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Monoclonal antibodies against the human lymphocyte differentiation antigen CD 76 bind to gangliosides

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Two monoclonal antibodies, HD 66 and CRIS-4, by which the new CD 76 B-cell-associated cluster was defined, bound to several gangliosides (sialic acid containing glycolipids) of different polarity. One of the gangliosides recognized by HD 66 could be identified as NeuAcα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc-β1-1'Cer. This antigen was enzymatically synthesized. Sialidase treatment of the ganglioside antigens abolished binding of HD 66 and CRIS-4.

2,6-Sialosyllactoneotetraosylceramide; Sialylation; CD 76; B lymphocyte; Immunostaining; Carbohydrate antigen

1. INTRODUCTION

Monoclonal antibodies (mAbs) directed against human leucocytes are classified in 78 clusters of differentiation (CD) by their binding profiles with various types of leucocytes and their recognition of identical antigens [1]. Two monoclonal antibodies, HD 66 and CRIS-4, define the recently designated CD 76 antigen [2]. This antigen is present on mature human B lymphocytes and some B-cell leukemias, a subpopulation of T lymphocytes, and some epithelial cell types [3]. We now show that both CD 76 mAb – though to different extents – recognize sialylated type 2 chain carbohydrate moieties of glycosphingolipids (GSLs) such as IV^{6}NeuAcnLc$_4$Cer. In contrast to CD 77 mAbs, which recognize the neutral GSL globotriaosylceramide (Gb$_3$) on activated B-cells [3], CD 76 mAbs bind to sialylated GSLs on resting B-cells. Thus CD 76 mAbs detect a further cell specific carbohydrate surface antigen on human leucocytes like CDw 60, CDw 65 and CD 77 mAbs [3–5].

2. MATERIALS AND METHODS

2.1. *Glycosphingolipids*

Lactoneotetraosylceramide was purified from human neutrophils by Folch partition [6], DEAE Sepharose chromatography [7] and finally by high-performance liquid chromatography (HPLC) as described below. A monosialoganglioside fraction was prepared from unseparated human peripheral white blood cells and purified by Folch partition and DEAE Sepharose chromatography. Monosialogangliosides were eluted from the DEAE Sepharose column with 20 mM ammonium acetate in methanol. 2,6-Sialosyllactoneotetraosylceramide (IV^{6}NeuAcnLc$_4$Cer) was prepared enzymatically as follows: a solution of 10 μ (8 nmol) lactoneotetraosylceramide in 200 μl methanol was taken to dryness at ambient temperature in a well of a 4-well cell culture plate (Nunc, Wiesbaden, FRG). The adsorbed GSL was incubated with 140 nmol [^{14}C]-CMP-*N*-acetyl-neuraminic acid (1 μCi/140 nmol) in 200 μl 100 mM Mes buffer (2-(*N*-morpholino)ethanesulfonic acid), pH 6.5, containing 0.02% (w/v) sodium azide, 2.5 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin and 5 mU of CMP-*N*-acetylneuraminate:β-D-galactosyl-1,4-*N*-acetyl-β-D-glucosamine α-2,6-*N*-acetylneuraminyltransferase (EC 2.4.99.1) (Boehringer Mannheim, Mannheim, FRG) at 37°C for 24 h. The incubation mixture was then removed and the remaining adsorbed GSL were extracted twice with 1 ml of methanol. The methanol extracts were dried, suspended under sonification in 5 ml of 0.1 M aqueous KCl and recombined with the incubation mixture. The solution was then purified on a Sep-Pak C_{18} cartridge (Millipore, Molsheim, France) as described [8]. Approximately 43700 cpm of the reaction product, corresponding to 4.8 μg of 2,6-sialosyllactoneotetraosylceramide (2,6-SPG) was obtained in the methanol eluate.

2.2. *Separation of GSL by HPLC*

HPLC of GSL was performed on a 16 × 500 mm column filled with Lichrosorb Si 60 (5 μm particles) (Merck, Darmstadt, FRG). A linear gradient from 2-propanol/*n*-hexane/water (55:42:3 to 55:25:20) within 10 h at a flow rate of 1.5 ml/min was used. 200 fractions of 4.5 ml were collected.

2.3. *Sialidase treatment*

Sialidase treatment of gangliosides separated on thin-layer plates (HPTLC Si 60, Merck, Darmstadt, FRG) was performed with 10 mU/ml sialidase from *Vibrio cholerae* (EC 3.2.1.18; Behring, Marburg, FRG) in a 0.1% aqueous solution of $CaCl_2$ at 37°C overnight. In addition, for sialidase treatment in vials, the gangliosides were sonicated briefly in 0.2 ml 0.1% $CaCl_2$ and then incubated with 0.2 U (0.2 ml) *Vibrio cholerae* neuraminidase overnight at 37°C. Incubation was stopped by addition of 2 ml chloroform/methanol (2:1 v/v) yielding two phases. The lower phase contained the asialogangliosides.

