human leukocytes is 9-O-acetyl G_{D3}, which, in addition to T lymphocytes, is also found in the lipid extracts of other leukocyte subsets, particularly granulocytes.

MATERIALS AND METHODS

Antibodies. MoAb UM4D4²⁸ was obtained by the Fourth Workshop on Human Leukocyte Differentiation Antigens (Vienna, Austria, 1989). G_{D3}-specific MoAb BP-G_{D3} also detecting other terminally disialylated neolacto gangliosides (B. Kniep, unpublished observation, April 1991) was purchased from Dr Pallmann Ltd, München, Germany). The mAb M-T32 was raised against T cells from patients suffering from chronic lymphocytic T-cell leukemia; MoAb M-T21 and MoAb M-T41 were obtained against a CD8⁺ T-cell clone.²² 9-O-Acetyl G_{D3}-specific MoAb RB 13-2²⁹ was a kind gift from Dr Kindler-Röhrborn, Universitätsklinikum Essen, Germany. The MoAb 1B2 specific for the terminal Galβ1-4GlcNAc-carbohydrate structure found in glycolipids of the neolacto series³⁰ was used as cell culture supernatant obtained from the 1B2 hybridoma clone (American Type Culture Collection, Rockville, MD) and was a kind gift from R. Schwartz-Albiez, DKFZ, Heidelberg, Germany.

Preparation of leukocytes. Human leukocytes were prepared from pooled buffy coat layers of citric acid, sodium citrate, sodium dihydrogen phosphate, glucose, and adenine hydrochloride

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(CPDA1) anticoagulated blood. Five hundred milliliters of this pool, which represents 20 to 30 buffy coats, were centrifuged at 3,200g for 20 minutes. The white blood cell layer (~100 mL) was mixed with 50 mL citric acid, sodium citrate, and glucose (ACD) stabilizer and 100 mL Gelafundin (gelatine, molecular weight 35,000; Braun, Melsungen, Germany), distributed into glass flasks, and filled up with the plasma that was obtained during the centrifugation. After sedimentation for 6 hours at room temperature or for 24 hours at 4°C, the layer containing white blood cells was collected, diluted with an equal volume of phosphate-buffered saline (PBS), and centrifuged at 3,200g for 30 minutes. The pellet was resuspended in lysis buffer (155 mmol/L NH₄Cl in 10 mmol/L phosphate, pH 7.4) at 0°C for 30 minutes to lyse residual erythrocytes. Cells were washed twice in PBS, counted, taken up in ice-cold methanol, and stored at -70°C.

Isolation of leukocyte subpopulations. For preparation of granulocytes, 500-mL pooled buffy coats were centrifuged at 3,200g for 20 minutes and the white blood cell layer (buffy coat of the pooled buffy coats, 100 mL) was mixed with 50 mL ACD stabilizer and 200 mL Gelafundin and sedimented 1 hour at 20°C in glass tubes. The supernatant containing all leukocytes and some red blood cells was centrifuged at 1,000g for 45 minutes, and the red blood cells in the pellet were lysed as previously described. The remaining cells were resuspended in PBS and centrifuged on a density gradient (Lymphoprep, Nycomed, Oslo, Norway). Granulocytes were recovered as pellet and washed.

Monocytes were separated from the mononuclear fraction by plastic adherence in the presence of 2% fetal calf serum (FCS) under lipopolysaccharide-free conditions, as described previously.³¹ T Lymphocytes were enriched from peripheral blood mononuclear cells as rosettes with (2-aminoethyl)-isothiuronium bromide-treated sheep erythrocytes and centrifugation through a Percoll layer (Pharmacia, Freiburg, Germany) of 1.080-g/mL density.

B lymphocytes were prepared from human tonsils. Tonsillar tissue was obtained from patients undergoing tonsillectomy for tonsil hyperplasia and was kindly provided by the ENT Department, University of Munich. Tonsils were cut into small pieces and minced through a stainless steel mesh. Mononuclear tonsil cells were obtained by Ficoll-Hypaque centrifugation. B lymphocytes were enriched from mononuclear cells as CD37 rosettes by the direct MoAb rosetting technique.³²

Normal human thymic tissue was obtained from pediatric patients undergoing corrective cardiac surgery and was kindly provided by the surgical department of Deutsches Herzzentrum, Munich. Thymic cells were prepared by teasing small pieces of fresh thymus through a stainless steel gauge into cold RPMI 1640 medium containing FCS and 0.1 mg/mL DNase.

All leukocyte subpopulations were more than 95% pure as demonstrated by phenotyping with fluorescein isothiocyanate (FITC)-labeled CD-MoAbs and cytofluorographic analysis. After washing with PBS, cell preparations were immediately taken up in ice-cold methanol and stored at -70°C.

Glycolipids. II³(Neu5Ac)₂-LacCer (G_{D3}) and 9-*O*-acetyl G_{D3} were prepared and analyzed from bovine buttermilk as previously described.²⁴ Disialosylparagloboside ([DSPG] IV³(Neu5Ac)₂-nLc₄Cer) and disialosyllacto-*N*-norhexaosylceramide ([DSnHC] VI³(Neu5Ac)₂-nLc₆Cer) were purified from pooled buffy coats as follows: A crude lipid extract was prepared as previously described³³ and partitioned according to the method of Folch et al.³⁴ Folch upper phase (UP) was dialyzed, lyophilized, taken up in 1 L chloroform-methanol-water (C-M-W) 30/60/8 (vol/vol/vol), and passed through a 3 × 20-cm DEAE Sepharose column (acetate form, Pharmacia, Freiburg, Germany). Noncharged lipids were eluted with 2.5 L C-M-W 30/60/8 (vol/vol/vol) and 2.5 L methanol. Gangliosides

were eluted consecutively, each with 2 L of 10, 20, 30, 50, 80, 150, and 300 mmol/L ammonium acetate in methanol, respectively. All disialogangliosides were found in the 50-mmol/L fraction. After desalting, the disialogangliosides were separated on a high-performance liquid chromatography (HPLC) column (16 × 500 mm, Knauer, Berlin, Germany) filled with Lichrosorb Si 60 5 μm silica. Elution was performed essentially as previously described³⁵ using a gradient from 2-propanol-hexane-water (iP-H-W) 55/42/3 (vol/vol/vol) to iP-H-W 70/10/20 in 600 minutes at a flow rate of 1.5 mL/min; 200 fractions were collected. DSPG and DSnHC peaked in fraction 138 and 152, respectively. Their structures were confirmed by fast atom bombardment mass spectrometry, immunostaining using mAb BP-GD3 (specific for G_{D3} and neolacto gangliosides with a terminal disialosyl group), and immunostaining of their desialylated backbones with mAb 1B2, which is specific for GSLs of the neolacto series. The asialo derivatives were identical with respect to their mobility on thin-layer chromatography (TLC) and immunostaining by MoAb 1B2 to paragloboside (nLc₄Cer) and lacto-*N*-norhexaosylceramide (nLc₆Cer), respectively.

