Glycoconjugate Journal (1993) **10**:395–405

Monoclonal antibody against a lactose epitope of glycosphingolipids binds to melanoma tumour cells

KAI DING[1], TOMAS EKBERG[2], JESPER ZEUTHEN[3], SUSANN TENEBERG[4], KARL-ANDERS KARLSSON[4], and ANDERS ROSÉN[5]*

[1] *Department of Tumor Immunology, The Wallenberg Laboratory, University of Lund, S-220 07 Lund, Sweden*
[2] *Department of Organic Chemistry 2, University of Lund, S-214 01 Lund, Sweden*
[3] *Department of Tumor Cell Biology, Danish Cancer Society, DK-2100 Copenhagen, Denmark*
[4] *Department of Medical Biochemistry, University of Gothenburg, S-400 33 Gothenburg, Sweden*
[5] *Department of Cell Biology, Faculty of Health Sciences, University of Linköping, S-581 85 Linköping, Sweden*

Received 19 November 1992 and revised 21 April 1993

Mice were immunized with a neoglycoprotein consisting of a chemically modified carbohydrate moiety (reductively aminated 3′-sialyllactose) linked to human serum albumin. By this procedure an antibody response to the normally non-immunogenic carbohydrate structure was obtained. Hybridomas were established, and monoclonal antibodies were selected in ELISA based on their binding to the saccharide hapten, or to a lactosylceramide-mimicking neoglycolipid, lactose-bis-sulfone. One of the selected antibodies, 2H4, was of particular interest, since it also bound to glycolipids present on melanoma cells. FACS analysis of a panel of 14 melanoma cell lines showed that the 2H4 antibody bound to the majority of these. In frozen, non-fixed sections or paraffin sections of biopsies the monoclonal antibody 2H4 stained melanoma cells, but not tumour infiltrating lymphocytes or normal skin. Detailed immunochemical analysis of 2H4, using thin layer chromatography revealed that it recognized an internal lactose epitope in several glycosphingolipids.

Keywords: monoclonal antibodies, anti-lactose, glycolipid, melanoma.

Abbreviations: BSA, bovine serum albumin; FACS, fluorescence activated cell sorter; ELISA, enzyme-linked immunosorbent assay; HSA, human serum albumin; LacCer, lactosylceramide; MAb, monoclonal antibody; PBS, phosphate buffered saline.

Normal melanocytes, the precursors of melanoma, predominantly contain GM_3 gangliosides, while GD_3 gangliosides increase only after neoplastic transformation [1–3]. When melanomas progress from low to high metastatic variants, there is a shift in the ganglioside GM_3:GD_3 ratio. Other gangliosides associated with melanocytes include *O*-acetylated GD_3, GM_2, and GD_2, but these constitute less than 10% of the total ganglioside content [4].

Gangliosides are notoriously poor immunogens, and vaccine studies in patients with malignant melanoma show that GD_2 elicits an antibody response only occasionally and that GD_3 does not induce antibody production, in contrast to GM_2 which is consistently immunogenic [5–7]. It is therefore important to find ways to modulate and improve the immunogenicity of gangliosides. Active immunization of melanoma patients with a melanoma cell vaccine could, interestingly, induce antibodies against GD_3. The immunogen was found to be an *O*-acetylated form of GD_3 [4]. Chemically modified gangliosides, for example, lactone forms of GM_3 and GD_3, are suggested to constitute melanoma-associated immunogens [8–11]. The results of this study show that the reductively aminated carbohydrate moiety of GM_3 conjugated to carrier is immunogenic in Balb/c mice; hybridomas could be established producing monoclonal antibodies with specificity for a lactose epitope present in naturally occurring glycosphingolipids.

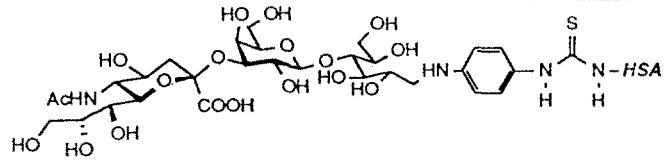
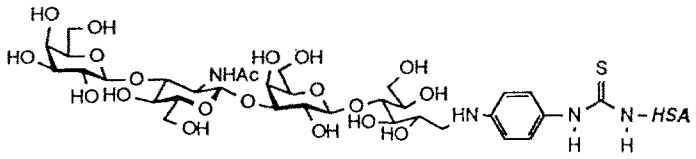
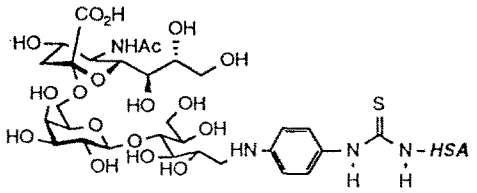
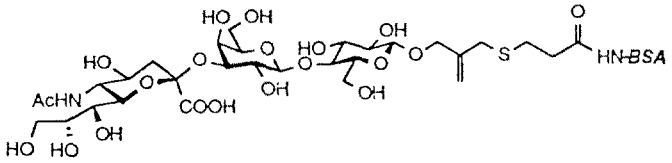
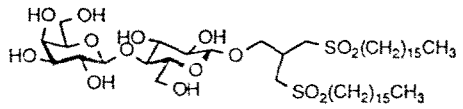
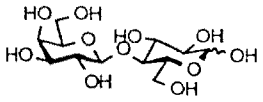
* To whom correspondence should be addressed.

Materials and methods

Glycolipids and glycoconjugates

The neoglycoprotein conjugates and saccharides used in this study are shown in Table 1. The immunogen, NeuAcα2-3Galβ1-3-D-Lyx, linked via acetylphenylenediamine to HSA and designated r3′-sialyllactose-HSA, containing 18 hapten molecules per HSA, the Galβ1-3GlcNAcβ1-3Galβ1-3-D-Lyx-HSA (r-lactotetraose-HSA), and the NeuAcα2-6Galβ1-3-DLyx-HSA (r6′-sialyllactose-HSA) were all purchased from BioCarb AB, Sweden. 3′-Sialyllactose-BSA

Table 1. Names and structures of glycoconjugates and carbohydrates.