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2.4. *Antibodies*

MAb 1B2 [9] detecting Galβ1-4GlcNAcβ1-R was a kind gift of Dr K. Skubitz (Minneapolis, MN, USA); mAbs HD 66 [10] and CRIS-4 (R.V.) were produced by the authors.

2.5. *Immunostaining of glycolipids*

Glycolipid antigens were detected on thin-layer chromatograms by immunostaining as described [11] with some modifications [12]. In brief, after separation of the GSLs on HPTLC plates, the plates were coated with polyisobutylmethacrylate, blocked with PBS/1% BSA, and incubated with the antibody (1/200 diluted ascites fluid) for 14 h at 4°C. After 4 washing steps, the HPTLC plate was treated with an 1/1000 diluted phosphatase-labeled goat anti-mouse IgM antibody (Medac, Hamburg, FRG) for 90 min at 37°C in the presence of 3% (w/v) polyethylene glycol 6000. The plate was washed 4 times and finally overlaid with 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 100 mM glycine-NaOH buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$. Glycolipid antigens are visualized by the appearance of a stable indigo-like stain within 30 min.

2.6. *GSL staining*

Staining of GSLs on HPTLC plates was performed using an immunochemical detection method (in preparation). Briefly, after their HPTLC separation, the GSLs were oxidized directly on the polyisobutylmethacrylate-coated HPTLC plate with sodium periodate, incubated first with digoxigenin hydrazide, and then with a phosphatase-labeled polyclonal antibody recognizing digoxigenin. Bound antibodies were made visible with BCIP as described above. All reagents for this assay were obtained from Boehringer Mannheim (Mannheim, FRG).

3. RESULTS AND DISCUSSION

Immunostaining patterns of mAb HD 66 and CRIS-4 are shown in fig.1. Both mAb reacted with several monosialogangliosides from human white blood cells. MAb CRIS-4 detected gangliosides of higher polarity than mAb HD 66. Some of the bands were shared by both mAb (marked with arrows). To characterize the antigen detected by HD 66, we fractionated the monosialogangliosides by HPLC as described in section 2 (elution profile not shown). Fraction no.113 contained only one HD 66-positive band (fig.2A). GSL staining showed that other gangliosides were not present in this fraction (fig.2B). After treatment with *Vibrio cholerae* sialidase applied directly on the HPTLC plate, HD 66 did not bind to the desialylated GSL antigen (fig.2C, lane a). However, the desialylated GSL antigen was stained by mAb 1B2 (fig.2C, lane b) which reacts with lactoneotetraosylceramide (nLc_4) and its higher type 2 chain homologues such as nLc_4, nLc_8, and nLc_{10} [9]. These GSLs have a common Galβ1-4GlcNAcβ1-R structure at the nonreducing end of their carbohydrate chain. We then treated an aliquot of HPLC fraction 113 with *Vibrio cholerae* sialidase and compared the product with nLc_4 by thin-layer chromatogram immunostaining using mAb 1B2 (fig.2D). The desialylated GSL from fraction 113, and nLc_4 cochromatographed on the HPTLC plate, suggesting that the sialidase-treated GSL of HPLC fraction 113 was possibly identical to nLc_4. To prove this

Fig.1. Immunostaining pattern of crude leucocyte monosialogangliosides separated on HPTLC plates in chloroform/methanol/water (50:40:10, v/v/v) containing 0.05% (w/v) $CaCl_2$; running time, 40 min. Immunostaining with mAb HD 66 (A) and mAb CRIS-4 (B). Bands stained by both mAb are marked with arrows.

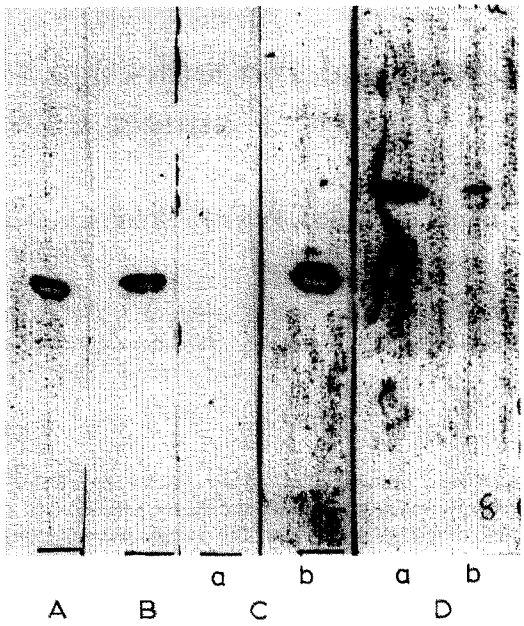

Fig.2. Reactivity of mAb HD 66 with a ganglioside of a HPLC-separated fraction (no.113) (see section 2) and the sialidase degradation product thereof. Solvent as described in fig.1. Running time for A,B,C: 40 min; for D: 50 min. (A) Immunostaining of HPLC fraction 113 with mAb HD 66 (B) total GSL staining (see section 2) of fraction 113 (C), immunostaining of fraction 113 with mAb HD 66 (lane a) and with mAb 1B2 after sialidase treatment on the plate (lane b). (D) Immunostaining with mAb 1B2 of desialylated fraction 113 (lane a) and approx. 200 ng lactoneotetraosylceramide (lane b).

