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The CDw65 Monoclonal Antibodies VIM-8 and VIM-11 Bind to the Neutral Glycolipid $V^3FucnLc_8Cer$

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At the IV[th] and V[th] Workshop on Human Leukocyte Differentiation Antigens a group of monoclonal antibodies recognizing myeloid cells was found to bind to the ganglioside X^3-NeuAcVII^{3}FucnLc$_{10}$Cer (VIM-2 dodecasaccharide). These antibodies were given the provisional cluster of differentiation designation CDw65. Three antibodies of this cluster (VIM-2, VIM-8, and VIM-11) have now been studied in detail at the molecular and the cellular level. Binding of VIM-2 is abolished after treatment of cells with *Vibrio cholerae* neuraminidase, whereas VIM-8 and VIM-11 show enhanced binding to neuraminidase-treated cells. We investigated binding of the three mAbs to glycolipid antigens with shorter carbohydrate chains. Distinct differences were observed in the binding of CDw65 antibodies to VIII^3-NeuAcV^3FucnLc$_8$Cer (VIM-2 decasaccharide). VIM-2 strongly bound to this antigen, whereas no binding was observed with the other two mAbs. Conversely, the asialoganglioside of the VIM-2 decasaccharide, V^3FucnLc$_8$Cer, was not recognized by VIM-2, but this antigen bound strongly VIM-8 and VIM-11. Thus, VIM-2 and the other CDw65 antibodies represented two different antigen specificities.

Key words: CDw65, CDw65s, VIM-2, VIM-8, VIM-11.

Many monoclonal antibodies (mAbs) which recognize myeloid cells bind to the carbohydrate part of glycolipids (GSLs) and glycoproteins from these cells (*1-3*). The most frequently observed type of antimyeloid carbohydrate specific mAbs is directed against the Lewis^x trisaccharide determinant Galβ1-4(αFuc1-3)GlcNAc-R located at the nonreducing end of the oligosaccharide chains of both glycoconjugates. These mAbs were grouped together into the cluster of differentiation 15 (CD15) and formed the first of the continuously growing number of clustered antibodies recognizing carbohydrate antigens. The structure of the GSLs bearing Lewis^x determinants was characterized mainly by Fukuda *et al.* as minor occurring molecules with long carbohydrate chains (*4*). During the V[th] Workshop and Conference on Human Leukocyte Differentiation Antigens (Boston, 1993), antibodies specific for the α-2,3 terminally sialylated forms of the CD15 antigens were grouped into a new separate cluster named CD15s. Other antimyeloid MAbs bound to even more polar GSLs (*2*). In an attempt to classify the antigen specificity of the latter mAbs, we found previously (*5*) that several mAbs bound to a GSL bearing a dodecasaccharide carbohydrate chain, X^3NeuAcVII^{3}FucnLc$_{10}$Cer, named VIM-2 dodecasaccharide (*6*). These mAbs were grouped into the CDw65 cluster of differentiation (*7*). Later it became evident that the CDw65 mAbs differed in their cellular and antigenic specificities: For example, after neuraminidase treatment of X^3NeuAcVII^{3}FucnLc$_{10}$Cer, binding of mAb VIM-2 was completely abolished but binding of mAb VIM-8 and VIM-11 was not impaired. Since all CDw65 mAbs, in spite of obvious differences, bound to X^3NeuAcVII^{3}FucnLc$_{10}$Cer, this ganglioside seemed not to be well suited to reveal fine differences in their antigen specificities. In this study, we reinvestigated the cellular and antigenic specificities of these three CDw65 mAbs.

MATERIALS AND METHODS

MAbs and Immunostaining—The mAbs VIM-2, VIM-8, VIM-11, and VIM-D5 were prepared as described (*8*). MAb CSLEX-1 (*9*) was purchased from UCLA (University of California, Los Angeles, USA). The isotype control mAb

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Abbreviations: nLc$_8$Cer, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer; V^3FucnLc$_8$Cer, Galβ1-4GlcNAcβ1-3Galβ1-4(αFuc1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer; VIII^{3}NeuAcV^3FucnLc$_8$Cer, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4(αFuc1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer, VIM-2 decasaccharide; X^3NeuAcVII^{3}FucnLc$_{10}$Cer, NeuAcα2-3Galβ1-4GlcNAcβ1-3Galβ1-4(αFuc1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1'Cer, VIM-2 dodecasaccharide; ATCC, American Type Culture Col-lection; BM-MNC, bone marrow mononuclear cells; C, chloroform; CD, cluster of differentiation; CDw, provisional cluster of differentiation; FAB-MS, fast atom bombardment mass spectrometry; FITC, fluoresceine isothiocyanate; GSL(s), glycosphingolipid(s); Lewis^x determinant, Galβ1-4(αFuc1-3)GlcNAc-; ME, methanol; mAb, monoclonal antibody; PMN, polymorphonuclear cells; TLC, thin layer chromatography; W, water.

1939-3G5 was obtained from the Myeloid Section of the V^th Workshop on Leukocyte Differentiation Antigens (Boston, 1993).