Lactosylceramide (LacCer) and nLc₄Cer were prepared from Folch lower phase of the buffy coat extract as previously described.³⁶ nLc₆Cer was obtained from 2,3 sialosyl lacto-*N*-norhexaosylceramide (VI³Neu5Ac-nLc₆Cer) by cleavage with *Vibrio cholerae* sialidase.³⁶

Purification of CDw60 glycolipid antigens from leukocytes. Unseparated leukocytes, 1.7 × 10¹² stored separately in 11 batches of 1 to 2 × 10¹¹ cells in methanol at -70°C before glycolipid purification, were stirred in batches in 500 mL methanol and sonified in 5 × 1-minute intervals in an ice bath using a Branson sonifier B-12 (Heinemann, Schwäbisch Gmünd, Germany) at 100 W. Cell homogenates were filtrated and the filter cakes were resuspended in 500 mL C-M 1/2 (vol/vol) and reextracted in the same manner; a third extraction was performed with C-M 2/1 (vol/vol). The lipid extracts were evaporated in vacuo and partitioned essentially according to the method of Folch et al.³⁴ using 100 mL C-M 2/1 and 20 mL water. The lower phases were partitioned again with the same volume of UP. By immunostaining (see below), it was found that the UP after this second partition (2nd UP) contained the vast majority of the CDw60-bearing glycolipids, whereas the bulk of the more polar glycolipids was concentrated in the first UP (1st UP). Some very polar CDw60 antigens were also found in the 1st UP, but their amount seemed to be very low. Therefore, the two UPs were not combined as usual and only the 2nd UP was used for further purification; it was evaporated and dialyzed for 72 hours against cold water (4°C). In our hands, this procedure proved to be more efficient for desalting of the 2nd UP than use of a C₁₈ reversed-phase column. The desalted 2nd UP was then passed over a DEAE Sepharose column (acetate form, 3 × 5 cm) washed with 1 L C-M-W 30/60/8 (vol/vol/vol) and 1 L methanol. Monosialogangliosides, disialogangliosides, trisialogangliosides, and polysialogangliosides were each eluted with 1 L of 20, 80, 150, and 500 mmol/L ammonium acetate in methanol, respectively, and the eluates were evaporated to dryness (25°C). Care was taken to avoid acidic conditions during evaporation by adding small portions of solid sodium hydrogen carbonate. The material was dialyzed, lyophilized, and stored at -70°C. The disialoganglioside fraction contained 208 μg lipid-bound sialic acid.

Glycolipid isolation from defined human leukocyte populations. Purified leukocytes (1 to 5 × 10⁸ cells) stored in 5 mL methanol at -70°C were extracted with 2 × 5 mL methanol for 5 minutes in an ultrasonic bath and twice with 5 mL C-M 2/1 (vol/vol). The extracts were then evaporated to dryness and partitioned in 5 mL C-M 2/1 and 1 mL water. The lower phase was reextracted with 3 mL M-W 1/1 (vol/vol); both UPs were combined and dried. Water-

soluble contaminants were removed by passage over Sep-Pak C18 cartridges (Millipore, Milford, MA) as previously described.³⁷

TLC. TLC analysis was performed on high-performance TLC (HPTLC) Silica Gel 60 plates (Merck, Darmstadt, Germany), with the running solvents being C-M-W 50/40/10 (vol/vol/vol) containing 0.05% calcium chloride (wt/vol) or C-M-W 120/70/17 and 0.02% calcium chloride; running time was 40 or 50 minutes.

TLC. Alkali-labile gangliosides were detected according to the method of Sonnino et al.³⁸ The ganglioside mixture was spotted as a small circle close to an edge of the HPTLC plate. After development in the first dimension, the plate was dried and treated with ammonia as described below; the plates were then run perpendicularly to the first dimension.

Ammonia treatment of gangliosides. Alkaline hydrolysis of gangliosides previously separated on HPTLC plates was performed by incubating the plates in a chamber saturated with concentrated aqueous ammonia for 17 hours at ambient temperature. Before a second TLC run (when a two-dimensional separation was to be performed), the ammonia-treated plate was dried for at least 1 hour in vacuo in the presence of P₂O₅.

TLC immunostaining. This method, originally described by Magnani et al.,³⁹ was performed as previously described⁴⁰ with some modifications.⁴¹ CDw60 MoAbs were used as 1:200-diluted ascites fluid in PBS containing 1% bovine serum albumin ([BSA] wt/vol) and 0.05% sodium azide (PBS/BSA). MoAb BP-GD3 was adjusted to 1 µg/mL antibody with PBS/BSA; MoAb RB13-2²⁹ was used as an undiluted culture supernatant.

Immunochemical detection of GSL on TLC. All reagents were purchased from Boehringer Mannheim, Mannheim, Germany. TLC digoxigenin-3-*O*-succinyl- ϵ -aminocaproic acid hydrazide (DIG) staining was performed essentially as previously described,⁴² but somewhat modified. To obtain an improved sensitivity, a 37-µmol/L solution of DIG was used and the anti-digoxigenin antibody (1:200 diluted) was allowed to incubate for 48 hours at 30°C. In a variant of the TLC DIG staining, the glycerol side chain of the neuraminic acid component of gangliosides was oxidized selectively (the other cis diol groups of the ganglioside molecule were not impaired)⁴³ using a 3-mmol/L solution of sodium periodate in PBS, pH 7.3. Oxidation was performed in the dark for 1 hour at 0°C. Total gangliosides were detected by TLC DIG staining after treatment with ammonia as described previously.

Mild base treatment of gangliosides. After TLC separation of the gangliosides, the plate was fixed with polyisobutylmethacrylate as previously described⁴⁰ and incubated with 100 mmol/L glycine-NaOH buffer, pH 10.0, at 37°C for 2 hours.

Quantification. For quantification of gangliosides after TLC, the resorcinol/HCl-sprayed HPTLC plates were heated for 30 minutes at 95°C and measured densitometrically in transmission mode at 580 nm⁴⁴ using a Shimadzu (Duisburg, Germany) dual-wavelength TLC Scanner CS9001 PC; 0.5 to 3.0 µg G_{D3} was used for calibration. Densitometric quantification of 9-*O*-acetyl G_{D3} after TLC immunostaining was performed using calibration curves obtained with 0.25 to 25 ng antigen and densitometric measurement of the indigo-like phosphatase stain at 600 nm in transmission mode. Peak area values and antigen levels showed a logarithmic relationship in a limited range (~1 to ≤20 ng antigen).