Entry	*Trivial name*	*Structure*
1	r3′-Sialyllactose-HSA	
2	r-Lactotetraose-HSA	
3	r6′-Sialyllactose-HSA	
4	3′-Sialyllactose-BSA	
5	Lac-bis-sulfone	
6	Lactose	

conjugate with a substitution degree of 30 3′-sialyllactose molecules per BSA was synthesized and provided by Dr Jan Dahmén, Symbicom AB, Lund, Sweden. Galβ1-4Glcβ1-OCH$_2$CH[CH$_2$SO$_2$(CH$_2$)$_{15}$CH$_3$]$_2$ [12] (designated Lac-bis-sulfone) was kindly supplied by Professor Göran Magnusson, Lund, Sweden. The glycosphingolipids (Table 4), were purified by us (Karlsson K-A and Teneberg S) according to previously described methods [13].

Immunization and monoclonal antibody production

Balb/c spleen cells were fused with the Sp2/0 myeloma fusion partner to generate hybridomas [14]. r3′-Sialyllactose-HSA (50 µg) mixed in Freund's complete adjuvant (Sigma) was injected subcutaneously. Three weeks later, the same dose of antigen was given in Freund's incomplete adjuvant; this was repeated twice with the same interval. One week after the last immunization, 5 µg of the antigen was injected intravenously without adjuvant, in 100 µl PBS (0.2 g KH_2PO_4, 2.9 g Na_2HPO_4:12H_2O, 0.2 g KCl, 8.0 g NaCl, in 1 l H_2O, pH 7.4). Four days later, the spleen was removed for hybridoma production. Three days after each immunization, the serum was tested by ELISA.

Tissue culture

Hybridoma cell lines were maintained in RPMI 1640 medium supplemented with 5% foetal calf serum, 100 IU penicillin per ml, 100 µg streptomycin per ml, and 1:50 dilution of hypoxanthin–thymidine (HT) supplement. All tissue culture media and supplements were from Gibco Ltd., Scotland.

ELISA screening

ELISAs were performed by first binding 100 µl (2 µg ml^{-1}) of r3′-sialyllactose-HSA or HSA in carbonate–bicarbonate, pH 9.6, buffer (1.59 g Na_2CO_3, 2.93 g, $NaHCO_3$, 0.2 g NaN_3 made up of 1 l H_2O), or 100 µl (5 µg ml^{-1}), Lac-bis-sulfone in methanol, to ELISA microplates (Immulon 2, Dynatech AG, Switzerland). After overnight incubation at 21 °C in a humidified atmosphere (for glycoprotein-plates), or in a ventilated hood (for glycolipid to allow drying), the plates were blocked by 1% BSA in PBS (composition see above) for 30 min. To each well were added 100 µl of hybridoma supernatant. The plates were incubated for 2 h at 22 °C, washed three times, and then incubated for 2 h with rabbit anti-mouse Ig alkaline phosphatase-conjugate (Dako A/S, Denmark). The conjugate was diluted 1:1000 in PBS containing 0.1% BSA, and incubated 2 h at 21 °C. The plates were washed three times with PBS containing 0.05% Tween 20 (Kebo, AB, Sweden). The phosphatase substrate (*p*-nitrophenyl phosphate, Sigma) was added and the plates incubated for 30 min at 37 °C. OD was measured at 405 nm in a Titertek Multiscan (Flow Labs Ltd., Scotland) photometer. Mouse sera from immune animals were included as positive controls, and were tested in dilutions 1:40 to 1:256 000. Pre-immune Balb/c mouse serum was used as a negative control.

Tests of hybridoma immunoglobulins (Igs)

To determine whether the hybridoma cultures were monoclonal or polyclonal, the hybridoma supernatants were subjected to agarose isoelectric focusing [15]; immunoglobulin bands were detected by specific antibodies against mouse Ig chains. To determine the Ig subclass, concentrated supernatants from the hybridomas were further analysed by Ouchterlony double immunodiffusion in agarose [16]. Goat antisera against mouse IgA, IgM, IgG1, IgG2 (Meloy Lab, USA), and against total mouse Ig (Dako A/S, Denmark), as well as a negative control serum, were applied in 1:10 dilution in the Ouchterlony wells. Purified mouse myeloma proteins (Bionetics, USA) were used as standards. The agarose slides were incubated overnight at 4 °C. For MAbs that did not give visible immunoprecipitates using the Ouchterlony method, we used commercial kits from Serotec, UK (agglutination of MAb-coated erythrocytes), or the dipstick method from Holland Biotechnology BV, The Netherlands.

Thin-layer chromatography

Two of the monoclonal antibodies were analysed in TLC with antibody overlay [17, 18]. Glycolipid mixtures of various origins prepared free of non-glycolipid contaminants [13], and pure reference substances isolated in the laboratory, were used for testing. The identities of the pure references were documented by mass spectrometry, NMR spectroscopy, and degradation methods (reviewed in [13]). The MAbs were used as undiluted supernatants. Bound antibody was detected by autoradiography, using rabbit anti-mouse antibody (Dako Code No. Z109) labelled with ^{125}I. The antibody–conjugate was diluted 1:100, to a final activity of 5×10^6 counts min^{-1} ml^{-1}.

FACS analysis

A panel of human melanoma cell lines and a mouse melanoma tumour B16 (Table 2) were used for immunofluorescence analysis in FACS VI (Becton Dickinson Immunocytometry Systems, USA) and software Consort 40. Immunofluorescence analysis was performed using hybridoma supernatant. Ascites fluids were avoided, since they could give false positive reactions. The cells were first washed three times in balanced salt solution (BSS). One million cells were incubated with MAb supernatant for 45 min at 4 °C. The positive control was an anti-GM_3-lactone MAb (DH2) [10], kindly provided by Dr S. Hakomori (The Biomembrane Institute, Seattle, WA, USA); purified mouse myeloma protein TEPC183 (IgM, κ) (Bionetics, USA), was used as a negative control. After three washes with BSS, the cells were incubated in a 1:30 dilution of FITC-rabbit anti-mouse immunoglobulin (Dako A/S, Denmark), for 45 min at 4 °C. The cells were mounted in 1 ml BSS after three final washes, and then analysed in the FACS.

