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Phage-Display Selection of a Human Single-Chain Fv Antibody Highly Specific for Melanoma and Breast Cancer Cells Using a Chemoenzymatically Synthesized G_{M3}−Carbohydrate Antigen

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Abstract: Overexpression of the cell-surface glycosphingolipid G_{M3} is associated with a number of different cancers, including those of the skin, colon, breast, and lung. Antibodies against the G_{M3} epitope have potential application as therapeutic agents in the treatment of these cancers. We describe the chemoenzymatic synthesis of two G_{M3}-derived reagents and their use in the panning of a phage-displayed human single-chain Fv (scFv) antibody library derived from the blood of cancer patients. Three scFv-phage clones, GM3A6, GM3A8, and GM3A15, were selected for recombinant expression and were characterized using BIAcore and flow cytometry. BIAcore measurements using the purified, soluble scFvs yielded dissociation constants (K_d) ranging from 4.2×10^{-7} to 2.1×10^{-5} M. Flow cytometry was used to evaluate the ability of each scFv to discriminate between normal human cells (human dermal fibroblast, HDFa), melanoma cells (HMV-1, M21, and C-8161), and breast cancer cells (BCM-1, BCM-2, and BMS). GM3A6 displayed cross-reactivity with normal cells, as well as tumor cells, and GM3A15 possessed little or no binding activity toward any of the cell lines tested. However, GM3A8 bound to five of the six tumor cell lines and showed no measurable reactivity against the HDFa cells. Hence, we have demonstrated that a synthetic G_{M3} panning reagent can be used to isolate a fully human scFv that is highly specific for native G_{M3} on the surface of tumor cells. The result is a significant step toward effective immunotherapies for the treatment of cancer.

The changes in glycosphingolipid (GSL) metabolism that occur in cancerous cells were first noted more than 30 years ago.[1] Since that time, substantial evidence has accumulated that now concretely associates major changes in GSL and glycoprotein expression and composition with oncogenic transformation.[2] Significantly, abnormal glycosylation and overexpression of GSLs and glycoproteins have been found in a wide range of cancerous tissues and have been shown to correlate with tumor progression, metastasis, and patient survival rates.[3] Although these GSLs are found on the surface of both normal cells and cancer cells, they are strongly immunogenic only when present on tumors owing to the formation of GSL "microdomains" when a threshold density level is exceeded. These clusters of GSL molecules are important not only from the standpoint of immunogenicity but also for the function of GSLs as adhesion molecules and as modulators of membrane signaling pathways.[4] Given the differences in GSL and glycoprotein markers between the normal and cancerous state, the development of monoclonal antibodies (mAbs) against carbohydrate epitopes for the treatment of cancer has generated considerable interest.

The most significant problem associated with immunotherapy is that mAbs are typically obtained from hybridomas of murine origin. Repeated administration of these mAbs invariably results in a human antimurine antibody (HAMA) response leading to undesirable side effects as well as accelerated clearance of the antibodies from the circulation.[5] Unfortunately, human hybridomas are difficult to prepare, often unstable, and generally secrete IgM antibody at low levels.[6] Antibody-engineering

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(1) (a) Mora, P. T.; Brady, R. O.; Bradley, R. M.; McFarland, V. W. *Proc. Natl. Acad. Sci.* **1969**, *63*, 1290−1296. (b) Hakomori, S.; Murakami, W. T. *Proc. Natl. Acad. Sci.* **1968**, *59*, 254−261.
(2) (a) Hounsell, E. F.; Young, M.; Davies, M. J. *Clin. Sci.* **1997**, *93*, 287−293. (b) Kim, Y. S.; Gum, J., Jr.; Brockhausen, I. *Glycoconjugate J.* **1996**, *13*, 693−707. (c) Bhavanandan, V. P. *Glycobiology* **1991**, *1*, 493−503. (d) Hakomori, S. *Adv. Cancer Res.* **1989**, *52*, 257−331. (e) Hakomori, S. *Sci. Am.* **1986**, *254*, 44−53.
(3) (a) Hakomori, S. *Cancer Res.* **1996**, *56*, 5309−5318. (b) Muramatsu, T. *Glycobiology* **1993**, *3*, 291−296.
(4) Hakomori, S.; Zhang, Y. *Chem. Biol.* **1997**, *4*, 97−104.

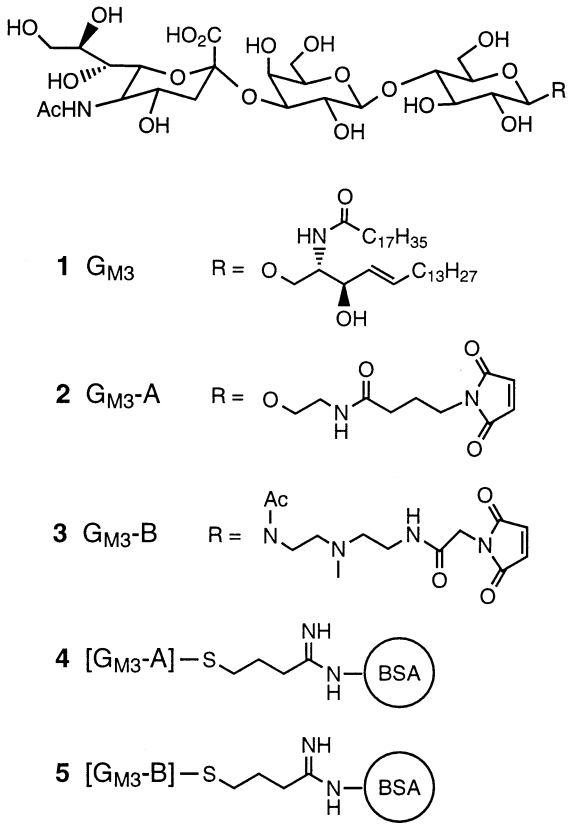
strategies aimed at humanization of murine mAbs, such as complementarity-determining region (CDR) grafting,[7] often do not lead to satisfactory clinical results. An alternative solution is to generate completely human antibodies using phage-display technology.[8] It is possible, for example, to create large antibody libraries from the peripheral blood lymphocytes of cancer patients in order to screen for antibodies specific for particular cancer antigens. We previously reported using this approach to identify single-chain Fv (scFv) antibody fragments specific for the carbohydrate antigens Lewis^x and sialyl Lewis^x.[9]

Overexpression of the ganglioside G_{M3} is associated with melanoma and a number of other cancer types, such as colon, breast, and lung.[10] It was previously demonstrated that the murine mAb DH2 recognized G_{M3} and inhibited the growth of melanoma cells in vitro and in vivo.[11] In addition, human IgM mAbs having various specificities for G_{D2}, G_{D3}, G_{M2}, and G_{M3} were isolated.[12] However, for the reasons outlined above, the use of these antibodies in a clinical setting is extremely limited. Herein, we describe the chemoenzymatic synthesis of G_{M3}-carbohydrate haptens that contain a linker moiety for attachment to a carrier protein and the use of these synthetic panning reagents to select human scFvs specific for G_{M3} from a phage-display library. BIAcore was used to measure the antibody affinities for the G_{M3} hapten itself, and flow cytometry was performed to evaluate scFv recognition of the intact antigen on the surface of melanoma and breast cancer cells. Significantly, our strategy allowed the isolation and characterization of a human scFv highly specific for three separate melanoma cell lines, HMV-1, M21, and C-8161, the latter of which is highly metastatic, and two breast cancer cell lines, BCM-1 and BCM-2, derived from stage IV breast cancer patients. Finally, the results suggested that GSL clustering may not be necessary to obtain anti-GSL antibodies that bind specifically to tumor cells.