RESULTS

Characterization of disialogangliosides from human blood leukocytes. A lipid extract from human leukocytes was separated by HPTLC and then stained with the MoAb M-T21 (CDw60); several immunoreactive bands were detected (Fig 1, lane a). The two upper spots in lane a are nonspecific since they were seen independently of the speci-

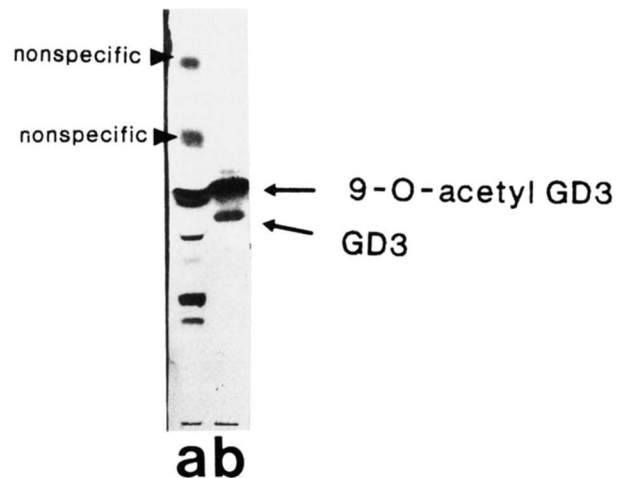


Fig 1. TLC immunostaining by MoAb M-T21 (CDw60) of a crude lipid extract (2nd UP, see the Materials and Methods) from 1.8×10^8 human leukocytes (lane a) and of a standard mixture containing 0.29 µg G_{D3} and 0.28 µg 9-*O*-acetyl G_{D3} (lane b). The glycolipids were separated on a HPTLC silica plate in C-M-W 50/40/10 (vol/vol/vol) containing 0.05% CaCl₂; running time, 40 minutes.

ficity of the applied antibody. We recently identified two M-T21⁺ gangliosides originating from bovine buttermilk (Fig 1, lane b) as the disialogangliosides G_{D3} and 9-*O*-acetyl G_{D3}.²⁴ The upper M-T21⁺ band in the leukocyte lipid extract (Fig 1, lane a) comigrated with 9-*O*-acetyl G_{D3} (Fig 1, lane b). Several additional M-T21 antigens were detected in leukocytes (Fig 1, lane a).

To confirm the presence of 9-*O*-acetyl G_{D3} in human leukocytes and to characterize the additional antigens recognized by MoAb M-T21, GSLs from whole-blood leukocytes were prepared. We started with 1.7×10^{12} leukocytes and used only a few separation steps to minimize the loss of *O*-acetylated gangliosides, which are supposed to be the carriers of the CDw60 carbohydrate epitope.²⁴ Purification of the GSLs was monitored using specific TLC immunostaining with MoAb M-T21, allowing the detection of 9-*O*-acetyl G_{D3} at an amount as low as 0.2 to 0.4 ng. In addition, a nonspecific immunochemical GSL detection method was used, namely TLC DIG stain.⁴² This method involves periodate oxidation of the GSL, which generates aldehyde groups; these are treated with DIG. The digoxigenin component is visualized by an antidigoxigenin antibody conjugated with alkaline phosphatase. TLC DIG staining is suitable especially for the detection of trace amounts of neutral glycolipids and gangliosides. Since the exocyclic (glycerol) side chain of neuraminic acid is more sensitive to periodate oxidation than other parts of the ganglioside molecules,⁴³ DIG staining of gangliosides can be performed using especially mild oxidation conditions (3 mmol/L periodate at 0°C and pH 7.3 for 60 minutes instead of 10 mmol/L periodate at 30°C and pH 5.5 for 30 minutes as in the standard procedure).⁴² In 9-*O*-acetylated gangliosides, oxidation of the exocyclic side chain of the terminal neuraminic acid is prevented. They are stained only after removal of the *O*-acetyl group with ammonia. Thus mild TLC DIG staining with

and without preceding ammonia treatment was used to screen for—and to support the presence of—9-*O*-acetylated gangliosides.

Nearly all of the antigens recognized by mAb M-T21 were found in the UP after the 2nd Folch solvent partition; the GSLs were then separated on a DEAE Sepharose column. M-T21 antigens (Fig 2) were found in the disialoganglioside fraction. Neither the bands at the positions of the M-T21 antigens nor any other disialogangliosides could be seen after TLC DIG staining (Fig 2, right). Only three faintly stained double bands were detected (only two of them can be seen in the photograph) by TLC DIG staining when a larger amount of the disialoganglioside fraction was analyzed (Fig 3, –). Immunostaining using MoAb BP-GD3 and comparison with standard glycolipids indicated that the upper double band represented G_{D3} , the lower double band DSnHC, and the double band between them (not visible on this photograph) DSPG, respectively (not shown). Treatment of this fraction with ammonia before TLC DIG staining showed several additional gangliosides (Fig 3, +), suggesting an abundance of alkali labile *O*-acetylated gangliosides. Immunostaining of the disialoganglioside fraction with MoAb M-T21 produced several bands, especially at higher antigen concentrations (Fig 4, – lanes). Ammonia treatment of disialogangliosides before immunostaining decreased both the number and intensity of the M-T21 immunostained bands (Fig 4, + lanes). Three double bands were recognized with MoAb M-T21 in the alkali-treated disialogangliosides; these comigrated with the disialogangliosides G_{D3} , DSPG, and DSnHC, respectively (Fig 4; ganglioside designations and structures are shown in Table 1). As will be demonstrated below, these three gangliosides were also stained by MoAb BP-GD3, which is specific for G_{D3} and for terminally disialylated gangliosides of the neolacto series such as DSPG and DSnHC.

To improve resolution of the disialogangliosides and to identify the degradation products and structural diversities of the *O*-acetylated CDw60 antigens, an aliquot of the disialoganglioside fraction was separated using two-dimensional TLC.³⁸ The disialogangliosides, containing alkali-stable as well as the alkali-labile *O*-acetylated forms, were chromatographed in the first dimension and then the TLC plate was exposed to ammonia vapor to remove the *O*-acetyl groups. The formerly *O*-acetylated gangliosides were then developed perpendicularly to the first dimension. The

deacetylated gangliosides were found at a position perpendicular to the position to which they had migrated in the first dimension in their *O*-acetylated form. The deacetylated gangliosides could then be compared with standard gangliosides. They were detected by immunostaining with MoAb BP-GD3 (Fig 5A); the components were designated with numbers (Fig 5B).

