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Characterization of a mouse monoclonal IgG3 antibody to the tumor-associated globo H structure produced by immunization with a synthetic glycoconjugate

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Globo H (Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc) is a carbohydrate structure that shows enhanced expression in many human carcinomas. From mice immunized with a globo H-KLH (keyhole limpet hemocyanin) synthetic conjugate an IgG3 monoclonal antibody (mAb VK-9) was derived that recognizes the globo H structure. Serological analysis showed that the minimal structure recognized by this mAb was the tetrasaccharide sequence Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Gal. An isomeric structure with an internal αGalNAc linkage was also recognized but less efficiently. mAb VK-9 did not react with many related structures, such as galactosylgloboside, globoside, H type 1, H type 2 blood group structures or fucosyl-gangliotetraosyl ceramide, but did react weakly with globo A ceramide. Not only did mAb VK-9 react with carbohydrate-protein conjugates but it could also recognize globo H-ceramide and human tumor cells expressing globo H. These results suggest that globo H-KLH could be explored as a vaccine in the treatment of carcinoma patients.

***Keywords*: monoclonal antibody, globo H, synthetic oligosaccharides, tumor antigen, glycolipid**

Introduction

Globo H is a globo-series carbohydrate structure originally identified as a glycolipid (Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1Cer) from human meconium [1] and teratocarcinoma [2]. Subsequently, it was also recognized as the antigen reacting with the mouse monoclonal antibody MBr1(IgM) which had been raised to the human breast cancer cell line MCF-7 [3, 4]. Globo H, as assessed by immunohistological analysis with mAb MBr 1, was found to be highly expressed in various types of carcinomas, *eg* breast, colon, lung and ovary and in small cell lung cancers [5]. Globo H can also be detected in some normal fetal [1] and adult epithelia [6]. In particular, it is found in human kidney and ureter [7, 8] and in erythrocytes of A_2 and O individuals [9]. More recently we have also detected globo H in the majority of carcinomas of the pancreas, stomach, prostate and uterine endometrium [10]. Because of the restricted distribution of globo H antigen, the therapeutic use of mAb MBr 1 has been tested in various strategies for the treatment of cancer with encouraging results [11, 12].

Based on this information we are exploring the possibility of developing globo H-based vaccines for the immunotherapy of cancer. These studies began with the complete synthesis of the globo H structure using the glycal assembly method [13, 14]. Subsequently, this oligosaccharide was coupled to immunogenic carriers, *eg* keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) and then tested in mice, together with the immunologic adjuvant QS21, for its ability to elicit an immune response [15]. It was found that the globo H-KLH conjugate, with QS21, was particularly effective in provoking the production of antibodies reacting with the globo H structure, as well as with human cancer cells.

We now describe the utilization of mice from this study to produce new mAbs reacting with the globo H. One IgG3 sub-class antibody (VK-9) was selected for detailed analysis. Even though this mAb was raised against a synthetic

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glycoconjugate it was able to bind to globo H-expressing human tumor cells.

Materials and methods

Materials

The glycoconjugates (oligosaccharide-protein conjugates and glycosphingolipids) used in this study and their source are listed in Table 1. The structure of the immunizing antigen (globo H-KLH) is shown in Figure 1 and the structures of the other compounds are given in Table 2. Allyl glycosides of globo H-related structures, which were used as inhibitors, are also listed in Table 2; they were prepared in this laboratory [15]. Globo H-ceramide and globo A-ceramide were isolated from meconium [1] and human kidney [7] and fucosyl gangliotetraosyl ceramide was from mouse small intestine [16]. Globoside was purchased from Sigma Chemical Co., St Louis, MO. Ovarian cyst mucins expressing A, B, H, and Lewis blood group determinants were described previously [17]. The main specificities expressed on the mucins are indicated in Table 2.

Mouse monoclonal antibody MBr1(IgM) was kindly provided by Drs Maria Colnaghi and Silvania Canevari (Istituto Nazionale Tumori, Milan). Antibodies to blood group specificities used as positive controls have been described [18].