Results and Discussion

Two G_{M3} haptens, **2** and **3**, which differ from each other only in the appended linker group, were designed (Chart 1). We examined different linkers on the basis of our previous work, particularly in the elicitation of immune responses, that showed the importance of the linker structure between hapten and carrier protein for aspects of recognition, although the effect is not yet well understood.[13] Thus, **2** incorporates the natural O-linked carbohydrate fragment that occurs in G_{M3} **1**, while **3** utilizes an acetylated *N*-glycosyl linkage that is less congruent to G_{M3}, but somewhat more stable. The synthesis of a similar *N*-linked G_{M3} hapten was recently reported.[14] Both linkers provide a tether that contains hydrogen-bonding functionalities, are much more hydrophilic than the fatty ceramide portion of G_{M3}, are approximately 10−12 Å in length, which is generally optimum for hapten recognition, and are terminated with a maleimide functionality. Since the haptens possess the distal sialic acid residue, using a linker that terminates in a carboxylic acid would not allow fully site-specific coupling to amine-containing partners. Hence, a maleimide moiety was chosen that readily reacts with either cysteine residues on proteins for the construction of panning reagents or sulfur functional groups on BIAcore sensor chips for the measurement of kinetic parameters.

Chart 1. Structures of Compounds under Discussion



The synthesis of G_{M3}-A was accomplished as shown (Scheme 1). Lactosyl bromide **6** was prepared from D-lactose as described in the literature.[15] Displacement of bromide from **6** with 2-azidoethanol in the presence of silver trifluoromethanesulfonate followed by deprotection of all acetyl groups gave the key intermediate **7**. In the enzymatic sialylation of **7**, CMP-sialic acid **9** was used as a sialyl donor and the reaction proceeded smoothly to completion at room temperature in the presence of excess α-2,3-sialyltransferase (α-2,3-SiaT) to provide **8**.[16] The final compound was prepared in two consecu-

(5) (a) Sakahara, H.; Saga, T.; Onodera, H.; Yao, Z.; Nakamoto, Y.; Zhang, M.; Sato, N.; Nakada, H.; Yamashina, I.; Endo, K. *Jpn. J. Cancer Res. (GANN)* **1997**, *88*, 895−899. (b) Dillman, R. O.; Beauregard, J. C.; Halpern, S. E.; Clutter, M. *J. Biol. Response Mod.* **1986**, **5**, 73−84. (c) Schroff, R. W.; Foon, K. A.; Beatty, S. M.; Olkham, R. K.; Morgan, A. C., Jr. *Cancer Res.* **1985**, *45*, 879−885. (d) Shawler, D. L.; Bartholomew, R. M.; Smith, L. M.; Dillman, R. O. *J. Immunol.* **1985**, *135*, 1530−1535.

(6) James, K. In *Handbook of Experimental Pharmacology: The Pharmacology of Monoclonal Antibodies*; Rosenberg, M., Moore, G. P., Eds.; Springer-Verlag: New York, 1994; Vol. 113, pp 3−19.

(7) (a) Daugherty, B. L.; DeMartino, J. A.; Law, M. F.; Kawka, D. W.; Singer, I. I.; Mark, G. E. *Nucleic Acids Res.* **1991**, *19*, 2471−2476. (b) Riechmann, L.; Clark, M.; Waldmann, H.; Winter, G. *Nature* **1988**, *332*, 323−327.

(8) (a) Graus, Y. F.; Verschurren, J. J.; Degenhardt, A.; van Breda Vriesman, P. J.; De Baets, M. H.; Posner, J. B.; Burton, D. R.; Dalmau, J. *J. Neuroimmunol.* **1998**, *82*, 200−209. (b) Henderikx, P.; Kandilogiannaki, M.; Petrarca, C.; von Mensdorff-Pouilly, S.; Hilgers, J. H.; Krambovitis, E.; Arends, J. W.; Hoogenboom, H. R. *Cancer Res.* **1998**, *58*, 4324−4332.

(9) Mao, S.; Gao, C.; Lo, L. C.-H.; Wirsching, P.; Wong, C.-H.; Janda, K. D. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6953−6958.

(10) Hoon, D. S.; Wang, Y.; Sze, L.; Kanda, H.; Watanabe, T.; Morrison, S. L.; Morton, D. L.; Irie, R. F. *Cancer Res.* **1993**, *53*, 5244−5250.

(11) Dohi, T.; Nores, G.; Hakomori, S. *Cancer Res.* **1988**, *48*, 5680−5685.

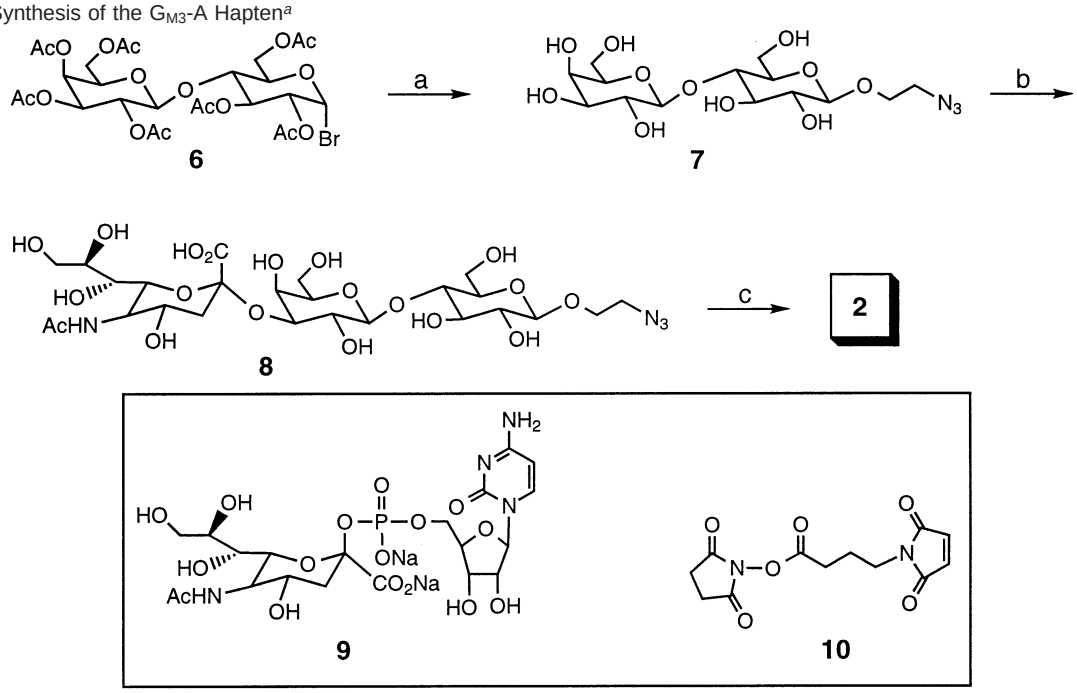
(12) (a) Nishinaka, Y.; Ravindranath, M. H.; Irie, R. F. *Cancer Res.* **1996**, *56*, 5666−5671. (b) Lloyd, K. O. *Ann. N. Y. Acad. Sci.* **1993**, *690*, 50−58. (c) Yamaguchi, H.; Furukawa, K.; Fortunato, S. R.; Livingston, P. O.; Lloyd, K. O.; Oettgen, H. F.; Old, L. J. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 3333−3337. (d) Furukawa, K.; Yamaguchi, H.; Oettgen, H. F.; Old, L. J.; Lloyd, K. O. *Cancer Res.* **1989**, *49*, 191−196. (e) Tai, T.; Paulson, J. C.; Cahan, L. D.; Irie, R. F. *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 5392−5396.