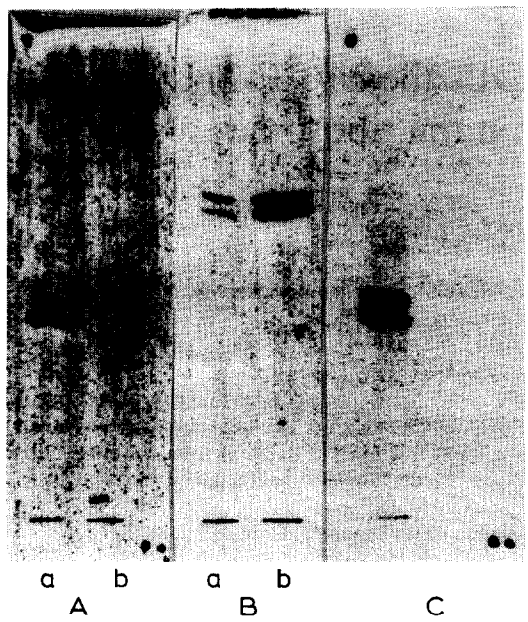

Fig.3. Immunostaining of enzymatically prepared (see section 2) 2,6-sialosyllactoneotetraosylceramide. Solvent and running time as described in fig.1. Antigens were 0.48 μg (4300 cpm) of 2,6-SPG (together with some starting material) (lane a) and 1 μg lactoneotetraosylceramide (lane b). Immunostaining with mAb HD 66 (A); immunostaining with mAb 1B2 (B); autoradiography of [^{14}C]NeuAc 2,6-SPG of A lane a (C).

and to check if sialylated nLc$_4$ was a HD 66 GSL antigen, we tested both known sialylated forms of nLc$_4$ for their reaction with HD 66. HD 66 did not bind to standard IV^{3}NeuAcnLc$_4$ (data not shown). However, enzymatically prepared IV^{6}NeuAcnLc$_4$ was detected by HD 66 (fig.3A). Immunostaining with mAb 1B2 showed the presence of some nLc$_4$ starting material in the enzymatically formed product (fig.3B, lane a). Comparison of an autoradiogram of the ^{14}C-labeled IV^{6}NeuAcnLc$_4$ (fig.3C) with its mAb HD 66 immunostain confirmed that IV^{6}NeuAcnLc$_4$ was one of the HD 66 antigens. Although the structures of the other HD 66-positive GSL antigens are not yet known, we obtained preliminary information about their partial structures (results not shown): neutral GSLs of the lactoneo series from leucocytes were separated on two small HPTLC plates. When these GSLs were sialylated directly on the two plates by 2,6-specific sialyltransferase and CMP-NeuAc, two common bands were detected by both mAb, HD 66 and CRIS-4. Mab CRIS-4 stained two additional bands in the more polar region of the chromatogram. These 4 bands corresponded to those of the lactoneo-neutral GSL immunostained by 1B2. This observation suggests that both mAbs detect – although to a different extent – gangliosides with a NeuAcα2-6Galβ1-4GlcNAcβ- terminal structure.

Another mAb, 1B9, recognizing the NeuAcα2-6Gal-structure has been previously described [13]. At this moment it is not clear if this mAb resembles the CD 76 mAbs in its reactivity.

HD 66 and CRIS-4 were classified into the CD 76 group due to their identical cell staining pattern [3]. Competitive binding inhibition in a cellular radioimmunoassay suggested that both mAbs react with the same or a closely associated epitope [14]. This study could now corroborate this observation. Since some monosialogangliosides with higher polarity are only detected by CRIS-4 and not by HD 66, it may be that the mAbs differ in their need for either a short (HD 66) or a long (CRIS-4) carbohydrate 'spacer' for optimal binding. HD 66 also reacted with surface-expressed proteins (85 and 67 kDa) of human B lymphocytes [14]. It is possible that the HD 66 epitope is also present on the carbohydrate moiety of glycoproteins. It is not clear at present whether the cell specificity of CD 76 mAb is due to GSL antigens, glycoprotein antigens, or both. The epitope specificity of CD 76 mAbs on glycoproteins is currently investigated.

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