Immunohistology

Sections (5 µm thick) of formalin-fixed, paraffin-embedded normal or tumour tissue were deparaffinized and rehydrated, or frozen non-fixed sections embedded in Tissue-Tek O.C.T. compound (Miles Inc., USA) were rinsed with distilled water, and equilibrated in Tris-buffered NaCl solution. They were subsequently incubated for 30 min with normal rabbit serum (Dako Code No. X902) diluted 1:5 in TBS, for blocking of non-specific background staining. The avidin–biotin–immunoperoxidase system involved a three-layer technique. The first layer, containing the MAb, was applied in the form of undiluted hybridoma supernatants. The second layer consisted of biotinylated rabbit $F(ab')_2$ anti-mouse immunoglobulins (Dako Code No. E413) diluted 1:200 in TBS. The third layer consisted of freshly prepared avidin/biotinylated horseradish peroxidase complex (ABComplex/HRP, Dako Code No. K355), prepared according to the manufacturer's instructions. The incubation times for the three layers were 30, 20, and 30 min, respectively. The peroxidase was revealed by a 15 min incubation with 3-amino-9-ethylcarbazole (AEC; 0.2 mg ml^{-1}) in 0.05 M sodium acetate buffer, pH 5.1; the slides were counterstained for 1 min with haematoxylin, and mounted in Aquamount.

ELISA inhibition

For IgG monoclonal antibodies (MAbs), the inhibition was carried out in glass tubes, at 4 °C for 18 h, in a ratio of 1:1

Table 2. Cell lines analysed in FACS.

Cell line	*Origin*	*Reference*
A375	Human malignant melanoma	ATCC CRL 1619[a]
SK-MEL-3	Human malignant melanoma	ATCC HTB 69
SK-MEL-24	Human malignant melanoma	ATCC HTB 71
SK-MEL-28	Human malignant melanoma	ATCC HTB 72
SK-MEL-31	Human malignant melanoma	ATCC HTB 73
746	Primary human malignant melanoma	[b]
RAH 2	Primary human malignant melanoma	[c]
RAH 3	Primary human malignant melanoma	[c]
RAH 4	Primary human malignant melanoma	[c]
RAH 5	Primary human malignant melanoma	[c]
RAH 6	Primary human malignant melanoma	[c]
MEL	Human malignant melanoma	[c]
RPMI 8322/M5	Human malignant melanoma	[c]
B16-F0/F1	Mouse melanoma	ATCC CRL 6322/6323
MP6	Human T cell hybridoma	[33]
B6	Human B cell line, IgM^+	[34]
BL41	Burkitt's lymphoma EBV^- cell line	[35]
BL41-E95-A	EBV converted BL41	[35]
Fibroblast	Human embryonal lung fibroblasts	[d]
HL-60	Human promyelocytic leukemia	ATCC CCL 240
THP-1	Human monocytic cell line	ATCC TIB 202

[a] ATCC, American Tissue Culture Collection.
[b] Danish Cancer Society, Copenhagen.
[c] RAH: Radiumhemmet, Karolinska Hospital, Stockholm.
[d] Hybridoma Group, Karolinska Institute, Stockholm.

by vol, of different inhibitors (sugars, glycoconjugates or synthetic glycoproteins, listed in Table 1) and the MAb. The MAb/inhibitor mixture was then added to the ELISA plates containing the immunogen (or control) to be tested, as described above. The IgM MAbs were tested in solid-phase inhibition by first coating ELISA plates with the potential inhibitor, in an overnight incubation at 21 °C. The plates were blocked with 1% BSA in PBS, and washed several times in PBS-Tween. The MAb supernatant was then added to all wells, and reacted with the solid phase inhibitors overnight at 4 °C. The subsequent steps were the same as for microplate ELISA.

$$\text{Inhibition (\%)} = \text{OD}_{405\,\text{nm}} \frac{(\text{No inhibitor} - \text{with inhibitor})}{(\text{No inhibitor})} \times 100$$

Inhibitor concentration at 50% inhibition is termed IC_{50} value. PBS buffer reacted with MAb supernatant at 1:1 by vol was used as a negative control (no inhibition).

Modelling

Molecular modelling was performed on a Silicon Graphics work station, using the program Biograf (Molecular Simulation, Ltd.) and the Dreiding 2 force field.

Results

Immunization

Antibody titres in the animal sera were followed by ELISA before and after r3′-sialyllactose-HSA immunization. The immune sera could be diluted up to 1:512000 before reaching background OD levels (Fig. 1(a)). Since most of this reactivity was directed against the HSA carrier protein (as expected), the sera were also tested against natural GM_3 ganglioside without carrier, dissolved in methanol and dried on to ELISA plates. The BSA in the blocking buffer was exchanged with gelatin, to circumvent any possible interference of anti-albumin antibodies (Fig. 1(b)). The immune sera reacted strongly in dilutions up to 1:6400. The pre-immune sera were consistently low in titre, but in all cases higher than background values.

Establishment of hybridomas and screening of specific clones in ELISA

The high-titered animals were selected for hybridoma production and, following establishment of growing cultures, ELISA was used for screening. Table 3 shows the results from ELISA analyses on stable hybridoma clones obtained after several reclonings. HSA-coated ELISA plates were always used as negative controls during the screening procedure. Two different neoglyconjugate target structures