(13) (a) Isomura, S.; Ashley, J. A.; Wirsching, P.; Janda, K. D. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 861−864. (b) Matsushita, M.; Hoffman, T. Z.; Ashley, J. A.; Zhou, B.; Wirsching, P.; Janda, K. D. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 87−90.

(14) Xue, J.; Pan, Y.; Guo, Z. *Tetrahedron Lett.* **2002**, *43*, 1599−1602.

(15) Dahmen, J.; Frejd, T.; Gronberg, G.; Lave, T.; Magnusson, G.; Noori, G. *Carbohydr. Res.* **1983**, *116*, 303−307.

Scheme 1. Synthesis of the G_{M3}-A Hapten[a]



Reagents and conditions: (a) (i) 2-azidoethanol, AgOTf, CH_2Cl_2, −78 °C to room temperature, 24 h, (ii) NaOMe, MeOH (70% yield for two steps); (b) **9**, α-2,3-SiaT, cacodylate buffer, pH 6.5, rt, 1 d (77% yield); (c) (i) H_2, 10% Pd/C, MeOH/H_2O (50/50), rt, 2 h, (ii) **10**, 0.1 M $NaHCO_3$/MeOH (50/50), rt, 1 h (42% yield for two steps).

tive steps. First, the terminal azido functionality was reduced by catalytic hydrogenation in methanol−water, then the resulting amine was coupled directly with the activated ester of maleimidobutyric acid in slightly basic solution, affording **2**.

The enzyme α-2,3-SiaT has been used previously for oligosaccharide synthesis[17] and generally transfers *N*-Ac-neuraminic acid (sialic acid) to the 3-position of terminal galactose or *N*-Ac-galactosamine residues. Several recent advances, such as the cloning of glycosyltransferases, development of a cofactor regeneration system, and the large-scale syntheses of nucleotide sugars have made the enzymatic approach especially attractive for the chemical glycosylation of certain problematic linkages. In fact, α-2,3-SiaT has been used in the synthesis of natural G_{M3}.[18] The advantages of using enzymatic glycosylation are that elaborate protection-deprotection steps are not required and the carbohydrates are obtained with only the correct, naturally occurring stereochemistry.

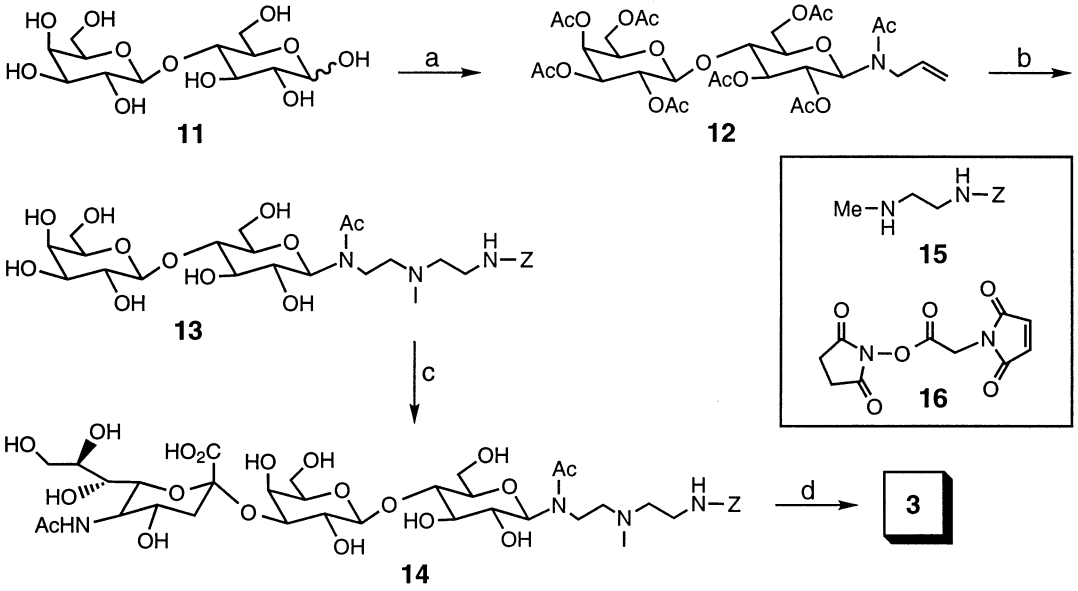
The synthesis of G_{M3}-B also proceeded smoothly (Scheme 2). Compound **12** was prepared from α-D-lactose **11** in neat allylamine at room temperature, a manner similar to the known procedure.[19] Ozonolysis of the terminal alkene at low temperature followed by reductive workup gave the aldehyde, which was directly coupled with secondary amine **15** in the presence of sodium cyanoborohydride, affording the peracetylated disaccharide. After deprotection of all acetyl groups, the intermediate **13** was obtained in good overall yield. Since *N*-lactosyl derivatives have not often been used in enzymatic sialylation,[20] we were pleased to observe that the sialyl group was successfully transferred from **9** to **13**, yielding compound **14** nearly quantitatively. The terminal benzyloxycarbonyl group was removed by catalytic hydrogenation and the resulting amine was coupled with activated ester **16** to provide hapten **3**.

We chose bovine serum albumin (BSA) as the protein for conjugation to form the panning reagents. To this end, we first treated BSA with Traut's reagent, which modifies lysine residues through an imidate ester bond to afford terminal thiols. The modified BSA was then directly coupled with **2** or **3** in phosphate buffer, pH 7.4, at room temperature and dialyzed to give **4** and **5**, respectively (Chart 1). The average numbers of carbohydrate epitopes attached to the modified BSA (17.0 for **2** and 10.2 for **3**) were determined by MALDI-TOF MS analysis.

The G_{M3}-A−BSA and G_{M3}-B−BSA conjugates were immobilized on a solid support and used to pan a previously constructed scFv-phage library derived from the blood samples of 20 human subjects diagnosed with a variety of cancers.[9] Over the course of four rounds of panning, phage titers increased from 1.8×10^5 to 1.4×10^9 (7800-fold enrichment) using **4** and from 5.9×10^5 to 1.6×10^8 (270-fold enrichment) using **5**. Eleven clones from the panning with **4** and 10 clones from using **5** were selected for sequence analysis (Tables 1 and 2). Several distinct groups of related antibodies emerged, and representative clones from each of these groups were chosen for BIAcore analysis. From **4**, one group comprised clones GM3A2, GM3A3, GM3A6, and GM3A11. A distinguishing feature arising from sequence analysis of these clones was that CDR L2 contained nine amino acids, rather than the more typical

(16) Blixt, O.; Brown, J.; Schur, M. J.; Wakarchuk, W.; Paulson, J. C. *J. Org. Chem.* **2001**, *66*, 2442−2448.
(17) (a) Wittmann, V.; Takayama, S.; Gong, K. W.; Weitz-Schmodt, G.; Wong, C.-H. *J. Org. Chem.* **1998**, *63*, 5137−5143. (b) Seitz, O.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 8766−8776. (c) Wong, C.-H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem. Int. Ed.* **1995**, *34*, 521−546. (d) Ichikawa, Y.; Lin, Y.-C.; Dumas, D. P.; Shen, G.-J.; Wong, C.-H. et al. *J. Am. Chem. Soc.* **1992**, *114*, 9283−9298. (e) Ichikawa, Y.; Shen, G.-J.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 4698−4700.
(18) (a) Liu, K.-C.; Danishefsky, S. J. *Chem. Eur. J.* **1996**, *2*, 1359−1362. (b) Liu, K. K.-C.; Danishefsky, S. J. *J. Am. Chem. Soc.* **1993**, *115*, 4933−4934. (c) Ito, Y.; Paulson, J. C. *J. Am. Chem. Soc.* **1993**, *115*, 1603−1605.
(19) (a) Ramon, D.; Rollin, P.; Klaffke, W. *Angew. Chem. Int. Ed.* **2000**, *39*, 396−398. (b) Spevak, W.; Dasgupta, F.; Hobbs, C. J.; Nagy, J. O. *J. Org. Chem.* **1996**, *61*, 3417−3422.
(20) Wittmann, V.; Datta, A. K.; Koeller, K. M.; Wong, C.-H. *Chem. Eur. J.* **2000**, *6*, 162−171.