Table 1. Synthetic glycoconjugates used in this study

Conjugate	*Carbohydrate: carrier ratio (molar)*	*Source*
Globo H – BSA (hexasaccharide)	19 : 1	Ref. 15
Le^y – BSA (pentasaccharide)	15 : 1	Ref. 33
Le^b – BSA (pentasaccharide)	20 : 1	Ref. 33
H type 1 – BSA (pentasaccharide)	8 : 1	V-labs, Inc.
H type 2 – PE (tetrasaccharide)	1 : 1	Unpublished[a]
B – PE (pentasaccharide)	1 : 1	Unpublished[a]
Globo H – Cer (hexasaccharide)	1 : 1	Ref. 13
Globo A-Cer (heptasaccharide)	1 : 1	Ref. 7
Gal Globo – Cer (hexasaccharide)	1 : 1	Ref. 14
Le^x – PAA (trisaccharide)	1 : 5	Glyco Tech Inc.
Le^a – PAA (trisaccharide)	1 : 5	Glyco Tech Inc.
H type 1 – PAA (trisaccharide)	1 : 5	Glyco Tech Inc.

[a]Synthesized by coupling the allyl glycoside of the oligosaccharide, after ozonolysis, to dipalmitoyl phosphatidyl-ethanolamine (PE) by reductive amination [34].

Immunization of mice and production of monoclonal antibodies

Mice (female CB6F1) were immunized subcutaneously with globo H-KLH conjugate (containing 10 μg of carbohydrate), together with the immunological adjuvant QS21 (10 μg), at 0, 1, 2, 22, and 35 weeks as described previously [15]. One mouse, which was producing high titers of IgM and IgG antibodies to globo H, was used for hybridoma production. Mouse myeloma cells (SP 2/0) were used for fusion with the mouse splenocytes following procedure of a Köhler and Milstein [19], as modified [20]. Hybridoma supernatants were screened by ELISA for reactivity to globo H-BSA and globo H ceramide using protein A – alkaline phosphatase as the second-step reagent. Eight positive clones from among the 1715 examined were detected. One of these hybridoma clones was subcloned twice by limiting dilution and was designated VK-9. mAb VK-9 was shown to be an IgG_3 (κ) antibody using a typing kit (Vymed, San Francisco, CA). Hybridoma VK-9 was cultured *in vitro* in RPMI – 10% FBS and the resulting antibody was purified by chromatography on protein A-agarose [21].

Serological methods

Reactivity of the antibody was tested on a panel of human cell lines by staining frozen sections of a cell pellet with mAb VK-9 and rabbit anti-mouse Ig-horse radish peroxide conjugate as described [18]. Cell surface reactivity was tested using a mixed hemagglutination assay as described previously [22].

Immunochemical assay methods

ELISA was carried out as described previously [23]. For direct assays the antigen (dissolved in water for protein conjugates and in ethanol for glycolipids) was coated to the wells of plastic microtiter plates by allowing the solution to dry at room temperature. The wells were then blocked with 2% BSA-PBS for 1 h and the test antibody (10 μl) was added. After incubation for 1 h at room temperature, the excess antibody was removed and the plate washed three times with 0.5% BSA-PBS. Antibody-antigen complexes were detected with protein A – alkaline phosphatase (Sigma Chemical Co., St Louis, MO) or rabbit anti-mouse IgG + IgM + IgA – alkaline phosphatase (Zymed) and incubation with p-nitrophenylphosphate. Color development was quantitated in a plate reader at 405 nm. For inhibition assays the test oligosaccharide was serially diluted in PBS and mixed with an equal aliquot of suitably diluted antibody ($0.2\ \mu g\ ml^{-1}$) in PBS – 0.5% BSA. After mixing, the samples were transferred to a series of wells in which

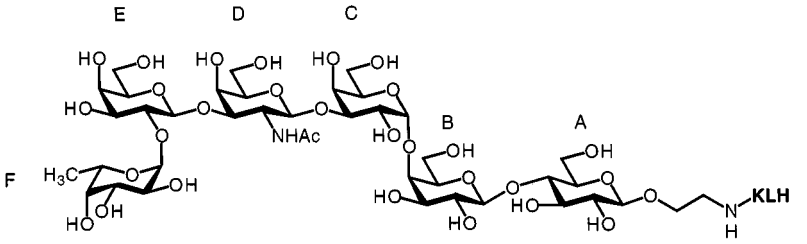

Figure 1. Structure of globo H-KLH conjugate used to immunize mice.