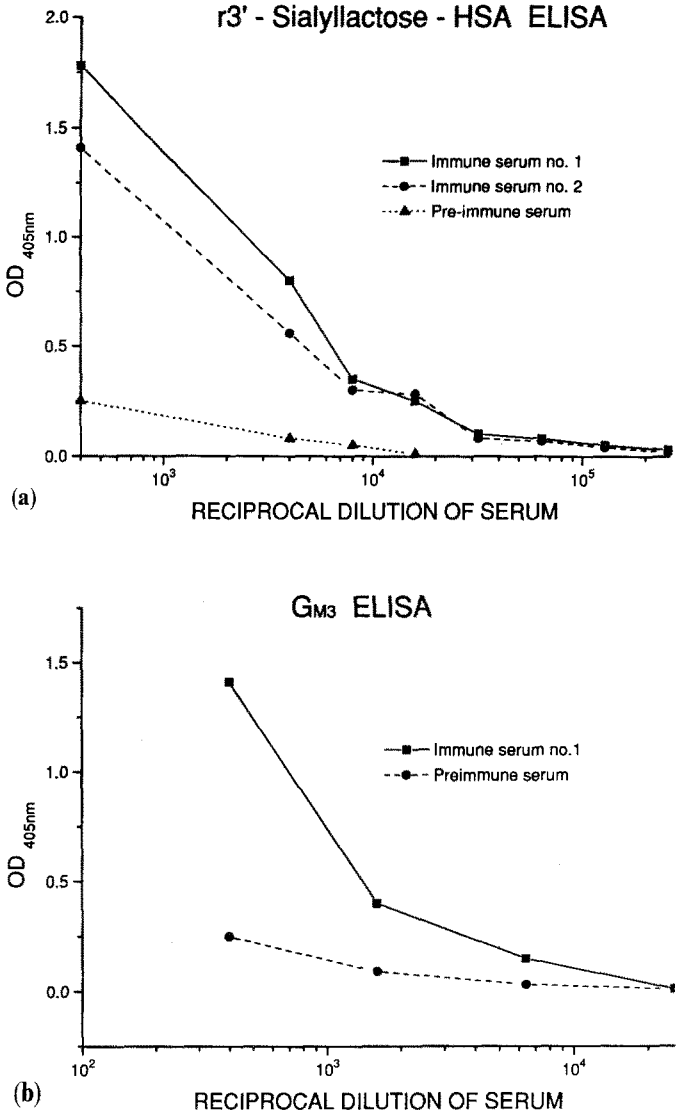


Figure 1. (a) Serum reactivity against the r3′-sialyllactose-HSA immunogen in ELISA before and after immunization. Sera were diluted from 1:400 to 1:256 000. Plates were coated with r3′-sialyllactose-HSA, and blocked with 1% BSA. (b) Titration of sera for specific GM_3 response. Plates were coated with GM_3-ganglioside in methanol by evaporation. 1% gelatin was used for blocking. Sera were diluted from 1:400 to 1:12 800.

were used for the initial analysis of these clones: (1) the immunogen, r3′-sialyllactose-HSA, and (2) Lac-bis-sulfone. We selected two representative clones for detailed characterization, 1H9A2B7F9 (abbreviated 1H9), which was positive for r3′-sialyllactose-HSA, but negative for Lac-bis-sulfone, and 2H4E10B4 (abbreviated 2H4), which was positive for Lac-bis-sulfone, but low reactive for r3′-sialyllactose-HSA. Isoelectric focusing analysis of 1H9 and 2H4 mAbs showed a restricted monoclonal pattern, and subclass determination revealed IgG1, κ and IgM, κ, respectively.

Table 3. Determination of antibody binding in ELISA: absorbance at 405 nm.

Clone	*r3′-Sialyllactose-HSA*[b]	*Lac-bis-sulfone*
2A4C8A4A1	1.33	1.22
2A4F11E11A1	1.11	1.34
1H9A2B7F8	0.54	0
1H9A2B7F9	1.58	0 (1H9)
2H4E10B4	0.47	1.50 (2H4)
2H4E10BE8	0.39	1.37
4D12D5	0.87	1.55
4D12D5B2	0	0.86
4D12D5BD6	0.40	1.52
4D12D5BE5	1.15	1.50
4D12D5BE7	1.08	1.53
4D12D5BG10	0.30	1.51
4D12G10	0.94	1.16
4D12G10D7	1.24	1.52
4D12G10E2	1.12	1.55
4D12G10E9	1.12	1.49
4D12G10F5	1.21	1.50
4D6D5	0.12	0.37
4D6D5C6	0.44	1.42
4D6D5C10	0.51	1.48
3G5E11	0.005	1.23
3G5E11A9	0	0.36

[a] All clones tested were selected for positivity in r3′-sialyllactose-HSA or Lac-bis-sulfone ELISAs, and were negative in HSA specific ELISA.

Characterization of binding patterns for two antibodies 2H4 and 1H9

1. Thin-layer chromatography. We were intrigued by the strong binding of 2H4 to the lactosylceramide resembling Lac-bis-sulfone (Table 3), and wondered whether it also reacted with naturally occurring glycolipids. To answer the question, several glycosphingolipids (Table 4) were separated by thin layer chromatography, and allowed to react with the antibodies in the overlay technique. As shown in Fig. 2 and summarized in Table 4, the antibody 2H4 seems to recognize lactose at an internal position. Several glycolipids were negative, including some of those shown in the chromatogram (Fig. 2). In lanes 2 and 3 of Fig. 2, the upper band (labelled 2) was lactosylceramide, which had long-chain (22–24 carbons) 2-hydroxy fatty acids (compound No. 2 in Table 4). The lower band labelled 2 had 16–18 carbon atom fatty acids. However, the bands labelled 1 in lane 1 with slightly higher mobility were negative; these correspond to lactosylceramide species with non-hydroxy fatty acids of 22–24 and 16–18 carbon atoms, respectively [19, 20]. Therefore, recognition of lactose is apparently dependent on the nature of the lipophilic part, a phenomenon which similarly has been found for several bacteria [19, 20]. The positive bands labelled 5 in the 3-sugar region of lanes 2

Table 4. Results from binding of antibodies 1H9 and 2H4 to glycolipids developed on thin layer plates (4 μg of each glycolipid were applied).

	Glycolipid structure	*1H9*	*^{2}H4*	*Tissue source*
1	Galβ4Glcβ Cer[a]	+	−	Human erythrocyte
2	Galβ4Glcβ Cer(h)[b]	−	+	Dog intestine
3	NeuAcα3Galβ4Glcβ Cer	−	−	Human brain
4	NeuGcα3Galβ4Glcβ Cer	−	−	Horse erythrocyte
5	Galα3Galβ4Glcβ Cer	+	+	Dog intestine
6	Galα4Galβ4Glcβ Cer	−	−	Human erythrocyte
7	GalNAcβ3Galα4Galβ4Glcβ Cer	−	−	Human erythrocyte
8	GalNAcα3GalNAcβ3Galα4Galβ4Glcβ Cer	−	−	Dog intestine
9	GalNAcβ4Galβ4Glcβ Cer	+	+	[c]
10	Galβ3GalNAcβ4Galβ4Glcβ Cer	++	++	Mouse faeces
11	Fucα2Galβ3GalNAcβ4Galβ4Glcβ Cer	−	+	Mouse intestine
12	Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ Cer	−	−	Human brain
13	NeuAcα3Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ Cer	−	−	Human brain

[a] Ceramide composed of sphingosine and non-hydroxy fatty acids.
[b] (h) means a ceramide composed mainly of phytosphingosine and hydroxy fatty acids.
[c] Produced by mild acid hydrolysis of GM2, GalNAcβ4(NeuAcα3)Galβ4Glcβ Cer, prepared from human brain.