Scheme 2. Synthesis of the G_{M3}-B Hapten[a]



Reagents and conditions: (a) (i) allylamine, rt, 70 h, (ii) Ac_2O, pyridine, 12 h (53% yield for two steps); (b) (i) O_3, MeOH, −78 °C to room temperature, then Me_2S, (ii) **15**, $NaCNBH_3$, AcOH (0.5 equiv), MeOH, rt, 24 h, (iii) NaOMe, MeOH (35% yield for three steps); (c) **9**, α-2,3-SiaT, cacodylate buffer, pH 6.5, rt, 1 d (65% yield); (d) (i) H_2, 10% Pd/C, MeOH/H_2O (50/50), rt, 2 h, (ii) **16**, 0.1 M $NaHCO_3$/MeOH (50/50), rt, 1 h (45% yield for two steps).

Table 1. Aligned Heavy-Chain (H) Amino Acid Sequences of Selected ScFv Clones

clone	H family	CDR H1	CDR H2	CDR H3
GM3A2	IGHV1	GYTFRNHAIS	WISGYNGNTNYAQRFQG	DLMNWGRFPLDY
GM3A3	IGHV1	GYTFRNHAIS	WISGYNGNTNYAQRFQG	DLMNWGRFPLDY
GM3A6	IGHV1	GYTFRNHAIS	WISGYNGNTNYAQRFQG	DLMNWGRFPLDY
GM3A11	IGHV1	GYTFRNHAIS	WISGYNGNTNYAQRFQG	DLMNWGRFPLDY
GM3A1	IGHV4	GASITSYYWN	YIHHRGASNYNPSLKS	TGTSSDIGRYNYVS
GM3A4	IGHV4	GMSITSSYWN	YVHYNGNTLSNPSLQS	WNGQNNAFDT
GM3A9	IGHV4	GMSITSSYWN	YVHYNGNTLSNPSLQS	WNGQNNAFDT
GM3A15	IGHV4	GMSITSSYWN	YVHYNGNTLSNPSLQS	WNGQNNAFDT
GM3A8	IGHV4	GGSISSSYWN	YVHYTGSTHYNPSLQS	WNGVNNAFDT
GM3A13	IGHV1	GYTFTSYDIN	WMNPNSGNTGYAQKFQG	ARYSGSYRPLDYYMDV
GM3A5	IGHV3	GFSFSSYAMT	TITARGDRTYNADSVKG	DSSYVDWPPYGLHV
GM3B	IGHV3	GFSFSSYAMT	TITARGDRTYNADSVKG	DSSYVDWPPYGLHV

Table 2. Aligned Light-Chain (L) Amino Acid Sequences of Selected ScFv Clones

clone	L family	CDR L1	CDR L2	CDR L3
GM3A2	IGLV5	TLRSGIDVGTYRI	KSDSDQKQG	MIWHSSAWV
GM3A3	IGLV5	TLRSGIDVGTYRI	KSDSDQKQG	MIWHSSAWV
GM3A6	IGLV5	TLRSGIDVGTYRI	KSDSDQKQG	MIWHSSAWV
GM3A11	IGLV5	TLRSGIDVGTYRI	KSDSDQKQG	MIWHSSAWV
GM3A1	IGLV2	TGTSSDIGRYNYVS	EVDKRPS	SSFGAGKV
GM3A4	IGLV2	SVISSDVGANKRVS	EVNKRRS	SSYTRTELL
GM3A9	IGLV2	SVISSDVGANKRVS	EVNKRRS	SSYTRTELL
GM3A15	IGLV2	SVISSDVGANKRVS	EVNKRRS	SSYTRTELL
GM3A8	IGKV1	RASQSISSYLN	AASRLRS	QQSYSTPVT
GM3A13	IGKV1	RASQRIDSFLN	AASSLQS	QQSYSTPLT
GM3A5	IGLV2	TGPSSDVGNYDYVS	DVSNRPS	SSYTGTLLL
GM3B	IGLV2	TGPSSDVGNYDYVS	DVSNRPS	SSYTGTLLL

seven. This structure has been observed previously but is considered rare.[21]

The soluble scFv GM3A6 was expressed in *Escherichia coli*, purified, and found to possess a dissociation constant (K_d) of 0.42 μM determined using BIAcore (Table 3). The second major sequence group included the identical clones GM3A4, GM3A9, and GM3A15 and the related clone GM3A1 [85% homology at the amino acid level, excluding the $(Gly_4Ser)_3$ linker]. BIAcore analysis of scFv GM3A15 revealed a moderate affinity for G_{M3} (K_d = 21 μM). Another clone, GM3A8, contained a heavy-chain sequence very similar to clones GM3A4, GM3A9, and GM3A15 but a light chain that more closely resembled clone GM3A13. On the basis of several factors such as expression level, enzyme-linked immunosorbent assay (ELISA) using G_{M3}-A−BSA (data not shown), and BIAcore (K_d = 1.2 μM), GM3A8 was considered, at this point, the most desirable scFv.

Interestingly, all 10 clones derived from conjugate **5** consisted of the same DNA sequence, and this sequence was also found in clone GM3A5. This suggested that, although linker differ-

(21) Kasaian, M. T.; Ikematsu, H.; Balow, J. E.; Casali, P. *J. Immunol.* **1994**, *152*, 3137−3151.

Table 3. Kinetic and Thermodynamic Parameters for Binding of scFvs to G_{M3}[a]

scFv	k_{on} (10^3 M^{-1} s^{-1})	k_{off} (10^4 s^{-1})	K_d (10^6 M)
GM3A6	2.1	8.9	0.42
GM3A8	0.44	5.3	1.2
GM3A15	4.9	1000	21

[a] The kinetic constants were measured by BIAcore using G_{M3}-A covalently attached to the BIAcore biosensor. The K_d was calculated ($K_d = k_{off}/k_{on}$) from the experimentally determined k_{on} (association rate constant) and k_{off} (dissociation rate constant) values.

ences between G_{M3}-A and G_{M3}-B afforded antibody populations that were on the whole quite distinct, some crossover in hapten recognition did occur in the panning process. Due to the comparatively low level of enrichment, suggestive of low affinity, and a lack of specificity by ELISA, no clones obtained using **5** were chosen for recombinant expression or BIAcore analysis. Notably, the results reflected many of our previous findings that linker structure can be critical during recognition processes, whether in vivo during an immune response or in vitro for panning procedures, suggesting that variations in the linkage between hapten and carrier should be evaluated for such experiments.[13]