The mAb 1B2, specific for the terminal Galβ1-4GlcNAc-carbohydrate structure found in glycolipids of the neolacto series (*10*), was used as cell culture supernatant obtained from the ATTC 1B2 hybridoma clone (American Type Culture Collection, Rockville, MD, USA). The specificity of mAb M-G54 (CD15), a friendly gift from Dr. E.P. Rieber, Dresden, FRG, has been characterized elsewhere (*2*). Thin layer chromatogram immunostaining was performed as described (*11*) with modifications (*2*). The reactivities of the CDw65 mAbs towards VIII³NeuAcV³FucnLc₈Cer and V³nLc₈Cer were measured by immunostaining followed by scanning of 8–400 ng of VIII³NeuAcV³FucnLc₈Cer before and after treatment with *Vibrio cholerae* neuraminidase (Boehringer Mannheim, Mannheim, FRG) at 37°C for 20 h in 0.1% (w/v) calcium chloride. The indigo-like stained bands were scanned with an incident light at 600 nm using a CS-9001PC Scanner (Shimadzu, Düsseldorf, FRG) in zig-zag mode. The areas were measured using the software version 2.0 supplied by the manufacturer.

Preparation of PBMC and Granulocytes, Cultivation of the HL60 Cell Line, Immunofluorescence Analysis and Enzyme Treatment of Cells—Cells: Bone marrow and peripheral blood mononuclear cells were isolated by buoyant density gradient centrifugation on Ficoll-Paque prior to use. Contaminating erythrocytes were removed by hypotonic lysis from the pelleted granulocyte fraction. The HL60 cell line was obtained from ATCC and cultured in RPMI 1640/10% FCS in continuous culture in a humidified atmosphere of 5% CO_2 at 37°C.

Immunofluorescence analysis: Fifty microliters of cells (10^7/ml) and 20 μl of mAb (10 μg/ml) were mixed and incubated for 30 min on ice, followed by incubation with 50 μl of FITC labeled F(ab')$_2$ fragments of sheep antimouse immunoglobulins (An der Grub, Kaumberg, Austria). Subsequent analysis was performed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA, USA).

Enzyme treatment of cells: 5×10^6 cells were incubated for 30 min with *V. cholerae* sialidase (10 mU/ml) at 37°C and then analyzed by flow cytometry.

Isolation of Glycolipids—The CDw65 glycolipid antigens were isolated from human spleens and from unseparated leukocytes. For the purification of the CDw65 GSLs from spleens, three fresh human spleens obtained from the local hospital were cut in small pieces and extracted successively with 5 liters each of chloroform/methanol (C/ME) 1 : 2, 1 : 1, and 2 : 1 (v/v). The residual tissue was lyophilized and extracted again as above. Presence of CDw65 GSL antigens was monitored after each separation step by thin layer chromatogram immunostaining using the mAb VIM-2. After evaporation of the solvent, the extract was taken up in water (W), sonicated, dialyzed and lyophilized. This material was partitioned according to Folch *et al.* (*12*). All CDw65 reactive GSLs were found in the Folch upper phase. It was dried, dissolved in C/ME 85 : 15 (v/v) and fractionated on a preparative silica column (3×10 cm, Iatrobeads 6RS 8060) with the following C/ME/W mixtures (v/v/v): 1 liter each of C/ME 2 : 1, C/ME 1 : 2, C/ME 1 : 4, ME, ME/W 4 : 1. The C/ME 1 : 4 fraction, which contained the bulk of the VIM-2 antigens, was evaporated to dryness, dissolved in C/ME/W 30 : 60 : 8 and passed through a DEAE-Sepharose column (3.5×10 cm, acetate form). After washing with 2 liter ME, gangliosides were eluted with 2 liters each of 0.01, 0.02, 0.03, 0.05, 0.08, 0.15, 0.3 M ammonium acetate in ME, and desalted on a preparative C-18 reversed phase column (1.6×50 cm, Bakerbond C-18, 40-μm particles) as described (*13*). The 0.01, 0.02, and 0.03 M ammonium acetate eluates contained the VIM-2 antigens. They were pooled and finally separated by HPLC using a 1.6×50 cm HPLC column containing Lichrosorb Si 60 5-μm particles (Merck, Darmstadt, FRG). Elution was performed with a linear gradient of 2-propanol/*n*-hexane/W from 55 : 42 : 3 (v/v/v) to 70 : 10 : 20 within 600 min at a flow rate of 1.5 ml/min, similarly as described (*14*). Two hundred fractions of 4.5 ml were collected and screened for VIM-2 antigens. HPLC fractions 140–170 showed mAb VIM-2 positive bands. GSLs from unseparated leukocytes were similarly processed (in this case without the Folch partition) starting with 1×10^{12} cells. A small sample of pure VIII³NeuAcV³FucnLc₈Cer was prepared and characterized as described (*15*).

Enzymatic Degradation—Glycosidase digestions were either performed in tubes or directly after thin layer chromatography (TLC) on polyisobutylmethacrylate-fixed silica plates (*11*). All glycosidase digestions in tubes were performed at 37°C under shaking. Incubations were stopped by adding 5 ml 0.88% aqueous KCl, followed by a brief sonication (5 min). The solutions were desalted on a Sep-Pak C-18 cartridge as described (*13*). The ME eluate, containing the GSLs and deoxytaurocholate, was then passed through a small (2 ml) DEAE-Sepharose column (acetate form) to remove the deoxytaurocholate. Sialidase treatment in tubes was carried out in 0.4 ml of 50 mM Na acetate buffer+1 mM $CaCl_2$ pH 5.5 with 0.2 units of *V. cholerae* neuraminidase [EC 3.2.1.18] (Behring, Marburg, FRG) for 16 h. β-Galactosidase digestion was performed in 0.4 ml of 50 mM Na citrate buffer pH 4.0 with 0.5 units of β-galactosidase from jack beans [EC 3.2.1.23] (Sigma, München, FRG) in the presence of 0.4 mg of deoxytaurocholate for 24 h. The incubation mixture with β-*N*-acetylglucosaminidase (jack beans [EC 3.2.1.30]; Sigma, Munich, FRG) contained 1 unit of the enzyme and 0.4 mg of deoxytaurocholate in 0.4 ml of Na-citrate pH 5.0. Digestion was stopped after 24 h. α-L-Fucosidase from bovine kidney [EC 3.2.1.51] was purchased from Sigma. The GSL sample was incubated with 0.1 unit of the α-L-fucosidase and 0.3 mg of deoxytaurocholate in 0.3 ml of 50 mM Na-citrate pH 5.0 for 24 h. Sialidase treatments of GSLs separated on TLC plates were performed after fixation with polyisobutylmethacrylate (*11*) using 10 mU/ml *V. cholerae* neuraminidase [EC 3.2.1.18] (Behring) in 0.1% calcium chloride at 37°C overnight.