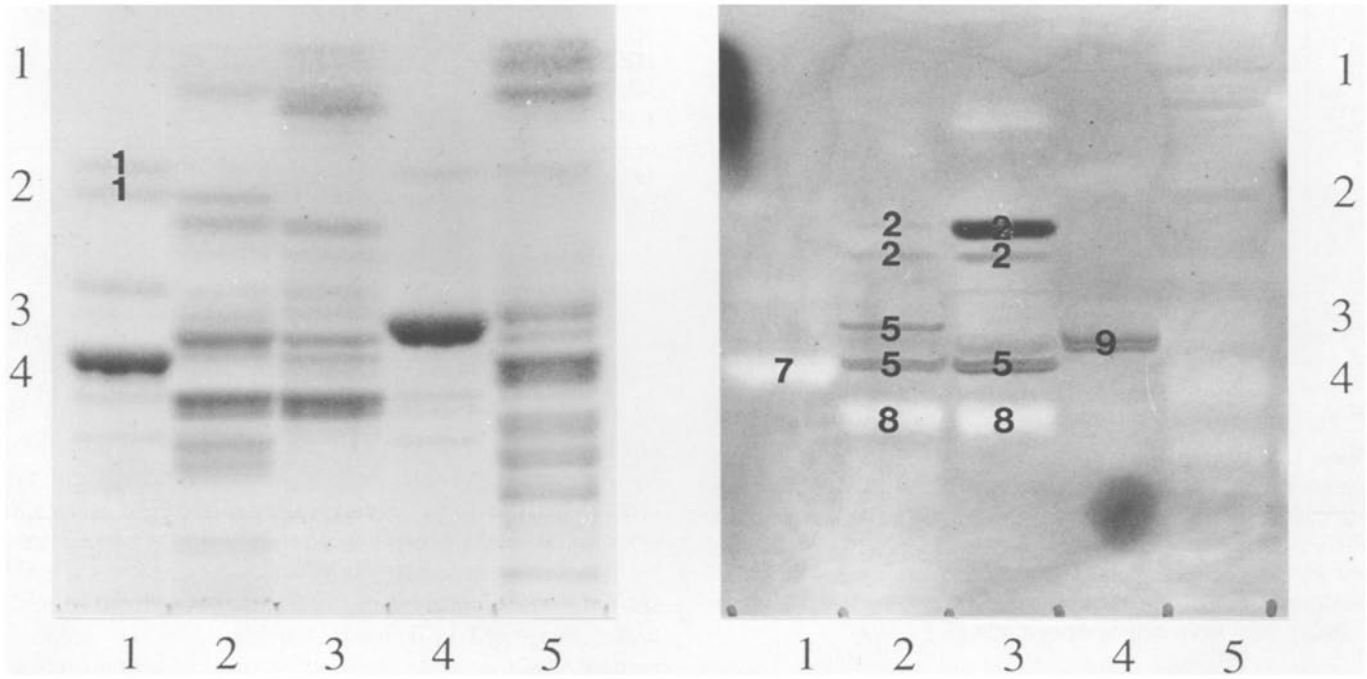


Figure 2. Thin layer chromatogram developed with anisaldehyde (left), and autoradiography after binding with antibody 2H4 and ^{125}I labelled anti-mouse antibody (right). Various non-acid glycolipid mixtures (40 μg) were applied to each lane. The glycolipid sources were: lane 1, human erythrocytes of blood group O, Le (a−b+); lane 2, dog intestine; lane 3, guinea-pig intestine; lane 4, guinea-pig erythrocytes; lane 5, rat intestine. Silica gel high performance thin layer chromatography Nanoplates (Merck) were developed with chloroform:methanol:water, 60:35:8 by vol; autoradiographs were exposed for 10 days.

and 3 was substance No. 5 (listed in Table 4), and the positive band in lane 4 was No. 9. As there was no binding to one-sugar glycolipids, the minimum requirement is lactose, Galβ4Glc. Remarkably, antibody 1H9, which was negative for Lac-bis-sulfone in the initial ELISA screening (Table 3), bound in a similar pattern, although weaker, according to the results from autoradiography (not shown).

2. Fluorescence activated cell sorter (FACS) analysis. The potent binding of the 2H4 Mab, in particular, to naturally occurring glycolipids, as found in the TLC analysis, prompted us to study the binding to live cells of various tissue lineages. Fourteen different melanoma cell lines and several non-melanoma cell lines (Table 2) were included in the study. Thirteen of the 14 melanoma cell lines were