To determine whether the scFvs could recognize the native G_{M3} epitope on the surface of tumor cells, the purified scFvs were used to stain three melanoma and three breast cancer cell lines, as well as normal dermal fibroblast (HDFa) cells as a nonmalignant cell control. The melanoma cell line HMV-1 has been characterized as poorly metastatic,[22] M21 is moderately metastatic,[23] and C-8161 is regarded as a highly metastatic cell line.[24] The three breast cancer cell lines, BCM-1, BCM-2, and BMS, were derived from circulating metastatic cells in the peripheral blood of stage IV breast cancer patients. Binding of anti-G_{M3} scFvs GM3A6, GM3A8, and GM3A15 to these cell lines was assessed using flow cytometry (Figure 1). It was found that clone GM3A15 displayed little or no binding activity toward any cells relative to antibody-free buffer controls. GM3A6 displayed low to moderate affinity toward four of the six tumor cell lines, but also appeared to bind to HDFa cells more strongly than the other two scFvs. Antibody cross-reactivity with normal cells is an unfavorable property for clinical applications of cancer immunotherapy. In contrast, scFv GM3A8 showed excellent specificity for tumor cells versus normal cells. Five of the six tumor cell lines were strongly stained by this scFv and, significantly, no measurable binding activity toward HDFa cells was detected. Hence, scFv GM3A8 is a candidate for future studies directed at killing tumor cells using a scFv−drug conjugate or by elaboration into a whole IgG format for complement-mediated and antibody-dependent cytotoxic pathways.

The results also demonstrated that a BSA carrier protein displaying the carbohydrate portion of a tumor-associated GSL could be used to isolate antibodies that recognized the intact, native GSL antigen on the cell surface. In the hapten design, it was not necessary to model the long, hydrophobic ceramide tails of G_{M3}, since these are generally concealed within the plasma membrane and not exposed for antibody binding, ensuring that the carbohydrate portion is presented on the cell surface. A hapten linker was designed that recapitulated this effect by appropriately attaching and presenting the G_{M3} trisaccharide on the surface of the carrier protein. It is likely that the hydrophilic nature of the hapten−linker construct of **4** precludes sequestration within the relatively hydrophobic protein matrix and therefore allows extensive access to the haptenic structure. Clearly, there was a difference between **4** and **5**, suggesting perhaps that the *N*-acetyl group and/or positively charged *N*-methyl group in the linker of **5** associate with the protein to cause some shielding of carbohydrate determinants. This might have resulted in the inability to isolate clones of adequate affinity using **5** as noted above.

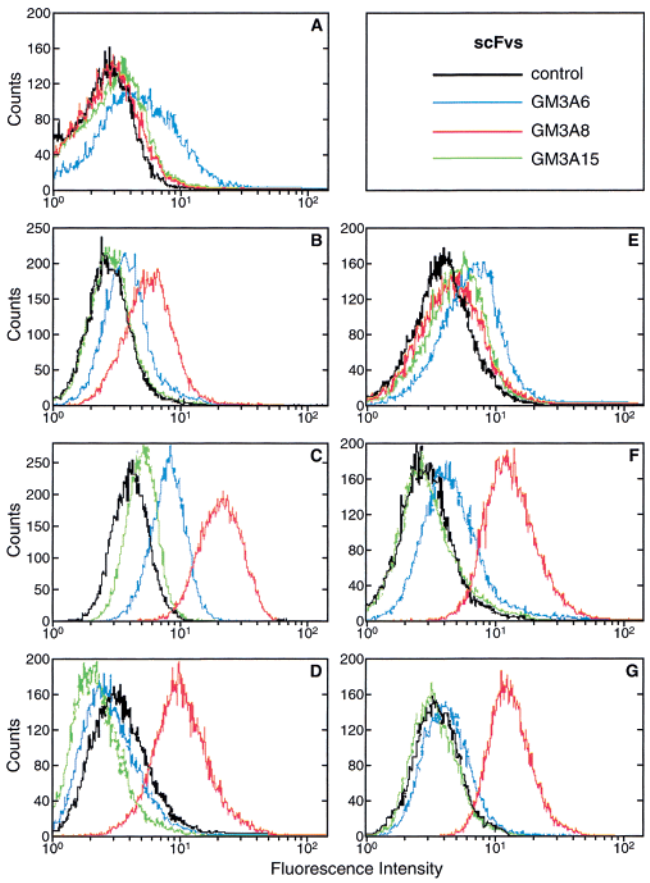


Figure 1. Analysis of the binding of anti-G_{M3} scFvs to various cell lines using flow cytometry: (A) normal HDFa cells; (B) melanoma, HMV-1; (C) melanoma, M21; (D) melanoma, C-8161; (E) breast cancer, BMS; (F) breast cancer, BCM-1; (G) breast cancer, BCM-2. Control = PBS.

Recent studies of carbohydrate anticancer vaccine designs indicated that multivalent display of the carbohydrate antigen Lewis^y in synthetic haptens was required for eliciting high antibody titers.[25] The antibodies produced in response to these vaccines were predominantly of the IgM class due to the chemical nature of the antigen and its reliance upon T cell-independent stimulation of B-cell activity. Elicitation of in vivo antibody responses against G_{M3} has also been particularly problematic. A biantennary hapten was recently used to obtain reasonable titers consisting of IgM and IgG antibodies.[26]

(22) Katagiri, Y.; Hayashi, Y.; Baba, I.; Suzuki, H.; Tanoue, K.; Yamazaki, H. *Cancer Res.* **1991**, *51*, 1286−1293.
(23) Schulz, G.; Staffileno, L. K.; Reisfeld, R. A.; Dennert, G. *J. Exp. Med.* **1985**, *161*, 1315−1325.
(24) Welch, D. R.; Bisi, J. E.; Miller, B. E.; Conaway, D.; Seftor, E. A.; Yohem, K. H.; Gilmore, L. B.; Seftor, R. E.; Nakajima, M.; Hendrix, M. J. *Int. J. Cancer* **1991**, *47*, 227−237.
(25) Kudryashov, V.; Glunz, P. W.; Williams, L. J.; Hintermann, S.; Danishefsky, S. J.; Lloyd, K. O. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3264−3269.
(26) Zou, W.; Abraham, M.; Gilbert, M.; Wakarchuk, W. W.; Jennings, H. J. *Glycoconjugate J.* **1999**, *16*, 507−515.

In contrast, our approach is based on a human scFv library and the isolation in vitro of preexisting antibodies that can bind carbohydrate epitopes. As in our previous work,[9] the coupling of a monovalent hapten to a carrier protein afforded a panning reagent that was sufficient to provide selection of the relevant antibody clones. Yet, it is also conceivable that some of the G_{M3} ligands coupled to lysine residues in close proximity on BSA mimic a GSL-like cell-surface "microdomain" or similar multivalent cluster. Regardless, when given a large library of possible clones, the panning reagent strategy provides what is functionally required to allow isolation of at least a few scFvs that have the desired characteristics of specificity and affinity for the native antigen. Consequently, in light of the simpler design, haptens and panning reagents based on other members of the GSL family and of carbohydrates present on glycoprotein and mucin structures will be much more synthetically accessible. In this way, a variety of scFvs should be available against virtually any tumor-associated carbohydrate.