Permethylation and Fast Atom Bombardment-Mass Spectrometry (FAB-MS)—Permethylation was carried out by a modification of the method described by Ciucanu and Kerek (*16*) as described in Ref. *17*. The permethylated samples were analyzed by positive-ion FAB mass spectrometry on a ZAB HF mass spectrometer (V.G. Analytical, Manchester, UK) as described (*18*).

RESULTS

Reactivity of CDw65 mAbs with Peripheral Blood Cells, Bone Marrow Mononuclear Cells, and the Myeloid Cell

Line HL-60—The CDw65 mAbs VIM-2, VIM-8, and VIM-11 were tested for binding to lymphocytes, granulocytes, and monocytes from three healthy donors and to bone marrow mononuclear cell fractions (BM-MNC) from two donors by flow cytometric analyses. We found two different reaction patterns with normal blood cells (Table I). Group 1 mAb (VIM-2) bound to both monocytes and polymorphonuclear cells (PMN), while group 2 mAbs (VIM-8, VIM-11) bound only to PMN. Antibodies from both groups bound significantly to BM-MNC. Binding of CDw65 mAbs to HL60 cells before and after sialidase treatment was also tested. The differences in the mAb binding before and after VCN treatment of HL60 cells allowed the same grouping of mAbs as already observed with white blood cells (Fig. 1). Binding of mAb VIM-2 was found to be abolished upon sialidase treatment. This was identical to the behaviour of the anti-sLe^x (CD15s) mAb CSLEX-1 (Fig. 1). In contrast, binding of mAbs VIM-8 and VIM-11 was enhanced upon sialidase treatment, similar to the anti-Le^x (CD15) mAb VIMD5 (Fig. 1).

Reactivity of CDw65 mAbs with Monosialogangliosides and Desialylated Monosialogangliosides—The glycolipid antigens detected with CDw65 mAb VIM-2 were purified from the lipid extracts of human spleens and also from unseparated human leukocytes. Figure 2 shows the immunostaining patterns of the three CDw65 mAbs, VIM-2, VIM-8, VIM-11, and of an Galβ1-4GlcNAc-R specific control antibody, 1B2, with the purified monosialoganglioside fraction (− lanes) from human leukocytes and with the *in situ* desialylated monosialoganglioside fraction (+ lanes). All of these antibodies stained multiple bands, suggesting that they recognized an epitope shared by several GSLs. The mAbs VIM-8 and VIM-11 showed identical patterns (Fig. 2, B and C). Both mAbs bound preferentially to the desialylated monosialogangliosides (Fig. 2, B and C, + lanes). In contrast, mAb VIM-2 bound strongly to the monosialoganglioside fraction (Fig. 2A, − lane) but showed poor binding when the plate had been treated with sialidase (Fig. 2A, + lane). The small band still visible in the sialidase-treated sample was due to incomplete desialylation, because after incubation of the same antigens with sialidase in tubes, VIM-2 antigenicity was completely destroyed (not shown). Several bands stained by mAb VIM-2 in the monosialoganglioside fraction (Fig. 2A, − lane) and by the mAbs VIM-8/VIM-11 in the desialylated monosialoganglioside fraction, respectively (Fig. 2, B and C, + lanes), showed identical chromatographic mobilities (Fig. 2, indicated by arrows). This led us suppose that several VIM-2 antigens were changed to VIM-8/VIM-11 antigens upon sialidase treatment.

Characterization of the VIM-2 Decasaccharide—To verify the assumption that VIM-2 antigens are converted to VIM-8/VIM-11 antigens upon sialidase treatment, we

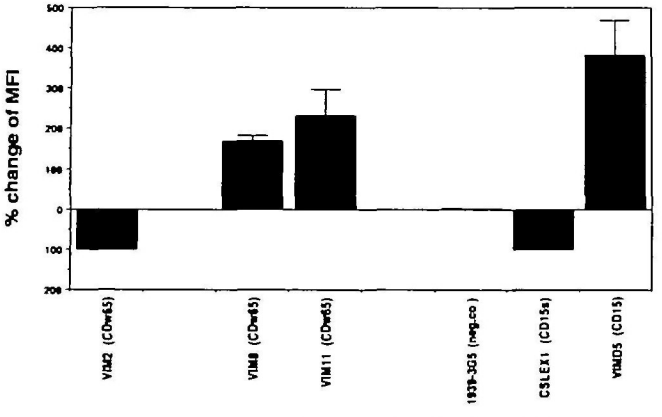

Fig. 1. **Sensitivity of CDw65 mAbs to *Vibrio cholerae* neuraminidase treatment in comparison to CD15 and CD15s mAbs and an isotype-matched control mAb.** Percent change in mean fluorescence intensity ± SEM (% MFI) upon enzyme treatment of HL60 cells is shown