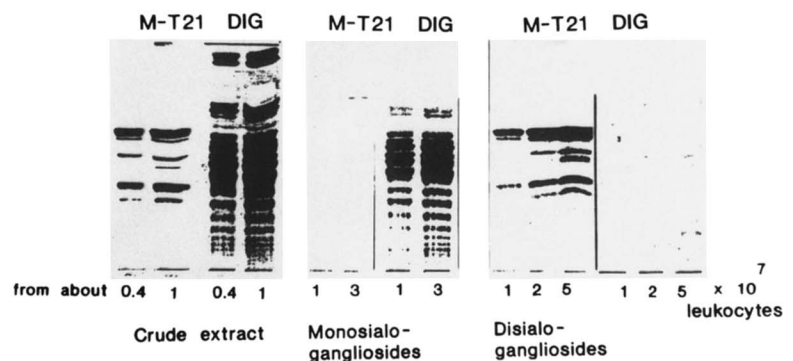
Because the chromatographic mobility of the alkali-stable gangliosides is not influenced by ammonia treatment, they appear on a line through the starting point. The components 1, 2, 9, 10, 13, and 14 (Fig 5B) were found on such a line (Fig 5A) and thus represented the alkali-stable gangliosides. Presumably due to a heterogeneity in their lipophilic moiety, the gangliosides appeared as double spots; the double spots 1 + 2, 9 + 10, and 13 + 14 (Fig 5B) migrated like the standard gangliosides G_{D3} , DSPG, and DSnHC, respectively (Fig 5A).

All gangliosides not found on the line through the starting point must have changed their mobility after ammonia treatment and were therefore considered to originate from alkali-labile (*O*-acetylated) forms. The presence of ganglioside lactones, which form another large group of alkali-labile gangliosides, could be excluded because disialoganglioside lactones would have been found in the monosialoganglioside or the neutral GSL fraction, respectively. Most of the disialogangliosides derived from alkali-labile forms were found on a line parallel to the line of alkali-resistant gangliosides (spots 5 + 6, 11 + 12, and 16 + 17 in Fig 5B). In addition, there were spots lying between the two lines (3, 4, and 15, difficult to see in this figure), and two spots (7 and 8) were located beyond the “line” of most of the alkali-labile gangliosides (Fig 5A).

When the disialogangliosides were separated as shown in Fig 5 but immunostained with mAb M-T21 (CDw60), essentially the same pattern of spots was obtained (not shown). This was in accordance with our earlier observations^{21,24} that MoAb M-T21 bound weakly to G_{D3} and other non-*O*-acetylated terminally disialylated GSLs of the neolacto series.

As the formerly *O*-acetylated and now deacetylated gangliosides could be immunostained with MoAb BP- G_{D3} , they all shared a terminal NeuAc α 2-8NeuAc α 2-3Gal β 1-4-group. The double spots 3 + 4, 5 + 6, and 7 + 8 appeared to be derived from different acetylated forms of ganglioside G_{D3} because their deacetylated form comigrated with G_{D3}

Fig 2. Relative abundance of CDw60 antigens in the crude lipid extract and the monosialoganglioside and disialoganglioside fraction of human leukocytes. GSLs corresponding to the indicated numbers of leukocytes were separated by TLC and (right panels) quantified using the DIG staining method or (left panels) immunostained with the CDw60 antibody M-T21. Solvent and running time are the same as in Fig 1.



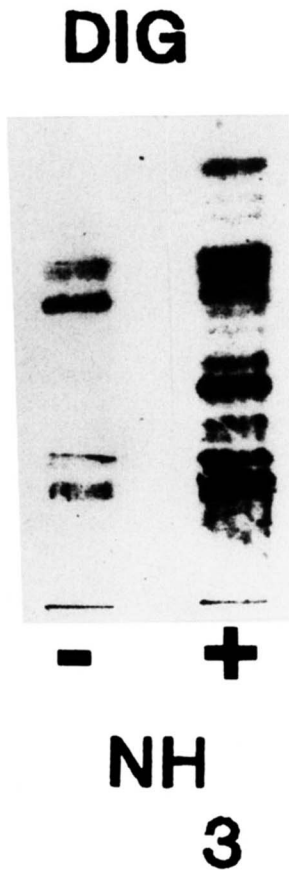


Fig 3. DIG staining of the purified disialogangliosides from human leukocytes without (–) and with (+) previous ammonia treatment of the HPTLC-separated antigens. Left and right lanes contained 0.8 and 0.2 μ g lipid-bound sialic acid, respectively; solvent as in Fig 1; running time, 50 minutes.

although the mobility of their acetylated forms was different. Similarly, the double spots 11 + 12 and 16 + 17 originated from DSPG and DSnHC, respectively (Fig 5).

Since the *O*-acetylated gangliosides were deacetylated during the two-dimensional TLC separation, a direct analysis of their immunologic properties could not be performed

after this step. On the other hand, the disialogangliosides, due to the existence of nonacetylated and several acetylated forms, would not be separable using one-dimensional TLC as shown in Fig 5. After TLC in the first dimension, G_{D3} comigrated with the acetylated form of DSPG (Fig 5, spots 1 and 11) and DSPG with the acetylated form of DSnHC (Fig 5, spots 9 and 16), respectively.

Indirect evidence for the identity of the main *O*-acetylated disialogangliosides was obtained as follows: by dropping perpendicular projections from the double spots 5 + 6, 11 + 12, and 16 + 17 derived from the most abundant *O*-acetylated gangliosides in the two-dimensional TLC (Fig 5), it is obvious that these GSLs were already separated from each other in the first-dimension chromatogram. Thus the projected six spots can be precisely aligned with and thereby most likely correspond to the specifically immunostained bands of *O*-acetylated gangliosides in one-dimensional TLC as shown in Fig 6G, right. Immunostaining of the disialogangliosides with MoAb RB13-2, an antibody specific for 9-*O*-acetyl G_{D3} ,²⁹ in fact detected three well-separated double bands (Fig 6G, right) located at the same positions as expected for the main *O*-acetylated disialogangliosides (Fig 5A). These three double bands were also seen in Fig 4 with MoAb M-T21 at a low antigen concentration (Fig 4, lane A, –).

The ganglioside doublets 5 + 6, 11 + 12, and 16 + 17 therefore appeared to originate from the 9-*O*-acetylated forms of G_{D3} , DSPG, and DSnHC, respectively. This conclusion was also confirmed by the relative stability of these GSLs to mild periodate oxidation. Periodate treatment of the disialogangliosides directly on the HPTLC plate had no effect on the MoAb RB13-2 staining pattern, indicating that this MoAb specifically detected not only 9-*O*-acetylated forms of G_{D3} but also 9-*O*-acetylated DSPG and DSnHC (not shown).