The scFv GM3A8 with its favorable binding properties for melanoma and breast tumor cells provides a solid foundation for further development. The in vitro affinity of the scFv is estimated to be comparable to a previously reported G_{M3}-binding murine IgG and anti-G_{M3} polyconal antibodies,[11,26] but with the significant advantages of possessing a human antibody sequence and having the ability to specifically recognize highly metastatic cancer cells. Moreover, the scFv format greatly facilitates genetic manipulations, such as mutagenesis for affinity enhancement, introduction of a cysteine residue for precise attachment of drugs, and grafting onto the appropriate immunoglobulin scaffold to enlist relevant effector functions. In future work, we will utilize GM3A8 variants bearing such modifications for preliminary analyses in cell culture. Some of these antibodies may then be worthy of subsequent evaluation against tumors in animal models and ultimately in clinical studies for treatment of human cancers.

Experimental Section

General Synthetic Methods. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Dichloromethane (CH_2Cl_2) was distilled from calcium hydride under N_2, and methanol (MeOH) was distilled from magnesium turnings and iodine under N_2. All reactions were run under an inert atmosphere with dry reagents and solvents and flame-dried glassware. Flash column chromatography was carried out using Merck 60 230−400 mesh silica gel. Thin-layer chromatography was performed using 0.25 mm silica gel coated Kieselgel 60 F_{254} plates and visualized using aqueous cerium molybdate or 5% sulfuric acid in ethanol with heating. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer at 500 and 125 MHz or a Bruker AMX-600 at 600 and 150 MHz, respectively, using the indicated solvents at 25 °C. Chemical shifts are reported in ppm on the δ scale. Compounds **10** and **16** were purchased from Sigma. The α-2,3-sialyltransferase-CMPNeuAc synthetase fusion protein,[27] CMP-sialic acid **9**,[16] and compound **15**[28] were prepared according to literature procedures.

Compound 7. A mixture of 2-azidoethanol (2.5 g, 28.8 mmol), 2,4,6-collidine (0.57 mL, 4.3 mmol), and 4 Å molecular sieves in dried dichloromethane was stirred for 30 min at room temperature under Ar. Silver trifluoromethanesulfonate (1.38 g, 5.4 mmol) was then added. The mixture was stirred for another 30 min in the dark and then cooled to −50 °C. Compound **6** (2.53 g, 3.6 mmol), dissolved in dried dichloromethane, was added slowly to the above mixture and allowed to warm to room temperature for 20 h in the dark. The yellowish-white precipitate was removed by filtration through a pad of Celite, and the colorless filtrate was concentrated and then purified by column chromatography ($CHCl_3$/MeOH, 30/1) to provide the peracetylated derivative (1.77 g, 70%). This was dissolved in anhydrous methanol and a catalytic amount of sodium methoxide (25wt % solution in methanol) was added (pH ∼ 10). The reaction was stirred at ambient temperature for 7 h. Amberlite IR-120 (plus) ion-exchange resin was added to neutralize the base, and then the solution was filtered and evaporated to dryness to give **7** as a fluffy solid (1.02 g, 99%). ^{1}H NMR (600 MHz, CD_3OD) δ: 4.37−4.35 (m, 2H), 4.03−4.00 (m, 1H), 3.93−3.91 (m, 1H), 3.86−3.69 (m, 5H), 3.61−3.42 (m, 8H), 3.28 (dd, J = 8.8, 7.9 Hz, 1H). ^{13}C NMR (150 MHz, CD_3OD) δ: 105.04, 104.28, 80.59, 77.02, 76.48, 76.34, 74.76, 74.66, 72.51, 70.25, 69.37, 62.46, 61.91, 52.00. HRMS (M + Na^+): calcd for $C_{14}H_{25}N_3O_{11}Na$ 434.1387, found 434.1380.

Compound 8. Azide **7** (0.23 g, 0.56 mmol) and CMP-sialic acid **9** (0.41 g, 0.65 mmol) were dissolved in sodium cacodylate (100 mL, 200 mM, pH 6.5) containing $MnCl_2$ (20 mM). The α-2,3-sialyltransferase-CMPNeuAc synthetase (12 U) was added and the reaction was slowly stirred at room temperature for 18 h. When all of the starting material was converted to product (TLC; EtOAc/HOAc/MeOH/H_2O, 45/20/20/15), the mixture was ultrafiltered through a 10 000 MWCO membrane. The filtrate was passed through Dowex anion resin (1-X8, 200−400 mesh, phosphate-form 4 × 10 cm) and lyophilized. The residue was loaded onto a column of Sephadex G15 (5 × 140 cm), equilibrated, and eluted with 5% *n*-BuOH in H_2O. Appropriate fractions were collected and lyophilized to give **8** (0.31 g, 0.43 mmol, 77%). ^{1}H NMR (600 MHz, D_2O) δ: 4.52 (m, 2H), 4.11−3.54 (m, 22H), 3.33 (t, J = 8.5 Hz, 1H), 2.75 (dd, J = 12.3, 4.4 Hz, 1H, H_{eq} of sialic acid), 2.02 (s, 3H, NAc), 1.79 (t, J = 11.8 Hz, 1H, H_{ax} of sialic acid). ^{13}C NMR (150 MHz, D_2O, trace acetone added as internal reference at δ 29.92) δ: 174.70, 173.61, 102.32, 101.87, 99.48, 77.84, 75.16, 74.85, 74.50, 73.99, 72.56, 72.44, 71.45, 69.05, 68.22, 68.04, 67.76, 67.14, 62.25, 60.71, 59.70, 51.36, 50.20, 39.31, 21.72. ESI (M + H^+) calcd for $C_{25}H_{42}N_4NaO_{19}$ 725, found 725.

Compound 2 (GM_3-A). A mixture of compound **8** (20 mg, 27.6 μmol) and 10% Pd/C (10 mg) in MeOH/H_2O (50/50) was hydrogenated at atmospheric pressure for 2 h at room temperature. The reaction mixture was filtered through a Celite pad and the filtrate was evaporated and dissolved in 0.1 M $NaHCO_3$ solution (0.5 mL). Compound **10** (15 mg, 55 μmol, suspended in 0.5 mL of MeOH) was added and the mixture was stirred at room temperature for 1 h, during which time the suspended **10** slowly dissolved, affording a clear solution. After concentration of the reaction mixture, the residue was purified by gel permeation chromatography (Sephadex G-15, 5% *n*-BuOH in H_2O) to give hapten **2** (10 mg, 42%) as a fluffy white solid after lyophilization. ^{1}H NMR (600 MHz, CD_3OD) δ: 6.82 (s, 2H), 4.43 (d, J = 7.5 Hz, 1H), 4.33 (d, J = 7.9 Hz, 1H), 4.05 (dd, J = 9.7, 3.1 Hz, 1H), 3.93−3.43 (m, 23H), 3.27 (t, J = 8.5 Hz, 1H), 2.87 (dd, J = 12.1, 3.8 Hz, 1H, H_{eq} of sialic acid), 2.21 (t, J = 7.5 Hz, 2H), 2.01 (s, 3H), 1.88 (q, J = 7.0 Hz, 2H), 1.73 (t, J = 11.8 Hz, 1H, H_{ax} of sialic acid). ^{13}C NMR (150 MHz, CD_3OD) δ: 175.51, 175.09, 174.93, 172.62, 135.43, 105.05, 104.28, 101.08, 80.79, 77.63, 77.06, 76.46, 76.18, 74.92, 74.74, 72.97, 70.81, 70.06, 69.68, 69.33, 68.96, 64.55, 62.72, 61.85, 53.94, 42.08, 40.64, 38.08, 34.10, 25.66, 22.61. HRMS (M + H^+): calcd for $C_{33}H_{51}N_3O_{22}Na$ 864.2862, found 864.2847.