Table 2. Carbohydrate structures examined in this study

Designation	*Structure*
1. Globo H (hexassacharide)	Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc-
2. Globo H (pentassacharide)	Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Gal-R[a]
3. Globo H (tetrasaccharide)	Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Gal-R
4. Globo H (α isomer)	Fucα1 → 2Galβ1 → 3GalNAcα1 → 3Galα1 → 4Galβ1 → 4Glc-R
5. Globo H	Fucα1 → 2Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc-R
6. Globo A	GalNAcα1 → 3(Fucα1 → 2)Galβ1 → 3GalNAcβ1 → 3Gal-α1 → 4Galβ1 → 4Glc-R′
7. Gal-globoside (SSEA-3 antigen)	Galβ1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc-R′
8. Globoside	GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc-R′
9. Fuc-gangliotetraose	Fucα1 → 2Galβ1 → 3GalNAcβ1 → 4Galβ1 → 4Glc-R′
10. H type 1	Fucα1 → 2Galβ1 → 3GlcNAcβ1 → 3Gal-
11. H type 2	Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Gal-
12. Le[x] blood group	Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → 3Gal-
13. Le[y] blood group	Fucα1 → 2Galβ1 → 4(Fucα1 → 3)GlcNAcβ1 → 3Gal-
14. Le[a] blood group	Galβ1 → 3(Fucα1 → 4)GlcNAcβ1 → 3Gal-
15. Le[b] blood group	Fucα1 → 2Galβ1 → 3(Fucα1 → 4)GlcNAcβ1 → 3Gal-

[a]R, allyl group, R′, ceramide

a fixed amount (50 ng per well) of globo H-ceramide had been absorbed. The plate was incubated and washed as described for the direct assay. Color development was compared with the value given by an equal concentration of uninhibited diluted antibody and the degree of inhibition (%) calculated as OD_{405} (uninhibited) – OD_{405} (inhibited) × 100/OD_{405} uninhibited.

Glycolipid isolation and analysis

The neutral glycolipid fraction was isolated from breast cancer cell line MCF-7 by extraction with chloroform: methanol and fractionation by DEAE-Sephadex chromatography [24] and from blood group O kidney [25] as described previously. Glycolipids were separated by thin layer chromatography and detected with orcinol or anisaldehyde reagents and by immunostaining with mAb VK-9 as described [24, 26], using horse radish peroxidase-conjugated secondary anti-mouse IgG antibodies.

Results

Derivation of mouse monoclonal antibody VK-9

Monoclonal antibody VK-9 was derived from a mouse immunized with globo H-KLH synthetic conjugate (Figure 1) using the procedure of Köhler and Milstein [19]. The hybridoma supernatants were screened against globo H-BSA and globo H-ceramide. Eight strongly positive clones were selected for subcloning and one of them (VK-9; IgG3/κ) was chosen for further study. Purified mAb was produced by *in vitro* culture of the VK-9 hybridoma and fractionation by protein A affinity chromatography [21].

Specificity analysis of mAb VK-9

The reactivity of mAb VK-9 was analyzed with a panel of synthetic glycoconjugates and glycolipids by direct ELISA (Table 1). Its reactivity (Figure 2A and B) was compared to that of mAb MBr1 (Figure 2C and D). Of the compounds