Minor alkali-labile disialogangliosides 7 + 8 (Fig 5) could only be tentatively identified. We recently detected a di-*O*-

Fig 4. Immunostaining by MoAb M-T21 (CDw60) of the purified disialogangliosides from human leukocytes without (–) and with (+) previous ammonia treatment. An amount corresponding to (A) 42 ng, (B) 0.12 μ g, (C) 0.25 μ g, (D) 0.42 μ g, and (E) 0.84 μ g lipid-bound sialic acid was stained. Standards: (F) G_{D3} , (G) mixture of DSPG (top) and DSnHC (bottom). Solvent and running time are as in Fig 3.

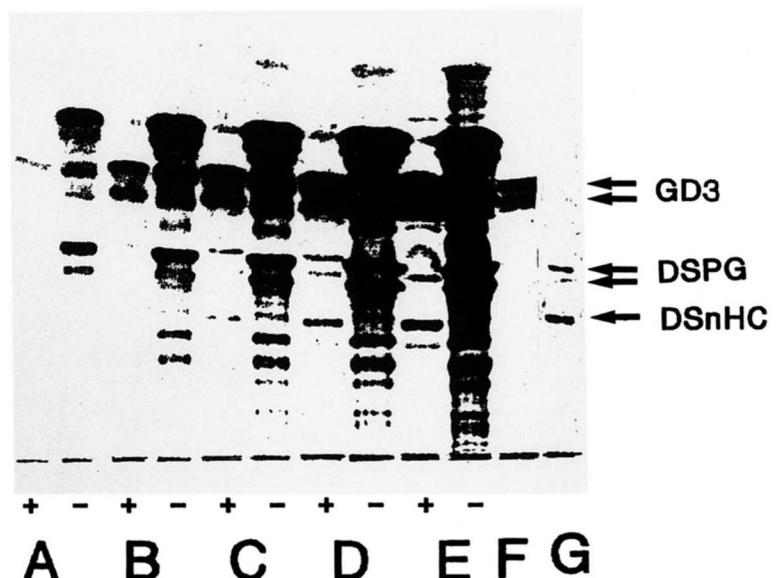


Table 1. Structures of Disialogangliosides From Human Leukocytes

Name	Designation*	Structure†	M-T21 Binding	UM4D4 Binding
G _{D3}	1, 2	(Neu5Ac) ₂ -LacCer	+	—
9- <i>O</i> -Ac-G _{D3}	5, 6	(Neu5, 9Ac ₂ -Neu5Ac)-LacCer	+	+
?	3, 4	Unknown, G _{D3} -derivate	—	—
di- <i>O</i> -Ac-G _{D3} ‡	7, 8	(Neu5, X, YAc ₂ -Neu5Ac)-LacCer	+	+
DSPG	9, 10	(Neu5Ac) ₂ -nLc ₄ Cer	+	—
9- <i>O</i> -Ac-DSPG	11, 12	(Neu5, 9Ac ₂ -Neu5Ac)-nLc ₄ Cer	+	+
DSnHC	13, 14	(Neu5Ac) ₂ -nLc ₆ Cer	+	—
9- <i>O</i> -Ac-DSnHC	16, 17	(Neu5, 9Ac ₂ -NeuAc)-nLc ₆ Cer	+	+

* Designation of spots in Fig 5B.

† Structure details in Appendix.

‡ Tentative identification due to the slightly higher mobility compared with the mono-*O*-acetylated sample. This behavior was very similar to a recently characterized di-*O*-acetylated G_{D3} from bovine buttermilk.²⁴

acetylated derivative of G_{D3} in bovine buttermilk,²⁴ which, compared with 9-*O*-acetyl G_{D3}, had a slightly increased mobility and also appeared to be recognized by CDw60 MoAbs. In analogy to this finding, ganglioside doublet 7 + 8 could be a di-*O*-acetylated G_{D3}.

The components 3 + 4 and 15 were also derived from alkali-labile forms of G_{D3} and DSnHC, respectively (Fig 5). The mobility of spots 3 + 4 was very similar to that of G_{D3} (spots 1 + 2), and they were therefore difficult to distinguish from each other. As will be shown below, the original alkali-labile form of spots 3 + 4 was presumably not recognized with CDw60 mAbs. Due to the dense conglomeration of

spots near G_{D3} (Fig 5A), the existence of additional *O*-acetylated derivatives of G_{D3} different from the three alkali-labile G_{D3} forms described here cannot be ruled out. The existence of such derivatives, very similar in their chromatographic mobility but not identical to G_{D3}, could be an explanation for the discrepancy observed when the relative amounts of *O*-acetylated forms in the whole disialoganglioside fraction, as shown in Figs 3 and 5, respectively, were compared. According to Fig 3, the majority of the disialogangliosides seemed to be *O*-acetylated, whereas similar amounts of acetylated and nonacetylated forms seemed to be present, as suggested in Fig 5. We supposed that a portion of the G_{D3}