Compound 12. This compound was prepared using a literature procedure.[19] Neat allylamine (15 mL) was added to the α-D-lactose monohydrate (2.0 g, 5.5 mmol), which gradually became a clear solution. This mixture was stirred at room temperature for 70 h, then

(27) Gilbert, M.; Bayer, R.; Cunningham, A. M.; DeFrees, S.; Gao, Y.; Watson, D. C.; Young, N. M.; Wakarchuk, W. W. *Nat. Biotechnol.* **1998**, *16*, 769−772.

(28) Li, Q.; Chu, D. T. W.; Claiborne, A.; Cooper, C. S.; Lee, C. M.; Raye, K.; Berst, K. B.; Donner, P.; Wang, W.; Hasvold, L.; Fung, A.; Ma, Z.; Tufano, M.; Flamm, R.; Shen, L. L.; Baranowski, J.; Nillius, A.; Alder, J.; Muelbroek, J.; Marsh, K.; Crowell, D.; Hui, Y.; Seif, L.; Melcher, L. M.; Henry, R.; Spanton, S.; Faghih, R.; Klein, L. L.; Tanaka, S. K.; Plattner, J. J. *J. Med. Chem.* **1996**, *39*, 3070−3088.

the solvent was removed by evaporation and the crude white solid was treated with toluene and evaporated three times. The flask was chilled in an ice bath, then pyridine (30 mL) and acetic anhydride (22 mL) were added, and the mixture was allowed to warm to room temperature overnight. The solvent was removed and the residue was taken up in $CHCl_3$, washed with 1 M HCl, 1 M $NaHCO_3$, and brine, and then dried over Na_2SO_4 and concentrated. The crude product was purified by column chromatography ($CHCl_3$/MeOH, 20/1) to give **12** as a white solid (2.1 g, 53%). ^{1}H NMR spectrum matched with that reported in the literature.[19] HRMS (M + Na^+): calcd for $C_{31}H_{43}NO_{18}$ 740.2372, found 740.2342. Mp: 85−90 °C (scinters).

Compound 13. Ozonized oxygen was bubbled into a solution of **12** (1.5 g, 2.1 mmol) in anhydrous MeOH at −78 °C until the blue color persisted (∼30 min). The excess ozonized oxygen was purged with the passage of Ar, then dimethyl sulfide (1 mL) was added at −78 °C, and the reaction mixture was warmed to room temperature by removal of the cooling bath. After 3 h, the solvent was removed by evaporation to give the aldehyde as a white solid, which was used for the subsequent reductive amination without purification. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 9.07 (s, CHO). HRMS (M + Na^+): calcd for $C_{30}H_{41}NO_{19}Na$ 742.2170; found 742.2136.

To compound **15** (0.43 g, 2.1 mmol), sodium cyanoborohydride (0.11 g, 1.7 mmol), and acetic acid (43 μL, 0.7 mmol) in anhydrous MeOH was added the above aldehyde (1.0 g, 1.4 mmol) dissolved in MeOH. After 3 h at room temperature, the solvent was removed by evaporation and the crude compound was directly purified by column chromatography ($CHCl_3$/MeOH, 10/1) to give the peracetylated intermediate as a white solid (0.4 g). HRMS (M + H^+) calcd for $C_{41}H_{58}N_3O_{20}$ 912.3613, found 912.3590. All acetyl groups were then removed using a catalytic amount of sodium methoxide (25wt % solution in methanol) in anhydrous methanol (pH ∼ 10) for 7 h to give **13** as a white solid (0.43 g, 35% from **12**). ^{1}H NMR (600 MHz, CD_3OD) δ: 7.36−7.29 (m, 5H), 5.07 (s, 2H), 4.81 (d, J = 8.8 Hz, 1H), 4.38 (d, J = 7.9 Hz, 1H), 3.92−3.27 (m, 14H), 3.25 (m, 2H), 2.87 (m, 1H), 2.62−2.50 (m, 3H), 2.36 (s, 3H, NCH_3), 2.19 (s, 3H, NAc). ^{13}C NMR (150 MHz, CD_3OD) δ: 175.64, 158.72, 138.33, 129.44, 128.95, 128.81, 105.07, 88.93, 80.37, 78.84, 77.03, 76.56, 74.75, 72.44, 71.28, 70.19, 67.42, 62.41, 61.89, 58.35, 57.09, 42.96, 40.38, 39.02, 22.14. HRMS (M + H^+): calcd for $C_{27}H_{44}N_3O_{13}$ 618.2874, found 618.2893.

Compound 14. This compound was prepared from **13** (20 mg, 32 μmol), **9** (26.5 mg, 49 μmol), and α-2,3-sialyltransferase-CMPNeuAc synthetase (12 U) following the same procedures as described for **8**, affording a fluffy white solid (20 mg, 65%). ^{1}H NMR (600 MHz, CD_3OD) δ: 7.37−7.31 (m, 5H), 5.11 (s, 2H), 4.44 (d, J = 7.9 Hz, 1H), 4.06 (dd, J = 9.7, 3.1 Hz, 1H), 3.92−3.37 (m, 22H), 3.14−3.05 (m, 4H), 2.87 (dd, J = 12.3, 4.4 Hz, 1H, H_{eq} of sialic acid), 2.76 (s, 3H, NCH_3), 2.22 (s, 3H, NAc), 2.02 (s, 3H), 1.74 (t, J = 11.8 Hz, 1H, H_{ax} of sialic acid). ^{13}C NMR (150 MHz, CD_3OD) δ: 174.58 (2), 173.91, 158.10, 137.14, 128.57, 128.16, 127.95, 104.11, 100.09, 87.49, 79.21, 77.89, 76.71, 76.12, 75.66, 73.96, 72.03, 70.34, 69.80, 69.12, 68.26, 68.00, 66.82, 66.74, 63.71, 61.67, 60.44, 57.34, 56.07, 52.99, 42.80, 41.50, 41.08, 21.63, 21.25; HRMS (M + H^+): calcd for $C_{38}H_{60}N_4O_{21}Na$ 931.3648, found 931.3657.

Compound 3 (GM_3-B). This compound was prepared from **14** (18 mg, 19.3 μmol) and **16** (9.6 mg, 38 μmol) using a procedure analogous to the preparation of hapten **2**, affording a fluffy white solid (8 mg, 45%). ^{1}H NMR (600 MHz, CD_3OD) δ: 6.82 (s, 2H), 4.46 (d, J = 7.9 Hz, 1H), 4.06 (dd, J = 9.7, 3.1 Hz, 1H), 3.96−3.48 (m, 24H), 3.26 (m, 4H), 2.87−2.85 (m, 4H), 2.23 (s, 3H), 2.01 (s, 3H), 1.73 (t, J = 11.8 Hz, 1H, H_{ax} of sialic acid). ^{13}C NMR (150 MHz, CD_3OD) δ: 175.51, 175.35, 174.91, 172.78, 172.45, 135.47, 105.05, 101.08, 88.54, 80.33, 78.95, 77.62, 77.13, 76.73, 74.93, 72.98, 71.41, 70.73, 70.06, 69.34, 68.97, 64.56, 62.70, 61.86, 57.48, 55.88, 53.94, 43.87, 42.10, 41.70, 41.54, 35.69, 22.60, 22.11. HRMS (M + H^+): calcd for $C_{36}H_{57}N_5O_{22}Na$ 934.3393, found 934.3396.

