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 immunochromatographic methods. All molecules containing the CDw60 determinant were found in the disialoganglioside fraction. They were O-acetylated derivatives of the gangliosides II2 (Neu5Ac)2-LacCer (Gb3), IV2 (Neu5Ac)2-LacCer (GD3), which had been isolated from bovine buttermilk. To date, this structure has mainly been reported as a melanoma-associated antigen in humans.

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-specific and is presumed to play an important role in membrane-mediated processes such as cell signaling and recognition events involved in cell differentiation, hormone receptor modulation, and cell-cell interaction. 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. Furthermore, GSLs, particularly gangliosides, and monoclonal antibodies (MoAbs), which recognize gangliosides, have been reported to control growth and function of lymphoid cells and phagocytes.

Since the structure and composition of the carbohydrate moieties of glycoconjugates change during ontogeny and functional differentiation of cells, the glycoconjugate patterns 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, the B-cell–specific CD71,7 and CD76,8-12 mAbs, and the T-cell–restricted CDw60 MoAbs. Previous studies have shown that CDw60 mAbs define new functional subpopulations of both the CD4+ and CD8+ T-cell subsets, and that they bind to several different disialogangliosides. Recently, we identified one of the target structures of CDw60 MoAbs as II2 (Neu5Ac, 9Ac2-Neu5Ac)-LacCer (9-O-acetyl GD3), which had been isolated from bovine buttermilk. To date, this structure has mainly been reported as a melanoma-associated antigen in humans.

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 Gd3, which, in addition to T lymphocytes, is also found in the lipid extracts of other leukocyte subsets, particularly granulocytes.

MATERIALS AND METHODS

Antibodies. MoAb UM4D4 was obtained by the Fourth Workshop on Human Leukocyte Differentiation Antigens (Vienna, Austria, 1989). Gd3-specific MoAb BP-Gd3 also detecting other terminally disialylated neolacto gangliosides (B. Kniep, unpublished observation, April 1991) was purchased from Dr Palfmann 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. 9-O-Acetyl Gd3-specific MoAb RB 13-2 was a kind gift from Dr Kindler-Röhrborn, Universitätsklinikum Essen, Germany. The MoAb 1B2 specific for the terminal Galα1–4GlcNAc–carbohydrate structure found in glycolipids of the neolacto series 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 adenosine hydrochloride.
(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 flask, 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 mM NaHCO3 in 10 mM 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. Lymphocytes were enriched from peripheral blood mononuclear cells as rosettes with (2-aminoethyl)ethanol-iodosidomitrone bromide-treated sheep erythrocytes and centrifugation through a Percoll layer (Pharmacia, Freiburg, Germany) of 1.080-dLmL density.

B lymphocytes were prepared from human tonsils. Tonsillar tissue was obtained from patients undergoing tonsillectomy for tonsillar 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.

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. II(5Neu5Ac)2-LacCer (GD3) and 9-O-acetylgalactosylceramide were prepared and analyzed from bovine buttermilk as previously described. Disialoganglioside II(5Neu5Ac)2-LacCer was purified from pooled buffy coats as follows: A crude lipid extract was prepared as previously described and partitioned according to the method of Folch et al. Folch upper phase (UP) was dialyzed, lyophilized, and taken up in 1 L chloroform-methanol-water (C-M-W) 30/60/80 (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 30/60/80 (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 mM/L ammonium acetate in methanol, respectively. All disialogangliosides were found in the 50-mM/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 described 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 DSNHPC peaked in fraction 138 and 152, respectively. Their structures were confirmed by fast atom bombardment mass spectrometry, immunostaining using mAb BP-GD3 (specific for GD3 and neolacto gangliosides with a terminal disialyl 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 (nLc2Cer) and lacto-N-norhexaosylceramide (nLc3Cer), respectively.