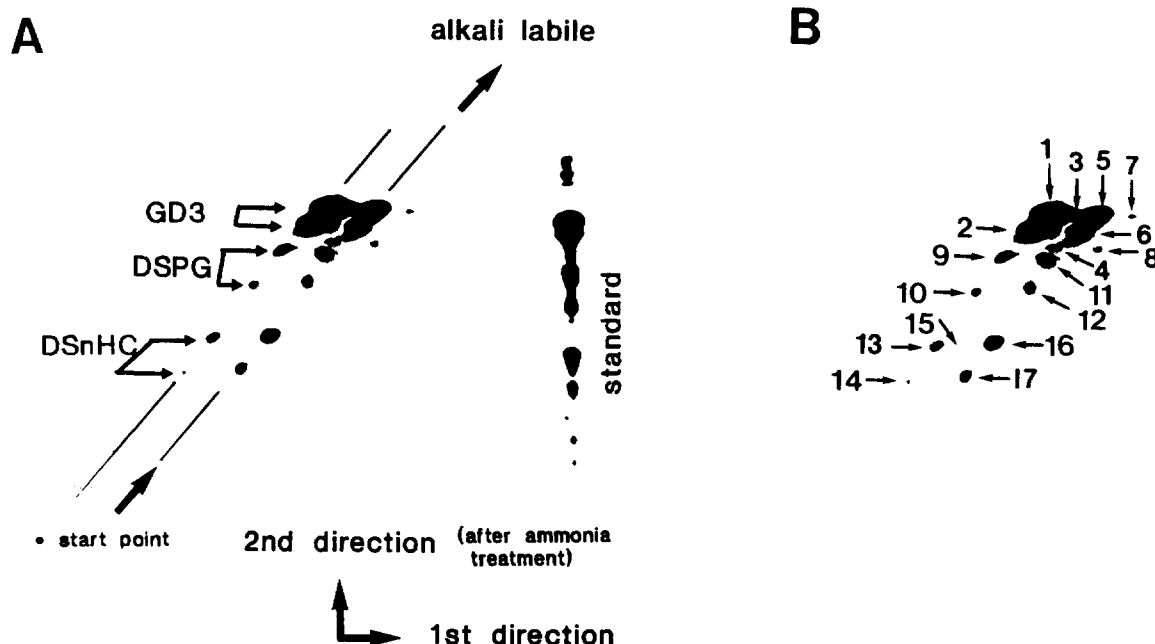


Fig 5. Two-dimensional HPTLC analysis for identification of alkali-labile disialogangliosides from human leukocytes. Chromatography was performed in the indicated directions with ammonia treatment after the first chromatography. The plate was placed in a chamber equilibrated with 17N ammonium hydroxide for 17 hours, dried for 1 hour over P₂O₅ using an oil pump, and then developed perpendicular to the first direction. Following the second chromatography, the gangliosides were immunostained with MoAb BP-GD3. Alkali-resistant gangliosides are located on a line through the origin; alkali-labile gangliosides are found beyond this line. The sample contained 0.42 μ g lipid-bound sialic acid. (A) Position of standard gangliosides is indicated by arrows; (B) spots are designated by numbers. Running solvent was in both directions C-M-W 120/70/17 (vol/vol/vol) containing 0.02% calcium chloride; running time was 50 minutes in each direction.

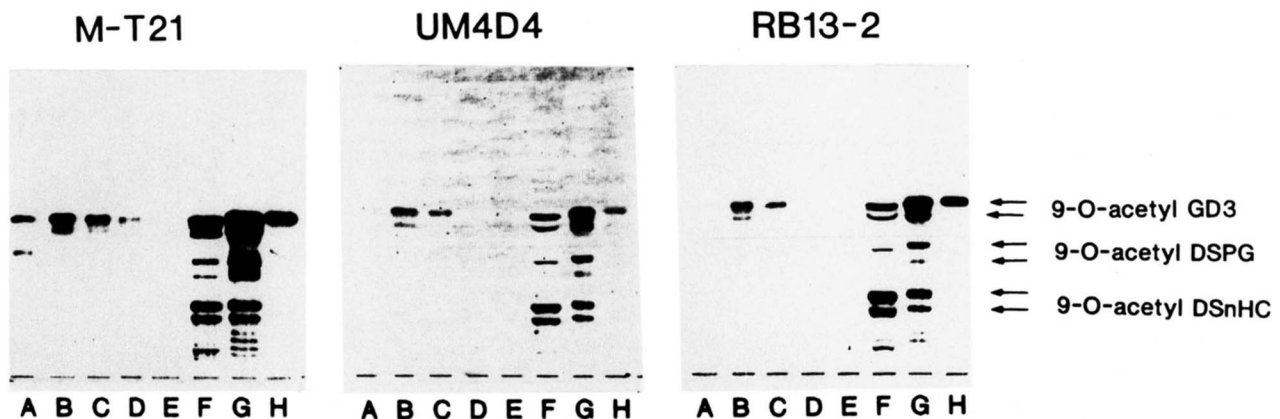


Fig 6. Immunostaining of lipid extracts from separated human leukocytes using (left) MoAb M-T21 (CDw60), (middle) MoAb UM4D4, and (right) reference MoAb RB13-2. Amounts of antigens are as follows (except for the M-T21 stain, since MoAb M-T21 needed only one fourth of the cell extracts used with MoAb UM4D4 and MoAb RB13-2 to obtain a distinct staining profile): (lanes A) 2×10^7 thymocytes; (lanes B) 2×10^7 T cells; (lanes C) 2×10^7 B cells; (lanes D) 1×10^7 monocytes; (lanes E) 3.2×10^8 platelets; (lanes F) 1×10^7 granulocytes; (lanes G) 1.7×10^8 unseparated leukocytes, purified disialoganglioside fraction; and (lanes H) 10 ng 9-*O*-acetyl G_{D3} standard (upper band in lanes H) and 37 ng G_{D3} (lower band in lanes H, only visible in the M-T21 stain). Running time was 40 minutes; solvent, C-M-W 50/40/10 (vol/vol/vol) containing 0.05% (wt/vol) calcium chloride.

spots 1 + 2, especially the part lying between the two lines in Fig 5A, might have originated from an unknown G_{D3} derivative. Such a derivative could in fact be detected after pretreatment of the TLC-separated disialogangliosides with mild alkali (pH 10 buffer) followed by immunostaining with a CDw60 MoAb (Fig 7A +). The new intensively stained band migrated just below 9-*O*-acetyl G_{D3} (Fig 7A +) and was clearly different from G_{D3} , since it could be detected with MoAb UM4D4, which binds only to *O*-acetylated forms of G_{D3} .²⁴ This new *O*-acetylated CDw60 antigen seemed to be derived from a G_{D3} derivative that was not detected by CDw60 mAbs. Recently, Manzi et al⁴⁵ reported for the first time the presence of 7-*O*-acetyl G_{D3} in human melanoma cells and reported evidence that 9-*O*-acetyl G_{D3} -specific MoAbs did not recognize the 7-*O*-acetylated isomer. In view of this finding together with the well-known tendency of the 7-*O*-acetyl group to migrate to the 9-position,⁴⁶ the alkali-labile unknown G_{D3} derivative could be a 7-*O*-acetylated G_{D3} .

Some other CDw60 antigens, presumably the long-chain analogs of the same types as described above such as $VIII^3(\text{Neu5Ac})_2\text{-nLc}_8\text{Cer}$ and its 9-*O*-acetylated form, may also be present in minor quantities in the disialoganglioside fraction of human leukocytes.

Antigen specificity of CDw60 MoAbs. We have previously shown that all CDw60 MoAbs bind preferentially to 9-*O*-acetyl G_{D3} isolated from bovine buttermilk and also to a di-*O*-acetylated form of G_{D3} .²⁴ This can now be extended to the long-chain analogs of 9-*O*-acetyl G_{D3} , 9-*O*-acetyl DSPG, and 9-*O*-acetyl DSnHC and probably also to 9-*O*-acetylated disialogangliosides having even longer carbohydrate chains. In general, all CDw60 MoAbs bound to 9-*O*-acetylated forms of G_{D3} and the 9-*O*-acetylated neolacto gangliosides DSPG and DSnHC, respectively; differences were found in their binding to nonacetylated gangliosides. Recently, we found that MoAb UM4D4 bound only to acet-

ylated G_{D3} , whereas the other CDw60 MoAbs, M-T21, M-T32, and M-T41 also showed some reactivity with nonacetylated G_{D3} , which was only seen at about 50- to 100-fold higher antigen concentrations than those needed for binding to 9-*O*-acetyl G_{D3} .²⁴ This reactivity was also observed for non-*O*-acetylated DSPG and DSnHC (Fig 4).