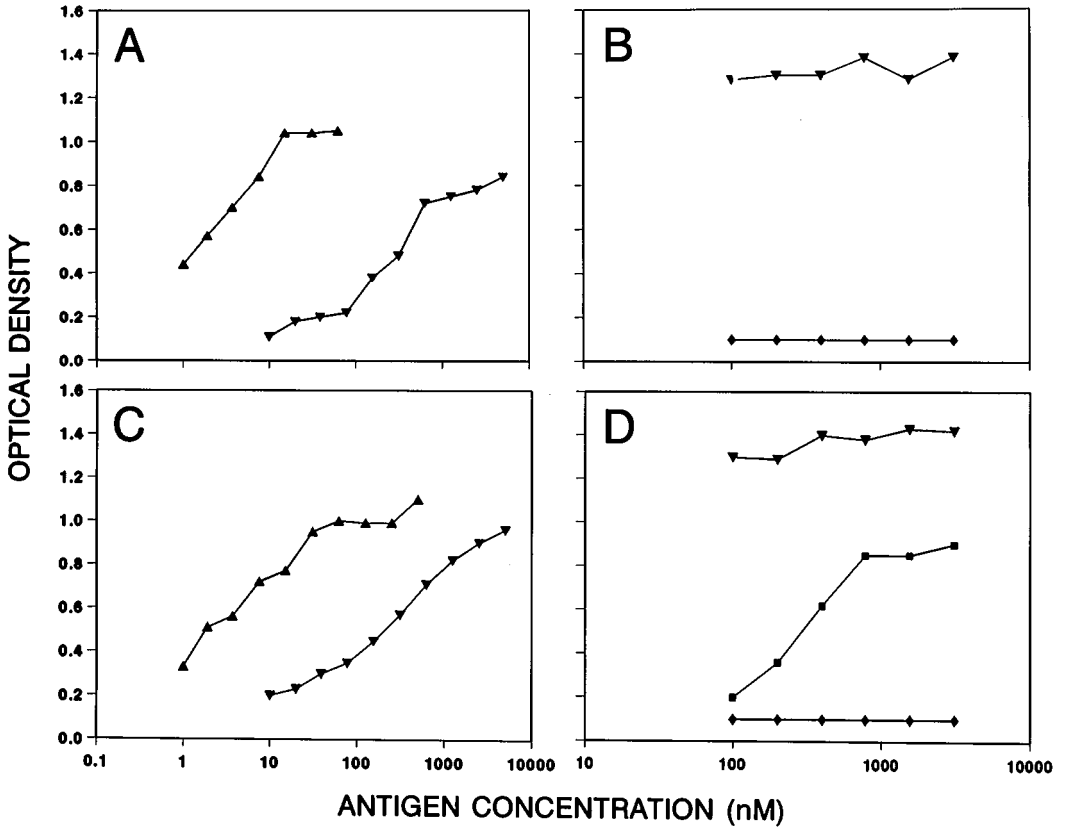

Figure 2. Reactivity of mAbs VK-9 and MBr1 with globo H-related glycolipids and glycoconjugates by ELISA. A: mAb VK-9 (0.5 μg ml^{-1}); B: mAb VK-9 (5 μg ml^{-1}); C: mAb MBr1 (0.1 μg ml^{-1}); D: mAb MBr1 (0.5 μg ml^{-1}). Antigens: ▲: globo H-BSA; ▼: globo H-Cer; ■: gal-globoside-Cer (SSEA-3). In panel B: ◆: globoside and other conjugates listed in Table 1; in panel D: ◆: globoside and other conjugates listed in Table 1, except gal-globoside-Cer.

tested, mAb VK-9 was most reactive against globo H-BSA and globo H-ceramide, as was mAb MBr1. When tested at a higher antibody concentration mAb MBr1 was also reactive with galactosylgloboside (Figure 2D), but mAb VK-9 did not react with this glycolipid or with any of the other compounds in this panel (Figure 2B). MAb VK-9 was also tested in inhibition assays with a series of shorter oligosaccharides with terminal globo H structures and two isomers of globo H hexasaccharides with α or β linkages between residues C and D or B and C respectively (Table 2 and Figure 1). All three compounds containing the four non-reducing terminal sugars of the globo H structure (Fucα1 → 2Galβ1 → 4GalNAcβ1 → 4Gal) were approximately equally efficient inhibitors (Figure 3). The isomer with an internal α-GalNAc linkage was less efficient and structures lacking the terminal fucose (galactosyl globoside) or the terminal Fucα1 → 2Gal residues (globoside) did not react with the antibody. By TLC-immunostaining mAb VK-9 was also tested for reactivity with globo A-ceramide and fucosyl-gangliotetraosyl ceramide (Figure 4). The antibody was not reactive with the ganglio series compound but did react with globo A-ceramide. Because of the limited availability of globo A-ceramide we were not able to quantitate the degree of reactivity but we estimate that the affinity of mAb VK-9 for globo A is 10–20 fold less than for the globo H structure. MAbs VK-9 reactivity were also assayed with a panel of natural mucins expressing A, B, H, Le^a, Le^b, Le^x, Le^y and precursor blood group epitopes. None of the antigens was reactive with mAb VK-9 (data not shown). The reactivity of the mAbs VK-9 with total neutral glycolipid fractions from rat small intestine [16], human kidney [7] and human blood group A, B, O erythrocytes and plasma was tested by TLC immunostaining (data not shown). The antibodies reacted with globo-H-cer and globo-A-cer as described above. In addition, a weak reactivity with some slow moving compounds, with about 8–10 sugar residues, was observed with the samples from the blood group A red blood cells. These compounds are most likely the blood group H and A type 3 core chain structures (extended blood group A type 2 chain structures) identified by Claussen *et al.* [27]. This cross reactivity is analogous to the weak binding to the α isomer of globo-H having an internal GalNAcα1-3 linkage (oligosaccharide 4) shown in Figure 3.