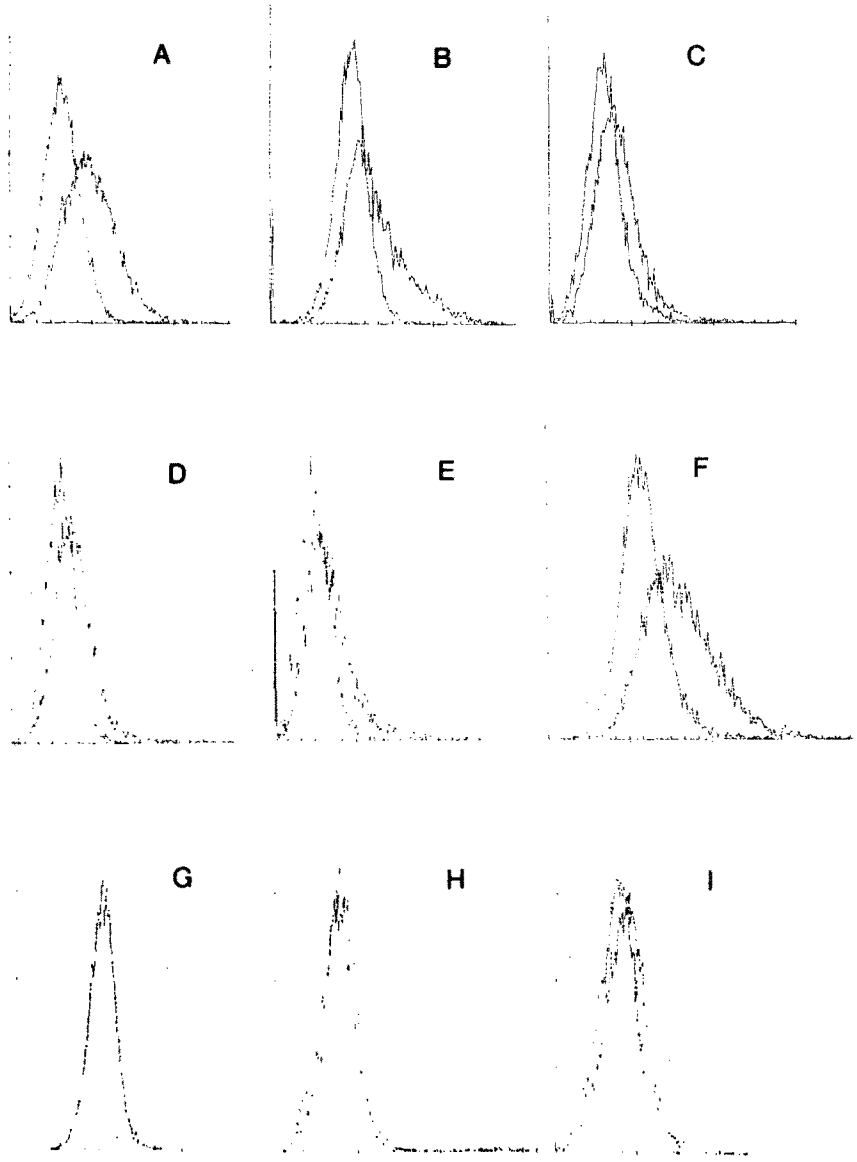


Figure 3. FACS analysis profiles of melanoma and control cell lines. The *y* axis denotes number of cells; the *x* axis expresses fluorescence intensity. 10000 cells were counted. The profiles of the 2H4 MAb stained cells are compared with the corresponding negative controls (BSS and FITC-conjugate). (a) A375 malignant melanoma; (b) SK-MEL-28 malignant melanoma; (c) B16 mouse melanoma; (d) RAH6 primary human melanoma; (e) M5 malignant melanoma; (f) MEL malignant melanoma; (g) SK-MEL-3 malignant melanoma; (h) THP-1 monocytic cell line; and (i) B6 B cell line.

positive in FACS, at varying intensities, with the 2H4 MAb, the only negative line was SK-MEL-3. The 1H9 MAb was completely negative. Figure 3 shows some representative positive and negative cell lines. The MP6 T-hybridoma, the B6 B lymphoblastoid cell line, and the monocytic cell line THP-1 were negative (Fig. 3). We found, however, positive reaction with the EBV-negative Burkitt's lymphoma B cell line BL41 and its EBV-transformed counterpart.

3. Immunohistology. The FACS experiments showing binding of 2H4 to *in vitro* grown cell lines were expanded to investigations on *in vivo* tumour biopsy material. The 2H4 MAb stained frozen non-fixed sections as well as paraffin sections of biopsies from patients with primary as well as metastatic melanomas. The immunohistological staining was stronger in frozen non-fixed sections than with fixed material. The reddish-brown staining was virtually absent from sections of normal human tonsil and skin tissue stained in parallel. Representative sections of tumour tissue showed the absence of positive staining on fibroblasts, stromal cells and tumour-infiltrating lymphoid cells. Staining was present in nervous tissue but absent from muscle tissue. Further immunohistological studies indicated that the 2H4 antibody was positive also for benign naevi, and normal melanocytes, but also that it reacted with normal squamous epithelial cells, in addition to melanoma tissues. In contrast

the 1H9 MAb did not stain non-fixed or fixed sections of melanoma or normal tissues.

4. Epitope characterization by competition ELISA. 1H9 MAb showed weak reactivity to naturally occurring glycolipids in TLC, and lacked binding to cells, in contrast to the 2H4 MAb. To compare the nature of the 1H9 epitope to that of 2H4, competition ELISAs were set up. Figure 4 shows that the immunogen (r3′-sialyllactose-HSA) completely inhibits binding to 1H9 MAb in a soluble competition ELISA, with an IC_{50} value of 2×10^{-9} M. 3′-Sialyllactose-BSA, which is linked to the carrier protein via a different spacer molecule [12], inhibited maximally only 40%; this may be because coupling of BSA to 3′-sialyllactose did not involve reductive amination and opening of the glucose ring. Neither free lactose nor Lac-bis-sulfone showed inhibition over 50% (Fig. 4(a)); this indicates that for 1H9 other residues besides lactose are more important in the epitope. Also, r-6′-sialyllactose-HSA inhibited reactivity of MAb 1H9 by not more than 70% (Fig. 4(b), and at a considerably higher IC_{50} value. The 2H4 IgM MAb was not used with soluble inhibitors, due to the decavalency of IgM, but in solid phase competition ELISA, Lac-bis-sulfone and lactose completely inhibited the reactivity of MAb 2H4, whereas GM_3 did not (not shown).

Molecular modelling

Computer-based molecular modelling was used to visualize the mobility at the reduced glucose residue (Fig. 5, Table 5). As shown, there are four different relatively low-energy conformers, based on the flexibility of the glucose residue (carbon atoms in black). Model A in Fig. 5 (a and b), represents the natural trisaccharide with glucose in the unreduced ring form (two different projections to visualize the four conformers). Models B and C are the four conformers (Table 5) which can result from rotations in the opened glucose ring. Thus, the modified lactose, with retained asymmetry of glucose at C2, C3, C4, and C5, may possibly adopt a conformation optimal for triggering the production of anti-lactose antibodies.