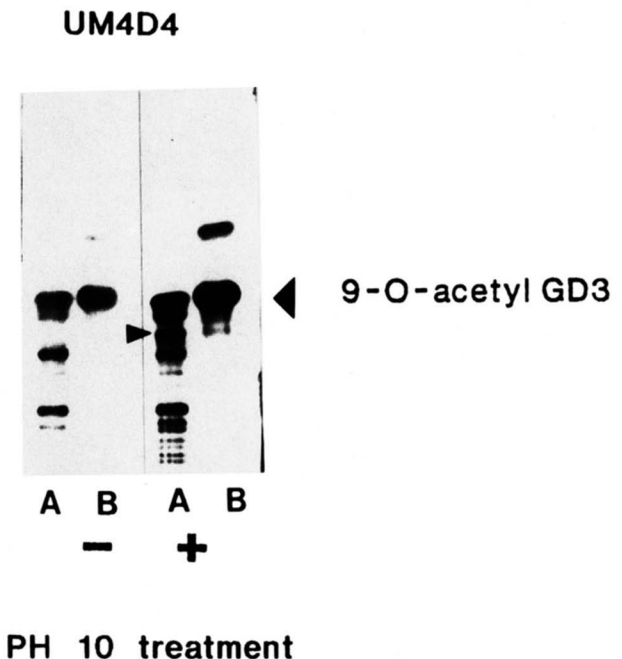


Fig 7. Immunostaining by MoAb UM4D4 of alkali-treated disialogangliosides from human leukocytes. Left panel (–), nontreated sample; right panel (+), sample treated with 100 mmol/L glycine-NaOH buffer, pH 10.0, at 37°C for 2 hours. Antigens were (A lanes) disialogangliosides from human leukocytes and (B lanes) 9-*O*-acetyl G_{D3} from bovine buttermilk.

The structures of the disialogangliosides identified in human leukocytes and their reactivity with CDw60 MoAbs are summarized in Table 1.

Expression of CDw60⁺ gangliosides by different human leukocyte populations. Having identified various CDw60-reactive gangliosides in unseparated human blood leukocytes, it remained to be shown whether they were differentially distributed in leukocyte subpopulations. This seemed an important question, particularly in view of the rather restricted cell surface expression of CDw60 antigens, which, among hematopoietic cells, are only found on functional subsets of T lymphocytes.²³ To this end, lipid extracts were prepared from various purified leukocyte populations and immunostained after TLC separation with the MoAbs M-T21 (Fig 6) (left) and UM4D4 (middle) and the 9-*O*-acetyl G_{D3} specific reference MoAb RB13-2 (right), respectively. As shown in Fig 6 (left), lipid extracts from thymocytes (lane A), T cells (lane B), B cells (lane C), and monocytes (lane D), and granulocytes (lane F) contained M-T21-reactive antigens. The most abundant CDw60 antigen found in all extracts comigrated with 9-*O*-acetyl G_{D3} (lane H). Granulocytes showed additional bands similar to those obtained with the whole disialoganglioside fraction of unseparated leukocytes (Fig 6, left, lane F). This was not surprising since it is known that granulocytes contain more than 90% of the glycolipids of all leukocytes.³⁶ Thymocytes appeared to contain G_{D3} (Fig 6, left, lane A). As 9-*O*-acetylated DSPG and G_{D3} migrate together on TLC (Fig 5B, spots 1 and 11 or 2 and 12), we confirmed the identity of the lower immunostained band in the lipid extract of thymocytes as G_{D3} by immunostaining with the BP-G_{D3} antibody (not shown). The presence of G_{D3} in thymocytes has been described.¹³ From the staining pattern it was evident that besides granulocytes (Fig 6, left, lane F), T cells (Fig 6, left, lane B) produced the strongest staining. Immunostaining of the same lipid extracts with MoAb UM4D4 (Fig 6, middle) showed bands in T cells, B cells, and granulocytes but not in thymocytes and monocytes as detected with MoAb M-T21. The serological preference of CDw60 mAb for a subpopulation of human T cells was partially reflected by the extent of their binding to lipid extracts from separated leukocytes. MoAb UM4D4 showed the most confined specificity. The fastest-moving ganglioside band comigrated with 9-*O*-acetyl G_{D3} (lane H). Binding of MoAb UM4D4 to 9-*O*-acetylated gangliosides was confirmed when we compared the UM4D4 pattern with that obtained with MoAb RB13-2 (Fig 6, right); both patterns were nearly identical.

The total amount of 9-*O*-acetyl G_{D3} per 10⁷ cells of the purified leukocytes is represented in Table 2.

DISCUSSION

This study presents an analysis of the structures and cellular distribution of the GSLs with CDw60 antigenic determinants in human leukocytes. A structural family of gangliosides was detected that so far has not been recognized in normal human leukocytes, namely the *O*-acetylated disialogangliosides of the neolacto series. The occurrence of these alkali-labile gangliosides in normal human leukocytes

Table 2. Amount of 9-*O*-Acetyl G_{D3} in the Lipid Extract of Human Leukocytes

Cell	ng 9- <i>O</i> -Acetyl G _{D3} /10 ⁷ Cells*
Thymocytes	1.7
T lymphocytes	27
B lymphocytes	8.7
Monocytes	<1
Granulocytes	>107

* Values obtained by densitometric scanning at 600 nm after TLC immunostaining using MoAb UM4D4.

might have been overlooked before, since the conventional purification procedures for GSLs often include mild alkaline hydrolysis for removal of phospholipids or ion-exchange chromatography with an eluent containing alkaline sodium acetate.

In the present investigation, we avoided alkaline hydrolysis and used neutral ammonium acetate as an eluent. Despite these precautions, it was not possible to quantify exactly the proportion of acetylated and nonacetylated forms of disialogangliosides originally present in leukocytes, since it could not be excluded that *O*-acetylated gangliosides were deacetylated during our glycolipid purification.

The total amount of disialogangliosides that could be isolated from leukocytes was rather low. In these experiments, 122 μg lipid-bound sialic acid was obtained from 10¹² unseparated blood leukocytes. This finding, together with the observation that several different gangliosides had identical mobilities, has so far hampered a more detailed analysis of these molecules.