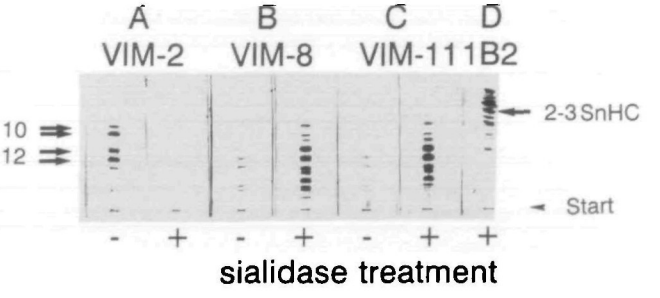

Fig. 2. **Immunostaining patterns of the complete monosialoganglioside fraction from unseparated leukocytes.** HPTLC Si 60 plates (Merck, Darmstadt, FRG) were developed in chloroform-methanol-water (50 : 47 14, v/v/v) containing 0.05% $CaCl_2$ for 45 min at ambient temperature and stained with the indicated mAbs before ("−" lanes) and after ("+" lanes) their treatment with *Vibrio cholerae* neuraminidase as described in "MATERIALS AND METHODS." Abbreviations are: 10, VIM-2 decasaccharide, VIII^{3}Neu5AcV^3-FucnLc$_4$Cer; 12, VIM-2 dodecasaccharide X^{3}NeuAcVII^{3}FucnLc$_{10}$Cer, 2-3 SnHC *position* of VI^{3}Neu5AcnLc$_6$Cer (mAb 1B2 detects its asialoganglioside nLc$_6$Cer)

TABLE I. **Reactivity of CDw65 mAbs with white blood cells (three donors) and BM-MNC (two donors) is shown.** Reactivity was determined by FACS analyses and is expressed as MFI ± SD (mean fluorescence intensity ± standard deviation) and % pos. ± SD (percent positive cell ± standard deviation) as compared to negative isotype control mAb 1939-3G5 and to CD15 mAb VIMD5 and CD15s mAb CSLEX1.

Antibody	Lymphocytes (n=3)		Monocytes (n=3)		Granulocytes (n=3)		BM-MNC (n=2)	
	% pos	MFI	% pos	MFI	% pos	MFI	% pos.	MFI
Control mAbs								
1939-3G5 (isotype control)	2±1	7±1	6±3	19±3	4±1	19±2	3±1	12±1
CSLEX1 (CD15s)	17±2	12±1	94±1	385±68	95±2	713±123	60±14	88±50
VIMD5 (CD15)	3±1	7±1	21±5	27±2	99±0	651±253	28±10	35±17
GROUP 1								
VIM-2 (CDw65)	2±1	7±0	89±7	126±49	99±0	457±136	64±15	89±44
GROUP 2								
VIM-8 (CDw65)	3±3	7±2	6±2	21±1	95±5	231±88	42±9	38±13
VIM-11	2±1	7±1	6±3	21±3	85±12	134±50	27±2	25±4

purified a VIM-2 antigen from the monosialoganglioside fractions of human spleen and from unseparated leukocytes as described in "MATERIALS AND METHODS." The VIM-2 binding HPLC fraction 156 from leukocytes and fraction 150 from spleen were selected for further characterization. The FAB-MS spectrum of permethylated fraction 150 and the proposed fragmentation scheme are shown in Fig. 3. Fraction 150 contained two compounds. The molecular ions of permethylated 1 are at m/z $MH^+=2,696$ and m/z $MNa^+=2,718$, indicating $NeuAc_1$-$HexNAc_3$-Hex_5-Cer. Permethylated 2 gave rise to m/z $MH^+=2,870$ and $MNa^+=2,892$, suggesting a $NeuAc_1$-$HexNAc_3$-Fuc_1-Hex_5-Cer composition. To determine which of the two compounds was the VIM-2 antigen and which monosaccharide moieties were required for antigen recognition, an aliquot of fraction 150 was subjected to treatment with either α-fucosidase or sialidase. As shown in Fig. 4, both enzymes destroyed the antigenicity with respect to mAb VIM-2 binding. The disappearance of VIM-2 binding upon α-fucosidase treatment indicated compound 2 to be the VIM-2 antigen.