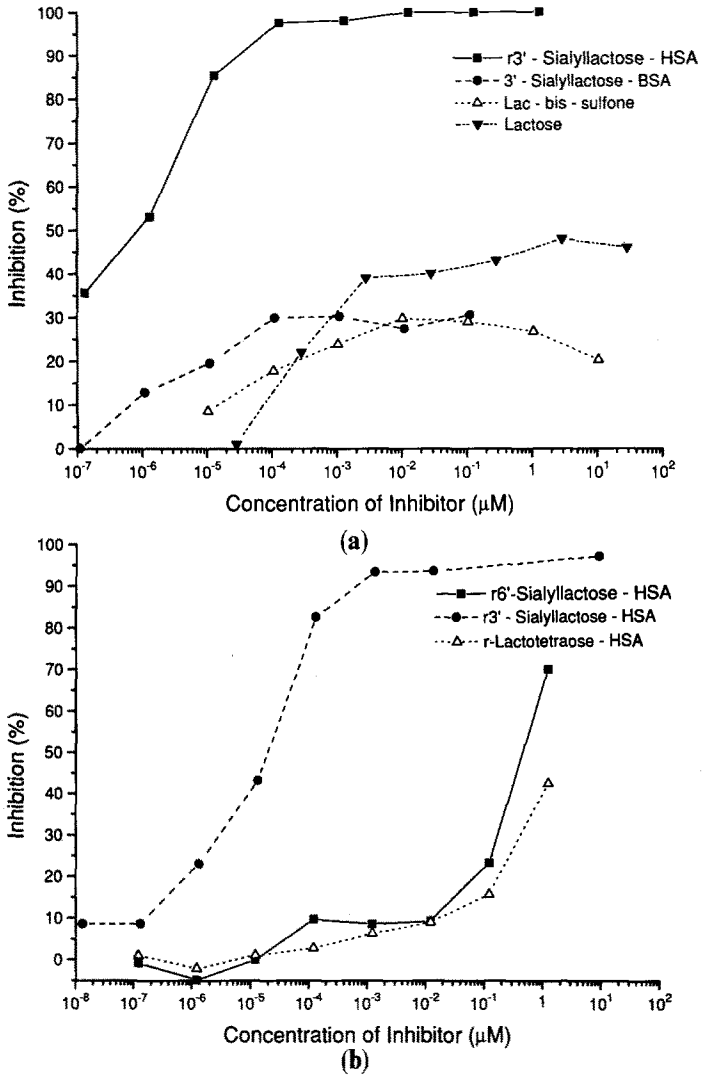


Figure 4. Epitope characterization by competition ELISA for the 1H9 MAb. The different inhibitors were added in serial dilutions to the 1H9 MAb for 18 h in glass tubes. The mixtures were then transferred and tested for binding on r3′-sialyllactose-HSA coated plates. The concentration is based on the single hapten concentration in conjugates having a high degree of hapten substitution. Calculation of percentage inhibition is described in the Materials and methods section. Parts (a) and (b) depict inhibition profiles from two different sets of experiments.

Discussion

Malignant transformation in melanocytes is accompanied by specific chromosomal aberrations, leading to a number of phenotypic changes [21, 22]. Aberrant carbohydrate structures, in glycoproteins as well as in glycolipids, presented on the cell membrane are well known to be strongly associated with malignant transformation [23]. These changes in normal glycosylation patterns are often the results of alterations in the degree of fucosylation and sialylation of the glycoproteins and glycolipids. For the analysis of these tumour-associated alterations, several mouse and human hybridomas, as well as EBV-transformed cell lines have been established and their antibodies

Table 5. Energies of four conformers of r3′-sialyllactose-HSA.[a]

Conformer No.	*Energy (total)*	*Energy (van der Waals)*
	kcal mol^{-1}	kcal mol^{-1}
1	121.4	83.9
2	126.0	81.0
3	122.2	85.7
4	119.5	85.1

[a] Trisaccharide and link region only.