To prepare monosialogangliosides (LacCer) and nLc2Cer, some very polar CDw60 antigens were also found in the 1st UP, but which 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 30/60/80 (vol/vol/vol) and 1 L methanol. Monosialogangliosides, disialgangliosides, trisialogangliosides, and polysialogangliosides were each eluted with 1 L of 20, 30, 150, and 500 mM/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 hydrocarbonate. The material was dialyzed, lyophilized, and stored at −70°C. The disialoganglioside fraction contained 208 μg lipid-bound sialic acid.

Glycolipid isolation from human leukocyte populations. Purified leukocytes (1 × 10^11 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-W 2/1 (vol/vol). The extracts were then evaporated to dryness and partitioned in 5 mL C-M-W 2/1 and 1 mL water. The lower phases were 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.48 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 perpendicular to the first dimension.

Ammonia treatment of gangliosides. Alkaline hydrolysis of gangliosides previously spotted 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.,49 was performed as previously described48 with some modifications.54 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 RB13-25 was used as an undiluted culture supernatant.

Immunochromatography of GSL on TLC. All reagents were purchased from Boehringer Mannheim, Mannheim, Germany. TLC digoxigenin-3-O-succinyl-r-aminoacaproic 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)54 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 described48 and incubated with 100 mmol/L glycine-NaOH buffer, pH 10.0, at 37°C for 2 hours.

Quantitation. 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 nm* using a Shimadzu (Duisburg, Germany) dual-wavelength TLC Scanner CS9001 PC. 0.5 to 3.0 μg GD3 was used for calibration. Densitometric quantification of 9-O-acetylated GD3 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 specificity of the applied antibody. We recently identified two M-T21+ gangliosides originating from bovine buttermilk (Fig 1, lane b) as the disialogangliosides Gm and 9-O-acetyl GD3.24 The upper M-T21+ band in the leukocyte lipid extract (Fig 1, lane a) comigrated with 9-O-acetyl GD3 (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 GD3 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 × 109 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 GD3 at an amount as low as 0.2 to 0.4 ng. In addition, a nonspecific immunochromatography 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
GSLs were then separated on a DEAE Sepharose column. Neither the bands at the positions of the M-T21 antigens nor any other disialogangliosides could be seen after 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 G1d2, 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 G12, 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 with MoAb BP-GD3, which is specific for G12 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 G13, 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 disialoanglioside lactones would have been found in the monosialoganglioside or the neutral GSL fraction, respectively. Most of the disialoangliosides 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 disialoangliosides 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 observations21,24 that MoAb M-T21 bound weakly to G13 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-G13, they all shared a terminal NeuAca2-8NeuAca2-3Galβ1-4 group. The double spots 3 + 4, 5 + 6, and 7 + 8 appeared to be derived from different acetylated forms of ganglioside G13 because their deacetylated form comigrated with G13.
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\textsubscript{D\textsubscript{3}} co-migrated 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\textsubscript{D\textsubscript{3}}, 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-T2 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\textsubscript{D\textsubscript{3}}, 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\textsubscript{D\textsubscript{3}} 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-
Table 1. Structures of Disialogangliosides From Human Leukocytes

<table>
<thead>
<tr>
<th>Name</th>
<th>Designation</th>
<th>Structure</th>
<th>M-T21 Binding</th>
<th>UM4D4 Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD3</td>
<td>1, 2</td>
<td>(Neu5Ac)₂-LacCer</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9-O-Ac-GD3</td>
<td>5, 6</td>
<td>(Neu5, 9Ac₂-Neu5Ac)-LacCer</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>?</td>
<td>3, 4</td>
<td>Unknown, GD₃-derivate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>di-O-Ac-GD3</td>
<td>7, 8</td>
<td>(Neu5, X, YAc₂-Neu5Ac)-LacCer</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DSPG</td>
<td>9, 10</td>
<td>(Neu5Ac₂,nLc₄Cer</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9-O-Ac-DSPG</td>
<td>11, 12</td>
<td>(Neu5, 9Ac₂-Neu5Ac)-nLc₄Cer</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DSnHC</td>
<td>13, 14</td>
<td>(Neu5Ac₂,nLc₄Cer</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9-O-Ac-DSnHC</td>
<td>16, 17</td>
<td>(Neu5, 9Ac₂-NeuAc)-nLc₄Cer</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* 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 GD₃ from bovine buttermilk.⁴⁴

acetylated derivative of GD₃ in bovine buttermilk,⁴⁴ which, compared with 9-O-acetyl GD₃, 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 GD₃.