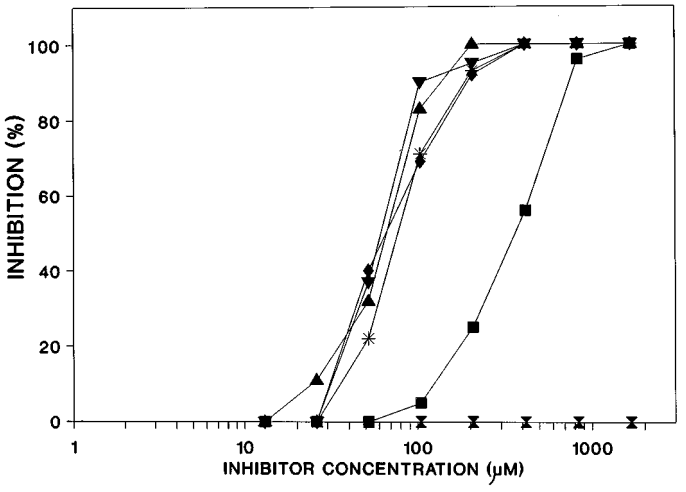

Figure 3. Specificity analysis of mAb VK-9 by inhibition with a panel of oligosaccharides. ∗ oligosaccharide 1; ▲oligosaccharide 2; ▼: oligosaccharide 3; ■ oligosaccharide 4; ◆ oligosaccharide 5; ⧗ globoside. See Table 2 for structures of oligosaccharides 1–5.

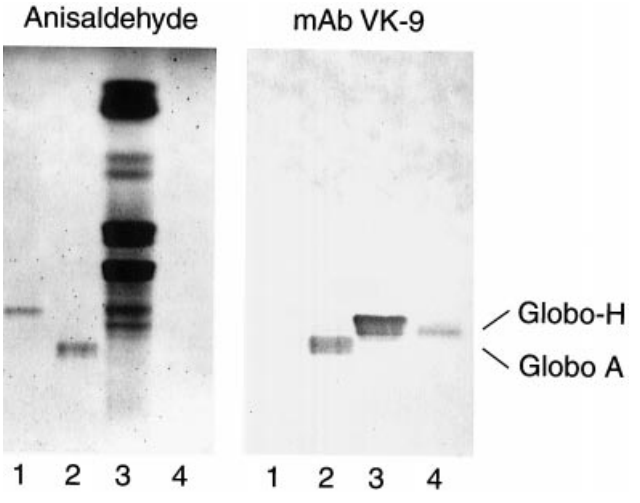

Figure 4. Reactivity of mAb VK-9 tested by TLC-immunostaining. A: glycolipids detected by anisaldehyde staining; B: glycolipids detected by immunostaining with mAb VK-9. Lane 1, fucosyl-gangliotetraosyl-Cer; lane 2, globo A-Cer (A-7-4); lane 3, total neutral glycolipids from blood group O pig kidneys; lane 4, globo H-Cer (H-6-4). Note that the amount of globo H-Cer applied in lane 4 was not sufficient to be detected with anisaldehyde but was detected by antibody staining.