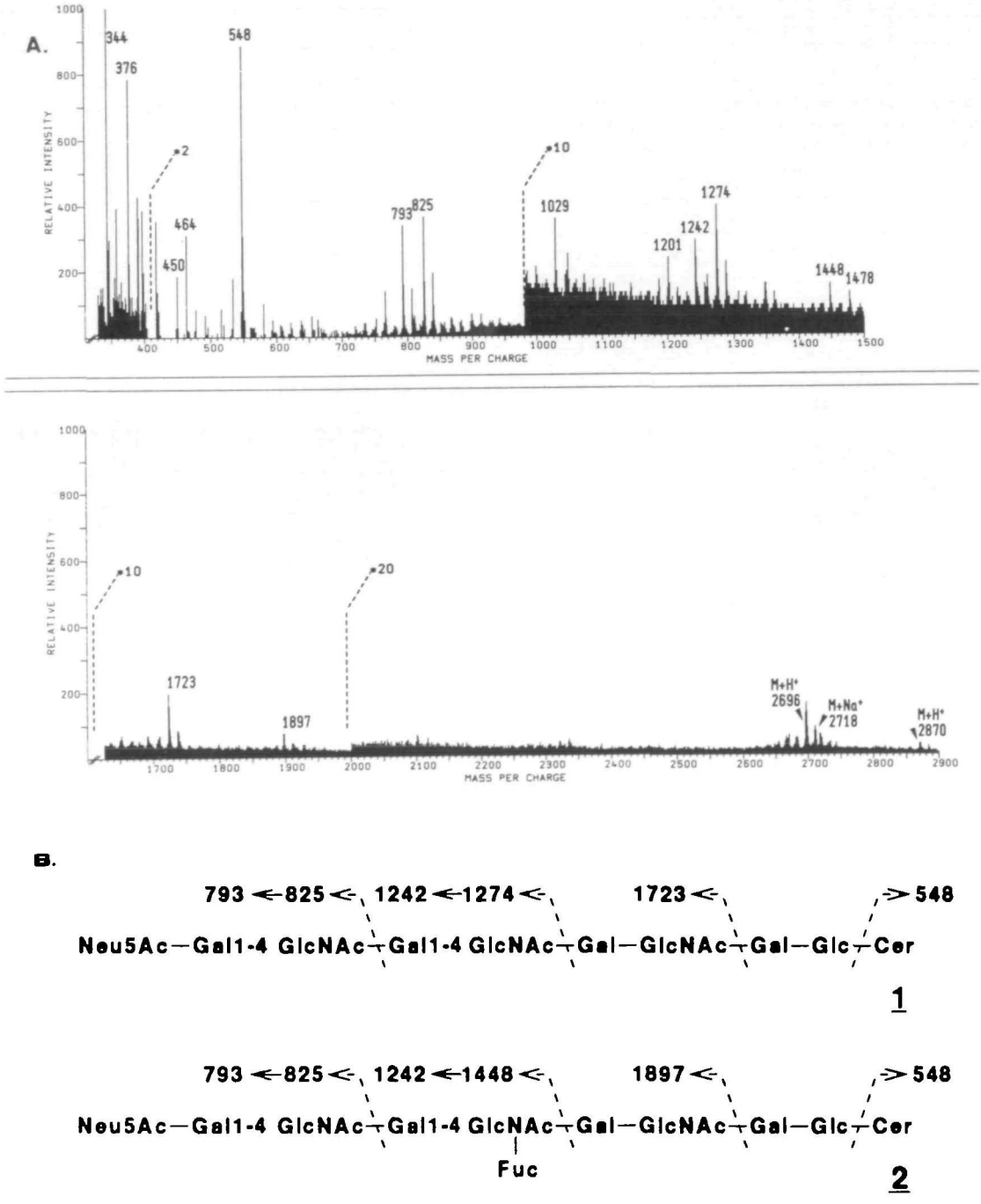

Fig. 3. (+)FAB-MS of the HPLC fraction 150 from human spleen after permethylation (A) and the fragmentation patterns of two gangliosides, whose structures have been assigned to this spectrum (B). VIII³Neu5AcnLc₄Cer(16 : 18) (*1*) and VIII³Neu5AcV³FucnLc₆-Cer(16 : 18) (*2*).

Whereas primary and secondary fragment ions of 1 point to a type 2 chain (=GSL of the neolacto series), the fragmentation of 2 did not allow an unambiguous assignment of the fragment ion m/z 1,242. It could be either a secondary ion originating from m/z 1,448 followed by the loss of a 1,3 bound fucose, or a degradation product of the pentasaccharide fragment m/z 1,274. To distinguish between these alternatives, aliquots of fractions 150 (spleen) and 156 (unseparated leukocytes) were digested sequentially with sialidase, β-galactosidase, and β-*N*-acetylglucosaminidase. The degradation product(s) could be immunostained with the mAB M-G54 (CD15) recognizing the Le^x structure (Fig. 4, lanes a and b). The degradation product from fraction 150 showed a doublet, that of fraction 156 only the lower band of it. As described by Fukuda *et al.* (*4*), such heterogeneity may be caused by different fatty acids of the ceramide moiety with identical carbohydrates. A similar doublet is seen at the same position in the neutral GSL standard mixture immunostained with the same mAb M-G54 (Fig. 5). This doublet corresponds to $V^3FucnLc_6Cer$ (N3), a heptaglycosylceramide that is strongly immunostained by all known CD15 mAbs (*2*, *4*). Therefore,

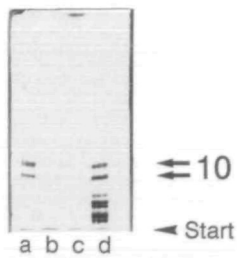

Fig. 4 **Immunostaining of glycosidase-treated gangliosides with VIM 2.** Solvent system: chloroform-methanol-water (50 : 40 · 10, v/v/v) containing 0 05% $CaCl_2$. Lane a, 0.5% of HPLC fraction 150 (spleen); lane b, 1% of fraction 150 after treatment with sialidase; lane c, 1% of fraction 150 after treatment with α-L-fucosidase; lane d, standard (unseparated monosialoganglioside fraction) Abbreviation: 10, VIM-2 decasaccharide, $VIII^3Neu5AcV^3FucnLc_8Cer$

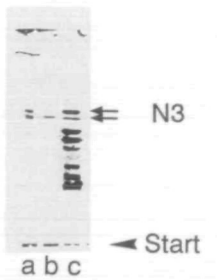

Fig. 5. **Immunostaining of the glycosidase-treated gangliosides with the anti-Le^x antibody M-G54.** Solvent system: see Fig 4 Lane a, HPLC fraction 150 (spleen) after neuraminidase, β-galactosidase and β-*N*-acetylglucosaminidase treatment, lane b, HPLC fraction 156 (unseparated leukocytes), sequentially degraded with the same glycosidases as fraction 150; lane c, neutral glycolipid fraction of unseparated human leukocytes, enriched for polar compounds by silica chromatography. The doublet at the top corresponds to N_3 ($V^3FucnLc_nCer$)