The components 3 + 4 and 15 were also derived from alkali-labile forms of GD₃ and DSnHC, respectively (Fig 5). The mobility of spots 3 + 4 was very similar to that of GD₃ (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 GD₃ (Fig 5A), the existence of additional O-acetylated derivatives of GD₃ different from the three alkali-labile GD₃ forms described here cannot be ruled out. The existence of such derivatives, very similar in their chromatographic mobility but not identical to GD₃, 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 GD₃

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 indicated by arrows; alkali-labile gangliosides are located on a line through the origin; alkali-labile gangliosides are found beyond this line. The sample contained 0.42 μg 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.
spots 1 + 2, especially the part lying between the two lines in Fig 5A, might have originated from an unknown GD3 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 GD3 (Fig 7A +) and was clearly different from GD3, since it could be detected with MoAb UM4D4, which binds only to O-acetylated forms of GD3. This new O-acetylated CDw60 antigen seemed to be derived from a GD3 derivative that was not detected by CDw60 mAbs. Recently, Manzi et al. reported for the first time the presence of 7-O-acetyl GD3 in human melanoma cells and reported evidence that 9-O-acetylated GD3-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, the alkali-labile unknown GD3 derivative could be a 7-O-acetylated GD3.

Some other CDw60 antigens, presumably the long-chain analogs of the same types as described above such as VIII(Neu5Ac)₂-LC₄Cer 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 GD3 isolated from bovine buttermilk and also to a di-O-acetylated form of GD3. This can now be extended to the long-chain analogs of 9-O-acetyl GD3, 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 GD3 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 acetylated GD3, whereas the other CDw60 MoAbs, M-T21, M-T32, and M-T41 also showed some reactivity with nonacetylated GD3, which was only seen at about 50- to 100-fold higher antigen concentrations than those needed for binding to 9-O-acetylated GD3. This reactivity was also observed for non-O-acetylated DSPG and DSNHC (Fig 4).
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 alkali-labile gangliosides in normal human leukocytes. This seemed summarized in Table 1.

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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 G3D and G3D 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 G3D and the unknown G3D 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 G3D), and the remaining 80% seemed to originate from the G3D derivative. This suggested that G3D, 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 G3D was possible. Staining with the 9-O-acetyl G3D-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.25 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 G3D 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 al27 identified 0-acetylated sialic acids in the hydrolysates of human B and T cells, and Holzhauser and Faillard46 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 UM4D424 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.26-49 Unlike other glycolipid antigens, which are preferentially localized at the cell surface, most of the UM4D4 antigens in human thyocytes were found in the cytoplasm.40 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 enrichment 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; Neu95, 9Ac5, 5-N-acetyl, 9-O-acetyl neuraminic acid; G3D, 11(Neu5Ac5)-LacCer, Neu5Ac
α2 → Neu5Acα2 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; 9-O-acetyl G3D, 11(Neu5,9Ac5,9Ac5)-LacCer, Neu5, 9Acα2 → 8Neu5Ac
α2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; 9-O-
Ac DSPG, 11(Neu5,9Ac5,9Ac5)-LacCer, Neu5,9Acα2 → 8Neu5Ac
α2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; DSnHC,
disialyllecto-N-norhexaosylceramide, V1
( Neu5Ac5)-LacCer, Neu5 Acα2 → 8Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; 9-O-Ac
4GlcNCerβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; dsHNC, disialyllecto-N-hexaosylceramide, V1
( Neu5Ac5)-LacCer, Neu5 Acα2 → 8Neu5Acα2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; LacCer, lactosylceramide, Galβ1 → 4Glcβ1 → 1'-ceramide; nLacCer, paragloboside, Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1'-ceramide; nLacCer, lacto-N-orvosylceramide, Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNCerβ1 → 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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