Reactivity of mAb VK-9 with mammalian cells

As mAb VK-9 was raised against a synthetic glycoconjugate it was of interest to determine its reactivity with natural targets. When analyzed by a mixed hemagglutination assay, mAb VK-9 was shown to react strongly with the cell surface of a globo H-expressing breast cancer cell line MCF-7 (determined by reactivity with mAb MBr1) but not with the globo H-negative melanoma cell line SK-MEL-28 (Figure 5). As tested by immunocytochemistry on frozen sections of a panel of human cancer cell lines, mAb VK-9 was found to be strongly reactive with MCF-7 cells and weakly reactive with ovarian cancer cell line SK-OV-4 and breast cancer cell line T47D; other cell lines tested (ovarian cancer: OV-CAR-3 and 2774; melanoma: SK-MEL-28 and SK-MEL-31; neuroblastoma: IMR-32 and SK-N-MG) were unreactive (data not shown). The antigen reacting with mAb VK-9 in MCF-7 cell was shown by TLC-immunostaining to be a neutral glycolipid migrating with synthetic globo H-ceramide (Figure 6); no glycolipids having longer carbohydrate chains were detected. No mAb VK9-reactive glycoproteins could be detected in MCF-7 cells by immunoprecipitation of [^{3}H]GlcN-labeled cell extracts (data not shown).

Discussion

The monoclonal antibody (VK-9) produced in this study has a specificity very similar to that of the polyclonal sera of the globo H-KLH immunized mice from which it was derived [15] and is therefore representative of the polyclonal response in these mice. Analysis of the fine specificity of mAb VK-9 showed that it requires the four non-reducing terminal sugars of globo H (Figure 1; residues C–F) for maximum reactivity. Removal of terminal fucose residue completely destroyed reactivity and change of the β-linkage between sugars D and C to an α-linkage resulted in a reduced reactivity. The antibody was also able to recognize, to a lesser degree, the globo H determinant when it was situated internally as a part of the globo A structure. Conformational analysis of the globo-A-ceramide structure has shown that the saccharide chain has a bent configuration with the terminal GalNAcα1-3 residue oriented close to the plasma membrane while the Fuc-Gal-GalNAc saccharide sequence is exposed to the external part of the cell [28]. This may explain the weak recognition of the globo A compound by mAb VK-9. Related structures, such as galactosyl-globoside, globoside and type 1H and type 2H epitopes, were unreactive. The specificity of mAb VK-9 differs slightly from that of the prototype MBr1 antibody which has a small degree of cross-reactivity with galactosyl-globoside (SSEA-3 antigen). When analyzed by TLC-immunostaining with extracts of MCF-7 cells, mAb VK-9 detected a glycolipid migrating with globo H-ceramide (Figure 6). No other reactive glycolipid was detected in MCF-7 cells and no glycoprotein or mucin reactive with mAb VK-9 was detected by immunoprecipitation of [^{3}H]GlcN-labeled cells. Although Perrone and coworkers [29] detected MBr1-reactive glycoproteins by Western blotting of tumor extracts, globo-series structures have not been identified on glycoproteins by definitive structural analysis. The possibility that the globo H structure is carried by both glycolipids and glycoproteins needs to be examined further.