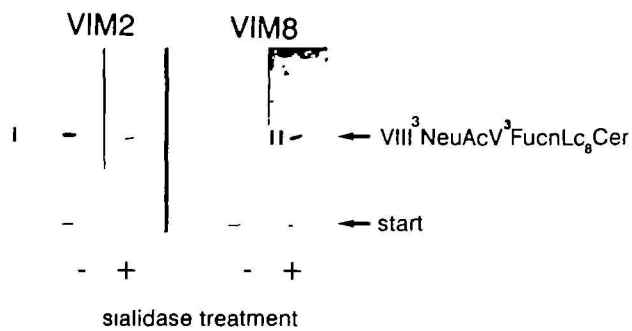

Fig. 6. **Conversion of VIM-2 antigen $VIII^3Neu5AcV^3FucnLc_8$-Cer in HPLC fraction 156 (unseparated leukocytes) to VIM-8 antigen $V^3FucnLc_4Cer$ upon *in situ* sialidase treatment.** An aliquot of 0.2% of fraction 156 (unseparated leukocytes) was developed in parallel on each of four 2.5×10 cm HPTLC silica plates in chloroform-methanol-water (50 · 47 · 14, v/v/v) containing 0.05% $CaCl_2$ (w/v) for 30 min. Two plates ("+") were pretreated with sialidase as described in "MATERIALS AND METHODS" before immunostaining

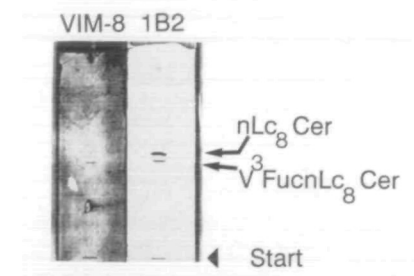

Fig 7 **Analysis of the *in vitro* sialidase-treated HPLC fraction 156 (unseparated leukocytes) with the mAbs 1B2 and VIM-8.** An aliquot of the antigens of HPLC fraction 156 was treated with *Vibrio cholerae* neuraminidase in tubes and desalted as described in "MATERIALS AND METHODS." The desialylated antigens were separated as described in Fig 6 and immunostained with mAbs VIM-8 and 1B2

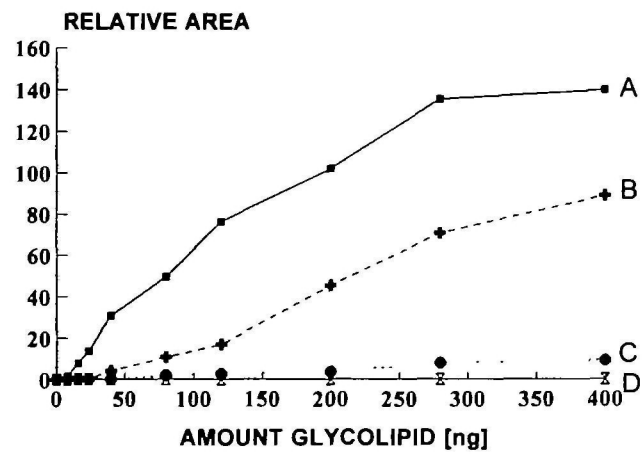

Fig 8. **Binding of the mAbs VIM-2 and VIM-8 to $VIII^3Neu$-$AcV^3FucnLc_8Cer$ and to $V^3FucnLc_4Cer$ as revealed by immunostaining.** The indicated amounts of $VIII^3NeuAcV^3FucnLc_8Cer$ were separated on four TLC plates as described in the legend of Fig. 2. Two of them were treated with *Vibrio cholerae* neuraminidase as described in "MATERIALS AND METHODS." A, binding of VIM-2 to $VIII^3$-$NeuAcV^3FucnLc_4Cer$; B, binding of mAb VIM-8 to *in situ* desialylated $VIII^3NeuAcV^3FucnLc_8Cer$; C, binding of mAb VIM-2 to desialylated $VIII^3NeuAcV^3FucnLc_8Cer$; D, binding of mAb VIM-8 to $VIII^3$-$NeuAcV^3FucnLc_4Cer$. Quantitation was performed by scanning as described in "MATERIALS AND METHODS "

compound 2 appeared to be identical to the VIM-2 decasaccharide VIII³NeuAcV³FucnLc₈Cer, first described by Macher *et al.* (*6*). Compound 1 was either a α2-3 or α2-6 sialylated form of nLc₈Cer. Since we were mainly interested in the reactivity of the asialogangliosides, the linkage of the sialic acid was not further analyzed.

Comparison of the Binding of the mAbs VIM-8 and VIM-11 to V³FucnLc₈Cer (Asialo-VIM-2 Decasaccharide) and to VIII³NeuAc V³FucnLc₈Cer (VIM-2 Decasaccharide)—An aliquot of HPLC fraction 156 from unseparated leukocytes containing the compounds 1 and 2 was immunostained with the mAbs VIM-2 and VIM-8 before and after treatment with sialidase (Fig. 6). MAb VIM-2 detected a single band in the immunostain, which nearly disappeared after sialidase treatment (+ lane). At the same time, mAb VIM-8 recognized a new band in the desialylated fraction (Fig. 6). This band might originate from either compound 1 or compound 2. To differentiate between these two possibilities, we modified the experiment shown in Fig. 6 by treating the mixture of compound 1 and compound 2 with sialidase before it was applicated to the TLC plates. This resulted in the formation of desialylated compound 1, nLc₈Cer, and desialylated compound 2, V³FucnLc₈Cer. It could now be expected that nLc₈Cer and V³FucnLc₈Cer would be separable from each other because V³FucnLc₈Cer contained an additional fucose rendering it more polar (=slower migrating). Aliquots of the desialylated antigens were then immunostained with the mAbs VIM-8 and 1B2 (Fig. 7).