Preparation of 4. To a solution of BSA (5 mg/mL) in 50 mM Tris buffer (pH 8.0) was added 2-iminothiolane-HCl (Traut's reagent; Pierce) (18 μL of a 0.25 M solution in H_2O). The mixture was shaken for 1 h at room temperature and then dialyzed twice against phosphate buffer (10 mM, pH 7.4). The solution was transferred to a microcentrifuge tube and hapten **2** (2.3 mg, 2.7 μmol), dissolved in phosphate buffer (100 μL), was added. After shaking for 4 h at room temperature, the mixture was dialyzed three times against phosphate buffer. The average number of hapten molecules **2** attached per molecule BSA was determined to be 17.0 by MALDI-TOF analysis.

Preparation of 5. This conjugate was prepared using hapten **3** and following the same procedure as for **4**. The average number of hapten molecules **3** attached to BSA was 10.2.

Preparation of scFv-phage. Construction of the cancer patient scFv-phage library has been described previously.[9] Approximately 2 × 10^9 cells from the library glycerol stock were used to inoculate 200 mL of Super Broth (SB) medium containing 2% glucose, 100 μg/mL carbenicillin, and 10 μg/mL tetracycline. The culture was shaken at 37 °C until OD_{600} ∼ 0.5, then ∼10^{12} colony-forming units (cfu) of VCSM13 helper phage were added. After a 30 min incubation at room temperature, the culture was diluted in 400 mL of SB (containing carbenicillin and tetracycline) and grown at 30 °C. After 2 h, 70 μg/mL kanamycin and 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) were added, and the culture was allowed to grow overnight. Phage particles were recovered from the culture medium by precipitating with 3% (w/v) NaCl and 4% (w/v) poly(ethyleneglycol) 8000.

Selection of scFv-phage. The library was subjected to four rounds of panning. Immunotubes (Maxisorb, Nunc) were coated overnight at 4 °C with 0.25 mL of 10 μg/mL G_{M3}-A−BSA or G_{M3}-B−BSA in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4). After blocking with BLOTTO (4% nonfat dry milk in PBS) for 1 h at 37 °C, ∼10^{12} cfu of scFv-phage in 2 mL PBS containing 1% milk and 3% BSA were added and incubated for 2 h with rocking at 37 °C. The tube was washed with PBS/ 0.1% Tween and PBS (twice for round 1, five times for round 2, and 20 times for subsequent rounds). Bound phage were eluted from the tube with 0.25 mL of 0.1 M glycine (pH 2.2) and neutralized with 15 μL of 2 M Tris base. Eluted phage were amplified by infecting fresh *E. coli* XL-1 Blue cells (Stratagene) and rescued as outlined in the preceding section. An initial panel of scFv-phage clones was selected using ELISA.

ELISA Methods. Relative affinities and specificity of scFv-phage and soluble scFvs were evaluated by ELISA. G_{M3}-A−BSA and G_{M3}-B−BSA were applied to a microtiter plate at a concentration of 5 μg/mL in PBS at 4 °C overnight. Unmodified BSA (5 μg/mL) was used as a negative control. After being washed three times with water, the wells were blocked with BLOTTO for 1 h at 37 °C. Following additional washing, for phage ELISA, 25 μL of scFv-phage suspension was added to each well, and the plate was incubated 1 h at 37 °C. After washing, 25 μL horseradish peroxidase (HRP)-conjugated anti-M13 mAb (Amersham Pharmacia) was then added for 30 min at 37 °C. For ELISA using soluble scFvs, 25 μL of purified scFv was added to each well, and the plate was incubated 1 h at 37 °C. Then, after washing, 25 μL of HRP-conjugated anti-Flag M2 mAb (Sigma) was added for 30 min at 37 °C. For detection, 50 μL/well of tetramethylbenzidine (TMB) substrate (Pierce) was added and the plate was incubated at room temperature. After adding 50 μL of 2 M H_2SO_4 to each well, the absorbance was read at 450 nm. Sequencing of clones was performed using an Applied Biosystems 377 automated DNA sequencer, and sequences were analyzed using the MacVector program and DNA Plot (http:// www.dnaplot.de).

Expression and Purification of scFvs. Three representative scFv G_{M3}-A clones (GM3A6, GM3A8, and GM3A15) were ligated into the expression vector pETFlag (derived from pET-15b, Novagen) and transformed into *E. coli* BL21 cells (Stratagene). Expression of soluble scFv was induced by growth of each culture overnight at 30 °C in SB containing 0.5 mM IPTG. The Flag-tagged scFvs were purified from

the growth media using anti-Flag M2 mAb affinity agarose (Sigma), with 0.1 M glycine (pH 2.7) as eluant. The yield of purified scFv typically varied from 0.3 to 3 mg/L, depending on the clone.

BIAcore Analysis. Binding kinetics was determined by surface plasmon resonance using a BIAcore 2000 instrument (Pharmacia).[29] Hapten **2** was immobilized on a CM5 sensor chip using the BIAcore surface thiol method, per the manufacturer's instructions. All measurements were conducted in HEPES-buffered saline with a flow rate of 10 μL/min. After each measurement, the chip surface was regenerated with 10 μL of 1 mM HCl.

Flow Cytometry. We used flow cytometry to analyze antibody reactivity toward three human melanoma cell lines: HMV-1, a poorly metastatic cell line;[22] M21, which is moderately metastatic;[23] and C-8161, a highly metastatic cell line.[24] We also tested three human breast cancer cell lines that were established from circulating metastatic cells isolated from peripheral blood samples of stage IV breast cancer patients. Blood samples were incubated with supramagnetic beads (Dynal) coated with an antibody against a human epithelial antigen (mAb Ber-Ep4). Tumor cells were recovered and expanded in culture. The cell lines established from these cultures, BCM-1, BCM-2, and BMS, exhibit adhesive, invasive, and migratory properties of highly metastatic tumor cells and will be described elsewhere. Normal human dermal fibroblast (HDFa) cells (Cascade Biologics, Inc.) were used as a nonmalignant cell control. The cells were harvested in PBS/EDTA and incubated with an anti-G_{M3} scFv (10 μg/mL in 0.5% BSA/PBS), or only 0.5% BSA/PBS without scFv as a negative control, for 45 min on ice. After washing twice in PBS/BSA and blocking with normal goat serum (10 min at 22 °C), the cells were incubated with anti-Flag M2 mAb (10 μg/mL) (Sigma) in PBS/BSA on ice for an additional 30 min. Finally, the cells were washed twice and incubated with FITC-labeled anti-mouse goat $F(ab')_2$ (Pierce) for 30 min on ice. After washing and counter-staining with propidium iodide (2 μg/mL) to identify and exclude dead cells, the samples were analyzed in a FACScan flow cytometer (Becton Dickinson).

(29) (a) Medaglia, M. V.; Fisher, R. J. *Protein−Protein Interact.* **2002**, 255−272. (b) Roder, U. W.; Markey, F. *Methods Mol. Med.* **1998**, *13*, 531−554. (c) Malmborg, A.-C.; Michaëlsson, A.; Ohlin, M.; Jansson, B.; Borrebaeck, C. A. K. *Scand. J. Immunol.* **1992**, *35*, 643−650. (d) Jönsson, U.; Fägerstam, L.; Ivarsson, B.; Johnsson, B.; Karlsson, R.; Lundh, K.; Löfås, S.; Persson, B.; Roos, H.; Rönnberg, I.; Sjölander, S.; Stenberg, B.; Ståhlberg, R.; Urbaniczky, C.; Östlin, H.; Malmqvist, M. *BioTechniques* **1991**, *11*, 620−627.

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Supporting Information Available: Copies of the ^{1}H NMR and ^{13}C NMR spectra for all prepared compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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