However, two-dimensional TLC combined with immunologic detection has allowed a first structural characterization of these antigens, since the parental molecules from which the acetylated derivatives originated could easily be identified. The acetylation site of most of these antigens could be determined using the reference mAb RB13-2, which is specific for 9-*O*-acetyl G_{D3}.²⁹ The presence of di-*O*-acetylated CDw60 antigens (spots 7 + 8 in Fig 5) remains to be determined.

According to the TLC DIG stain, only a minority of the disialogangliosides were not *O*-acetylated (Fig 3), whereas two-dimensional TLC analysis seemed to indicate that a significant proportion of the disialogangliosides, especially of G_{D3}, was not *O*-acetylated (Fig 5). These contradictory results led us to detect another acetylated G_{D3} derivative in human leukocytes. Due to the existence of only a small amount of non-*O*-acetylated G_{D3} in the disialoganglioside fraction of leukocytes (Fig 3), the majority of the large G_{D3} spots (Fig 5, spots 1 and 2) was supposed to originate from an unknown derivative of G_{D3} after treatment with ammonia. The chromatographic mobility of this G_{D3} derivative could only be slightly greater than that of G_{D3}. Thus the characteristic decrease in their chromatographic mobility after ammonia treatment, the most frequently used screening method for the detection of *O*-acetylated gangliosides, was not suitable for the detection of this acetylated G_{D3} derivative. However, its presence could be unequivocally

demonstrated after its alkali-induced rearrangement to a CDw60 antigen (Fig 6). The resulting antigen was detected with MoAb UM4D4 and was therefore *O*-acetylated.²⁴ The relative amount of unchanged G_{D3} and G_{D3} released from the unknown *O*-acetylated derivative after ammonia treatment, respectively, was estimated from the M-T21 immunostaining pattern. Separation and staining were performed at the same time as the separation shown in Fig 5. Due to the weaker binding of MoAb M-T21 compared with BP-GD3, the double spots originating from unchanged G_{D3} and the unknown G_{D3} derivative, respectively, were somewhat easier to distinguish (not shown). Only about 20% of spots 1 + 2 were found on the line of the alkali-stable gangliosides (unchanged G_{D3}), and the remaining 80% seemed to originate from the G_{D3} derivative. This suggested that G_{D3}, in contrast to its *O*-acetylated forms, was a minor-occurring disialoganglioside in human leukocytes.

CDw60 antigens were then analyzed in separated leukocyte populations. With the exception of granulocytes, the CDw60 immunostaining patterns were simple, so that in this case quick identification of the major CDw60 antigen as 9-*O*-acetyl G_{D3} was possible. Staining with the 9-*O*-acetyl G_{D3}-specific reference antibody RB13-2 confirmed that the CDw60 MoAbs detected preferentially 9-*O*-acetylated disialogangliosides (Fig 6). T cells and granulocytes contained most of the CDw60 antigens; however, by immunofluorescence surface staining CDw60 antigens are detected on T lymphocytes, but not on other cells.²³ The reason for this is not yet clear. It may be due to varying degrees of accessibility of the CDw60 determinants in the cell membrane of the separate leukocyte subsets or to a disparate localization of the CDw60 antigens within cells of different lineages. 9-*O*-acetyl G_{D3} has been described as a melanoma-associated antigen in humans,²⁵⁻²⁷ but so far it has not been reported to be a leukocyte antigen. In keeping with the presence of *O*-acetylated gangliosides in lymphocytes, Kamerling et al⁴⁷ identified *O*-acetylated sialic acids in the hydrolysates of human B and T cells, and Holzhauser and Faillard⁴⁸ found 9-*O*-acetyl neuraminic acid in trace quantities after mild acid treatment of normal lymphocytes and in large quantities after the same treatment in lymphocytes of patients with melanomas and mammary carcinomas.

With respect to the function of CDw60 antigens on cells of the immune system, a number of studies have been performed with the MoAb UM4D4²⁸ that suggest an important role for these antigens in T-cell activation. It has been claimed that the UM4D4 antigen is involved in a novel pathway of human T-lymphocyte activation and that it could play a role in the pathogenesis of autoimmune diseases such as rheumatoid arthritis.^{28,49} Unlike other glycolipid antigens, which are preferentially localized at the cell surface, most of the UM4D4 antigens in human thymocytes were found in the cytoplasm.⁵⁰ The mechanism leading to a selective enrichment or proliferation of UM4D4⁺ T cells in the synovial compartment and to their activation is not known. An interesting hypothesis⁵⁰ postulates the existence of a lectin-like adhesion receptor, eg, a lectin specific for *O*-acetylated gangliosides, which could mediate both enrich-

ment of CDw60⁺ T cells in the synovium and their permanent activation in this compartment.

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APPENDIX

Neu5Ac, 5-*N*-acetyl neuraminic acid; Neu9S, 9Ac₂, 5-*N*-acetyl, 9-*O*-acetyl neuraminic acid; G_{D3}, II³(Neu5Ac)₂-LacCer, Neu5Ac α₂ → 8Neu5Acα₂ → 3Galβ1 → 4Glcβ1 → 1'-ceramide; 9-*O*-acetyl G_{D3}, II³(Neu5,9Ac₂-Neu5Ac)-LacCer, Neu5, 9Ac₂α₂ → 8Neu5-Acα₂ → 3Galβ1 → 4Glcβ1 → 1'-ceramide; DSPG, disialosylparagloboside, IV³(Neu5Ac)₂-nLc₄Cer, Neu5Acα₂ → 8Neu5Acα₂ → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; 9-*O*-Ac DSPG, IV³(Neu5,9Ac₂-Neu5Ac)-nLc₄Cer, Neu5,9Ac₂α₂ → 8Neu5Acα₂ → 3Galβ1 → 4BlcNAcβ1 → 3Galβ1 → 4Blcβ1 → 1'-ceramide; DSnHC, disialosyllacto-*N*-norhexaosylceramide, VI³-(Neu5Ac)₂-nLc₄Cer, Neu5Acα₂ → 8Neu5Acα₂ → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; 9-*O*-Ac DSnHC, VI³(Neu5,9Ac₂-Neu5Ac)-nLc₄Cer, Neu5,9Ac₂α₂ → 8Neu5Acα₂ → 3Galβ1 → 4BlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; LacCer, lactosylceramide, Galβ1 → 4Glcβ1 → 1'-ceramide; nLc₄Cer, paragloboside, Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; nLc₆Cer, lacto-*N*-norhexaosylceramide, Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide.

Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature⁵¹; however, the suffix -OseCer is replaced by -Cer.

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