The plate stained by mAb 1B2 (specific for a terminal Galβ1-4GlcNAc-structure) showed two bands (Fig. 7). This confirmed that both of the desialylated antigens contained a terminal nonsubstituted Galβ1-4GlcNAc-structure. It also confirmed the location of the fucose branch of desialylated compound 2 at an inner lactosamine unit, since otherwise mAb 1B2 would not have bound. The same was suggested by the FAB-MS data (Fig. 3), since no tetrasaccharide ion $NeuAc_1Hex_1HexNAc_1Fuc_1$ at $m/z=999$ for sLe^x or sLe^a was detected. The upper band is the less polar of the products of the sialidase treatment, nLc₈Cer, and the slower migrating band is therefore the VIM-8 antigen V³FucnLc₈Cer. Staining of both antigens with mAb VIM-8 (Fig. 7) showed only the slower migrating antigen. Thus, VIM-8 bound to V³FucnLc₈Cer and not to nLc₈Cer.

The immunostaining pattern in Fig. 2 also suggested that the desialylated compound 1 and other nonfucosylated type 2 chain GSLs were not identical with the VIM-8 antigen, since a comparative staining of the desialylated monosialoganglioside fraction with mAb VIM-8 (Fig. 2B, "+") and mAb 1B2 (Fig. 2D) resulted in completely different patterns.

The binding of the mAbs VIM-2 and VIM-8 to different amounts of VIII³NeuAcV³FucnLc₈Cer before and after treatment of this antigen directly on the thin layer plate with *V. cholerae* sialidase is shown in Fig. 8. The binding of mAb VIM-2 (Fig. 8A) was nearly abolished after treatment of VIII³NeuAcV³FucnLc₈Cer with sialidase (Fig. 8C). In contrast, there was no binding of mAb VIM-8 to VIII³NeuAcV³FucnLc₈Cer (Fig. 8D). However, mAb VIM-8 bound strongly to the desialylated antigen (Fig. 8B).

DISCUSSION

In this investigation, we addressed the question of the heterogeneity of three CDw65 mAbs. Originally, all CDw65 mAbs were found to bind to X³NeuAcVII³FucnLc₁₀Cer (*5*), the VIM-2 dodecasaccharide (*6*). Binding of myeloid antibodies to this antigen was useful to distinguish CDw65 antibodies from the other antimyeloid carbohydrate specific mAbs (Table II). However, different cellular binding suggested that not all mAbs which bound to the VIM-2 dodecasaccharide behaved identically.

To evaluate the differences, we checked first the cellular reactivity pattern of our three CDw65 mAbs. In contrast to VIM-8 and VIM-11, VIM-2 bound not only to granulocytes but also to monocytes (Table I). This resembles the broader cellular specificity of the sLe^x specific mAbs compared to Le^x binding mAbs (Table I). The differences in binding of CDw65 mAbs to myeloid cells might thus reflect cell lineage-specific differences in the respective levels of sialylation of the CDw65 antigens. This hypothesis was confirmed by the observation that sialidase treatment of HL60 cells completely abrogated binding of mAb VIM-2, whereas the other two CDw65 mAbs showed enhanced reactivity with these cells. This again is similar to observa-

TABLE II. **Structures of myeloid carbohydrate antigens.**

MAb	Antigens recognized	Structure (epitope)
1B2	Lacto type 2	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-
VIMD5	CD15 antigens	Galβ1-4GlcNAcβ- 3 \| 1 Fucα
CSLEX1	Sialylated CD15 antigens	NeuAcα2-3Galβ1-4GlcNAc- 3 \| 1 Fucα
VIM-8, VIM-11	CDw65 antigens	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc- 3 \| 1 Fucα
VIM-2	Sialylated CDw65 antigens	NeuAcα2-3Galβ1-4GlcNAcβ1-3-Galβ1-4GlcNAc- 3 \| 1 Fucα

tions with sLe^x and Le^x mAbs (Fig. 1). Thus, on the basis of both, differences in cell binding profiles as well as sensitivity to sialidase treatment of cells, two distinct specificities of the CDw65 mAbs under study were defined.

To examine whether these differences are due to different antigen specificities of the CDw65 mAbs, we studied mAb binding to a GSL antigen which was shorter by one lactosamine unit than the original CDw65 antigen, the VIM-2 dodecasaccharide, namely, to the VIM-2 decasaccharide and to its desialylated form. VIM-8 and VIM-11 failed to bind to the sialylated decasaccharide, but it was still recognized by VIM-2. Conversely, the desialylated decasaccharide was no longer recognized by VIM-2 but was well recognized by VIM-8 and VIM-11.

One would expect that all VIM-8/VIM-11 antigens should also be recognized by mAb 1B2 since they have a terminal Galβ1-4GlcNAc-structure. The patterns represented in Fig. 2 suggested that this was not the case. MAb 1B2 showed a strong preference for nLc_6Cer (Fig. 2, here designated as "position of 2-3 SnHC"), but it also bound fairly well to nLc_4Cer (not shown), nLc_8Cer and $V^3FucnLc_8Cer$ (Fig. 7), to a lower extent to $VIII^3FucnLc_{10}Cer$ (Fig. 2, "12"), and even less to the same antigen types with longer carbohydrate chains (Fig. 2D). These latter were, however, the main VIM-8/VIM-11 antigens (Fig. 2). The decreasing sensitivity of mAb 1B2 to antigens with increasing carbohydrate chain lengths may be explained by the fact that mAb 1B2 was raised against purified nLc_6Cer (*10*).