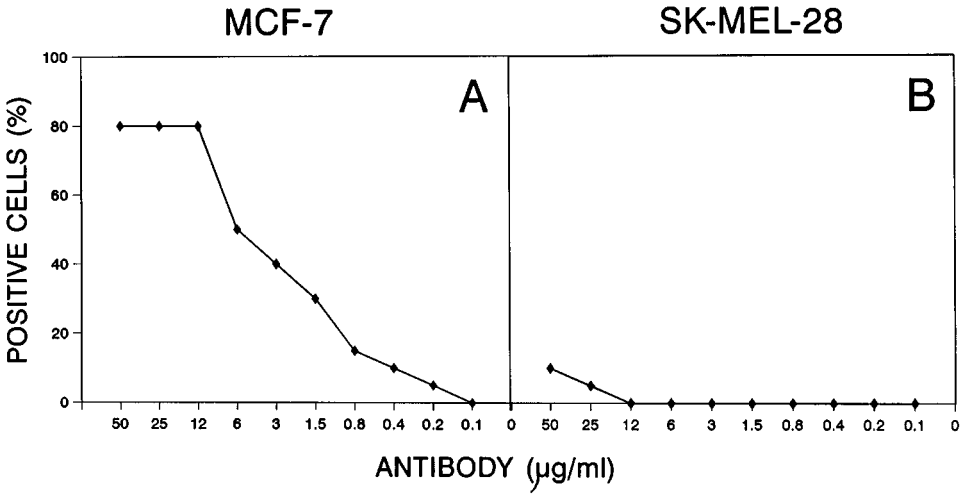

Figure 5. Cell surface reactivity of mAb VK-9 with globo H-positive (MCF-7) and globo H-negative (SK-MEL-28) cells assayed with protein A-MHA rosetting method. SK-MEL-28 cells were positive with a control mAb (R24) in the same experiment.

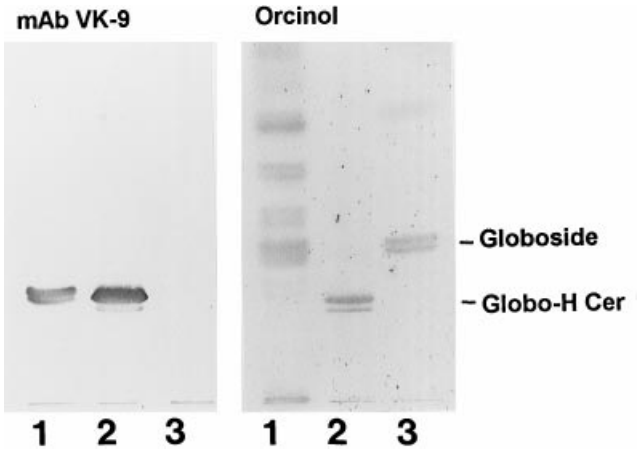

Figure 6. Reactivity of mAb VK-9 with glycolipids from MCF-7 cells analyzed by TLC-immunostaining. 1: neutral glycolipid fraction from MCF-7 cells; 2: globo H-Cer standard; 3: globoside standard. Left-hand panel: immunostaining with mAb VK-9; right-hand panel: glycolipids detected by orcinol-H_2SO_4 reagent.

In considering glycoconjugates as potential anti-tumor vaccines it is important to demonstrate that the antibodies generated react with the natural form of the antigen on tumor cells as well with the immunizing antigen. For globo H-KLH this criterion has been met both with respect to the whole polyclonal response [15] as well as with the derived monoclonal antibody. Previous experiences in producing an anti-tumor cell response by immunizing with synthetic glycoconjugates have been mixed. Although many successes have been reported (reviewed in [30, 31]), in an earlier study we were unable to produce an anti-tumor cell response by immunizing mice with a Le^y-serum albumin conjugate [32]. A number of differences between the present and previous study may explain this discrepancy, *ie* KLH vs HSA as a carrier and the use of QS21 as an adjuvant in this study as compared to no adjuvant in the earlier study. The nature of the carbohydrate hapten (globo H vs Le^y) does not seem to be important as we have also recently developed anti-tumor cell-reactive antibodies by immunization of mice with Le^y-KLH and QS21 [35].

MAb VK-9 will be useful for the immunochemical and immunohistological analysis for globo H-containing glycoconjugates. It may also be a suitable agent for use in the passive immunotherapy of carcinomas. Moreover, the ability of synthetic globo H-protein conjugates to induce a tumor cell-reactive antibody response encourages the use of such conjugates as vaccines in the immunotherapy of globo H-bearing tumors. Whether or not the expression of globo H on a number of normal tissues, particularly kidney, will limit the use of such an approach remains to be determined. It is generally believed that the restricted location of carbohydrate antigens in normal tissues, *eg* on the luminal surface of ducts, limits their accessibility to the immune system. A clinical trial using globo H-KLH vaccine which is presently underway in prostate cancer patients (P.O. Livingston, H.J. Scher *et al.* – work in progress) should help to clarify this issue.

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