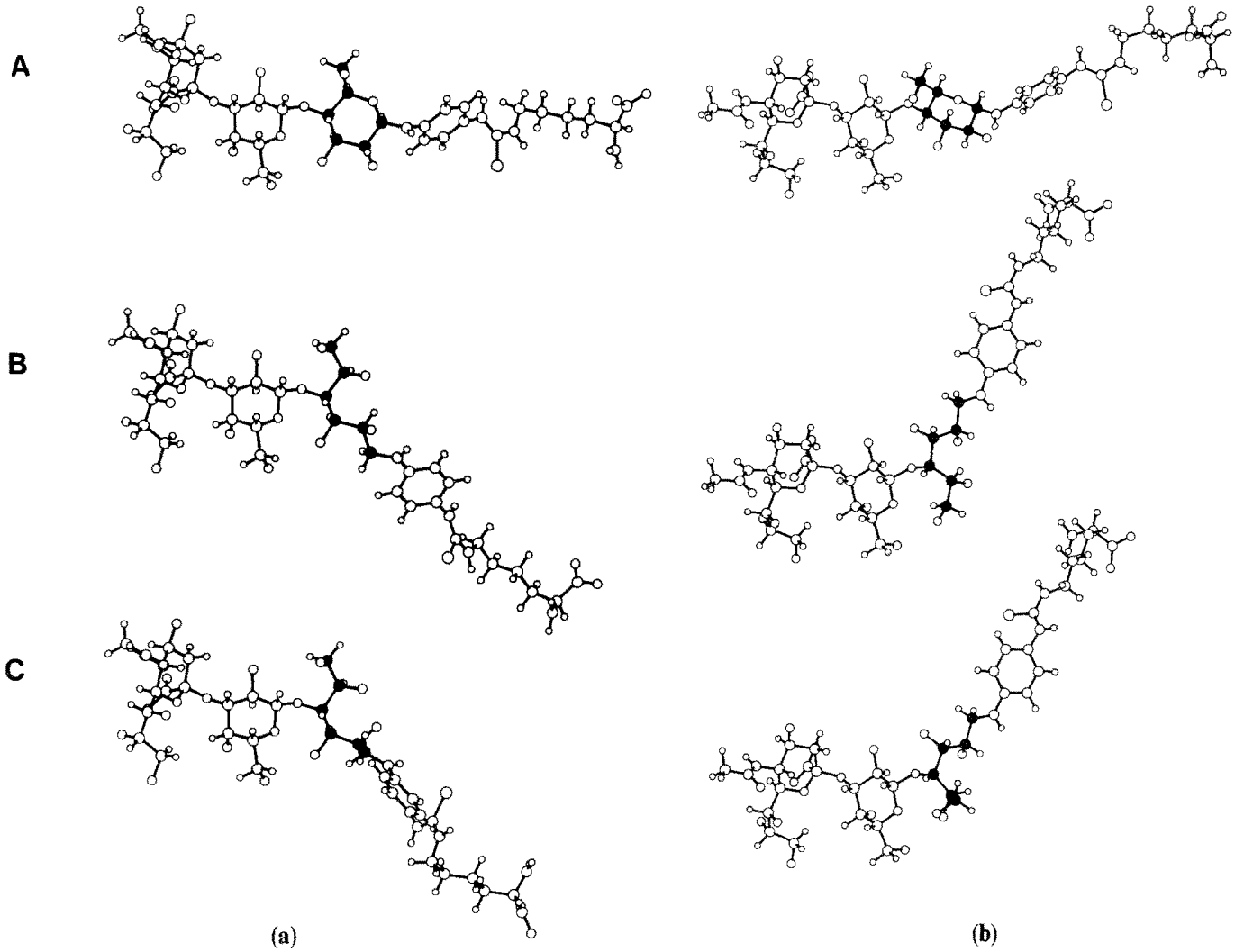


Figure 5. (a) Molecular models showing two (B and C) of the four low-energy conformers of the trisaccharide and linker region of r3′-sialyllactose-HSA (Table 1). The upper sequence (A) is the projection of the comparable sequence with non-reduced glucose. The carbon atoms of the non-reduced and reduced glucose unit have been indicated in black. All three models were orientated with C1, C3, and C5 of galactose (to the left of the labelled sugar moiety) in the plane of the paper, and then rotated $-30°$ around the x axis. B is conformer 1 and C is conformer 2 of Table 5. (b) Molecular models showing two (B and C) of the four low-energy conformers of r3′-sialyllactose-HSA as in part (a), except that rotation was around the y axis.

characterized [24]. These antibodies have potential applications as both diagnostic and therapeutic reagents [9, 25]. In addition, immune surveillance against the melanoma may be potentiated by whole-cell vaccine, mainly by inducing antibodies against the ganglioside structure *O*-acetyl-GD_3, which has been shown to cross-react with GD_3 [4]. However, there are a number of serious difficulties associated with the use of glycolipids, such as gangliosides, as target structures for immunotherapy. Gangliosides from human melanomas may have immunomodulatory activity, and could down-regulate the responses of T cells to IL-2 [26]. In addition, gangliosides are shed from cell surfaces and exert immunosuppressive effects [27, 28]. It has been suggested [29] that at the high cell-surface densities of certain gangliosides, formation of lactone structures may occur, such as the formation of GM_3 lactones. The lactone form itself may in fact constitute the actual immunogen [29, 30]. We found strong support for this idea in a parallel study [31], where we used a synthetic GM_3 lactam conjugate designed to emulate the natural GM_3 lactone. Strong immune response was obtained and several anti-lactam/anti-lactone monoclonal antibodies were isolated.

The results reported here, from studies using mouse and human melanoma cell lines and tumour material, suggest that an internal lactose epitope, recognized by the 2H4 MAb, is stably expressed on the cell surface. Interestingly, the recognition of internal lactose epitopes by this MAb is similar to the binding specificity of certain bacteria to the

internal lactose moiety in glycolipids [19, 20]. LacCer glycolipids are also expressed during myeloid differentiation, and one anti-LacCer MAb, previously described [32], was derived after immunization with whole cells from an acute non-lymphoblastic leukemia patient. Whether this MAb has a similar epitope preference to 2H4 is not known. We consider the binding specificity of the 2H4 MAb against LacCer to be of particular interest, since anti-LacCer antibodies are reported to be prevalent in colorectal cancer patients, but not in healthy donors [33]. It would be important to confirm this study, as well as to analyse further whether melanoma patients express similar antibodies against the internal LacCer epitope.

An altered LacCer epitope is obviously expressed on malignant melanoma cells, since the 2H4 MAb bound to the majority of human melanoma cell lines tested, mouse B16 melanoma cells, and human tumour material, but did not bind tumour infiltrating lymphocytes or normal tissue. The DH2 MAb (anti-GM_3/GM_3-lactone) [9] binds to a structure on melanoma cells clearly different from that recognized by the 2H4 MAb; the DH2 MAb exhibits preferential binding to the lactone form of GM_3. DH2-binding shows marked fluctuations when tested during different growth phases of B16 melanoma cells (Dr M. Jondal, personal communication), indicating that the degree of lactonization of GM_3 varies considerably.

Interestingly, the 2H4 antibody did not bind to GM_3 in the TLC experiments (compound No. 3 in Table 4) or in the FACS study (as evidenced from its positive binding pattern compared with the anti-GM_3 DH2 MAb); instead, the positive glycolipids (Fig. 2, right panel) have sequences which lack sialic acid, and have a minimum requirement of two sugars (lactose). The results from competition ELISA support the suggestion that the epitopes include internal lactose residues. Anti-carbohydrate antibodies generally recognize terminal sequences [24] and therefore the present 2H4 antibody may be considered unusual in its ability to recognize non-terminal residues (Table 4). Reductive amination of the glucose moiety in the 3′-sialyllactose results in a more mobile structure, and therefore must be considered a stronger immunogen than the corresponding natural structure. It will be important to investigate whether this response against 'non-self' modified carbohydrate moieties is a general phenomenon.

Acknowledgements

Many thanks to Ms Gunilla Kjellström, Ms Irma Jansson and Ms Dorrit Lützhøft for expert technical support, and to Mr Bernd Briese for FACS analysis. We also thank Assistant Professor Jonas Ångström for the molecular modelling and Professor Göran Magnusson for valuable discussions. This study was supported by the Swedish Cancer Foundation, the Danish Cancer Society (Grant No. 74-26/92), the Swedish Medical Research Council (Grant No. 3967) and funds from Karolinska Institute, Symbicom AB and AB Astra.

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