According to the results of the present study, the mAbs VIM-8 and VIM-11 (Fig. 2, B and C) on one hand and mAb VIM-2 (Fig. 2A) on the other hand showed such different binding behaviour that grouping of these mAbs in one cluster of differentiation is no longer justified. As the antigen specificities of the pairs VIM-2 and VIM-8/VIM-11 were comparable with the specificities of CD15s and CD15 specific for sialylated and nonsialylated antigens, we propose that mAb VIM-2 should be excluded from the CDw65 cluster and placed in a separate group, which could become the basis for a new cluster named CDw65s.

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REFERENCES

1. Uemura, K., Macher, B.A., DeGregorio, M., Scudder, P., Buehler, J., Knapp, W., and Feizi, T. (1985) Glycosphingolipid carriers of carbohydrate antigens of human myeloid cells recognized by monoclonal antibodies. *Biochim. Biophys. Acta* **846**, 26-32
2. Kniep, B. and Mühlradt, P.F. (1987) Monoclonal antibodies of the myeloid panel recognizing glycosphingolipids in *Leucocyte Typing III* (McMichael, A.J., ed.) pp. 619-621, Oxford University Press, Oxford
3. Feizi, T. (1985) Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens. *Nature* **314**, 53-57
4. Fukuda, M.N., Dell, A., Oates, J.E., Wu, P., Klock, J.C., and Fukuda, M. (1985) Structures of glycosphingolipids isolated from human granulocytes. *J. Biol. Chem.* **260**, 1067-1082
5. Kniep, B., Peter-Katalinic, J., and Mühlradt, P.F. (1989) Glycosphingolipid-recognizing antibodies of the myeloid section in *Leucocyte Typing IV* (Knapp, W., Dörken, B., Gilks, W.R., Rieber, E.P., Schmidt, R.E., Stein, H., and van dem Borne, A.E.G.Kr., eds.) pp. 877-879, Oxford University Press, Oxford
6. Macher, B.A., Buehler, J., Scudder, P., Knapp, W., and Feizi, T. (1988) A novel carbohydrate differentiation antigen on fucogangliosides of human myeloid cells recognized by monoclonal antibody VIM-2. *J. Biol. Chem.* **263**, 10186-10191
7. Stockinger, H. (1989) Cluster Report: CDw65 in *Leucocyte Typing IV* (Knapp, W., Dörken, B., Gilks, W.R., Rieber, E.P., Schmidt, R.E., Stein, H., and van dem Borne, A.E.G.Kr., eds.) pp. 836-838, Oxford University Press, Oxford
8. Knapp, W., Majdic, O., Stockinger, H., Bettelheim, P., Liszka, K., Köller, U., and Peschel, C. (1984) Monoclonal antibodies to human myelomonocyte antigens in the diagnosis of acute myeloid leukemia. *Med. Oncol. Tumor Pharmacother.* **1**, 257-262
9. Fukushima, K., Hirota, M., Terasaki, P.I., Wakisaka, A., Togashi, H., Chia, D., Suyama, N., Fukushi, Y., Nudelman, E., and Hakomori, S. (1984) Characterization of sialosylated $Lewis^x$ as a new tumor-associated antigen. *Cancer Res.* **44**, 5279-5285
10. Young, W.W., Jr., Portoukalian, J., and Hakomori, S. (1981) Two monoclonal anticarbohydrate antibodies directed to glycosphingolipids with a lacto-*N*-glycosyl type II chain. *J. Biol. Chem.* **256**, 10967-10972
11. Bethke, U., Müthing, J., Schauder, B., Conradt, P., and Mühlradt, P.F. (1986) An improved semi-quantitative enzyme immunostaining procedure for glycosphingolipid antigens on high performance thin-layer chromatograms. *J. Immunol. Methods* **89**, 111-116
12. Folch, J., Arsove, S., and Meath, J.A. (1951) Isolation of brain strandin, a new type of large molecule tissue component. *J. Biol. Chem.* **191**, 819-831
13. Williams, M.A. and McCluer, R.H. (1980) The use of Sep-Pak C_{18} cartridges during the isolation of gangliosides. *J. Neurochem.* **35**, 266-269
14. Watanabe, K. and Arao, Y. (1981) A new solvent system for the separation of neutral glycosphingolipids. *J. Lipid Res.* **22**, 1020-1024
15. Müthing, J., Spanbroek, R., Peter-Katalinic, J., Hanisch, H., Hanski, C., Hasegawa, A., Unland, F., Lehmann, J., Tschesche, H., and Egge, H. (1996) Isolation and structural characterization of fucosylated gangliosides with linear poly-*N*-acetyllactosaminyl chains from human granulocytes. *Glycobiology*, in press
16. Ciucanu, I. and Kerek, F. (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **131**, 209-217
17. Egge, H. and Peter-Katalinic, J. (1987) Fast atom bombardment mass spectrometry for structure elucidation of glycoconjugates. *Mass Spectrom. Rev.* **6**, 331-339
18. Peter-Katalinic, J. and Egge, H. (1990) Desorption mass spectrometry of glycosphingolipids. *Methods Enzymol.* **193**, 713-733