Efficient Metabolic Engineering of GM3 on Tumor Cells by *N*-Phenylacetyl-D-mannosamine†

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ABSTRACT: Abnormal carbohydrates expressed on tumor cells, which are termed tumor-associated carbohydrate antigens (TACAs), are potential targets for the development of cancer vaccines. However, immune tolerance to TACAs has severely hindered progress in this area. To overcome this problem, we have developed a novel immunotherapeutic strategy based on synthetic cancer vaccines and metabolic engineering of TACAs on tumor cells. One critical step of this new strategy is metabolic engineering of cancer, namely, to induce expression of an artificial form of a TACA by supplying tumors with an artificial monosaccharide precursor. To identify the proper precursor for this application, *N*-propionyl, *N*-butanoyl, *N*-isobutanoyl, and *N*-phenylacetyl derivatives of D-mannosamine were synthesized, and their efficiency as biosynthetic precursors in modifying sialic acid and inducing expression of modified forms of GM3 antigen on tumor cells was investigated. For this purpose, tumor cells were incubated with different *N*-acyl-D-mannosamines, and modified forms of GM3 expressed on tumor cells were analyzed by flow cytometry using antigen-specific antisera. *N*-Phenylacetyl-D-mannosamine was efficiently incorporated in a time- and dose-dependent manner to bioengineer GM3 expression by several tumor cell lines, including K562, SKMEL-28, and B16-F0. Moreover, these tumor cell lines also exhibited ManPAc-dependent sensitivity to cytotoxicity mediated by anti-PAcGM3 immune serum and complement. These results provide an important validation for this novel therapeutic strategy. Because *N*-phenylacetyl GM3−protein conjugates are particularly immunogenic, the combination of an *N*-phenylacetyl GM3 conjugate vaccine with systemic *N*-phenylacetyl-D-mannosamine treatment is a promising immunotherapy for future development and application to melanoma and other GM3-bearing tumors.

Sialic acids are unique nine-carbon acidic monosaccharides ubiquitously distributed in higher animals and humans (*1*). The most common form of sialic acids is *N*-acetyl-D-neuraminic acid (Neu5Ac,[1] also known as *N*-acetylsialic acid). Neu5Ac is a fascinating sugar in that it usually appears at the nonreducing end of cell surface glycans and thus plays forefront roles in various biological functions such as cell proliferation, immune recognition, and pathogenic invasion (*2*−*8*).

It is also well established that numerous tumors overexpress Neu5Ac, and the altered cell surface carbohydrate profiles are closely correlated with carcinogenesis and tumor metastasis (*9*−*12*). Abnormal glycans expressed on tumor cells, termed tumor-associated carbohydrate antigens (TACAs), are important molecular templates for the design of new cancer therapies (*13*), such as cancer vaccines (*14*−*20*). Since the early 1970s, a plethora of TACAs (*10*, *14*, *15*, *17*, *21*), many of which are sialylated oligosaccharides such as GM3 antigen (*22*), have been characterized, but only a limited number exhibit promise for cancer vaccine development (*14*, *23*−*33*). The majority, including the TACAs that are most abundantly expressed on tumors, fail to induce immune responses in cancer patients. The problem of immune tolerance reflects the prior survival of tumors within patients and suggests that these TACAs are not good targets for immunotherapy of cancer. Traditional approaches to improving the immunogenicity of carbohydrate antigens, such as conjugation with a carrier molecule and/or combination with a potent adjuvant, proved to be successful with only a few TACAs (*14*, *24*−*33*). Thus, immune tolerance to TACAs remains a major obstacle in the development of carbohydrate-based cancer vaccines.

To overcome this problem, we have explored a new strategy for cancer immunotherapy (Figure 1) (*34*). The first step is to immunize cancer patients with a vaccine consisting

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[1] Abbreviations: BuGM3, *N*-butanoyl GM3; Gal*p*, D-galactopyranose; Glc*p*, D-glucopyranose; GM3, Neu5Acα(2→3)Gal*p*β(1→4)Glc*p*, a tumor-associated antigen; ^{i}BuGM3, *N*-isobutanoyl GM3; KLH, keyhole limpet hemocyanin; MFVs, mean fluorescence values; ManNAc, *N*-acetyl-D-mannosamine; ManNBu, *N*-butanoyl-D-mannosamine; ManNiBu, *N*-isobutanoyl-D-mannosamine; ManNPAc, *N*-phenylacetyl-D-mannosamine; ManNPr, *N*-propionyl-D-mannosamine; ManNR, unnatural *N*-acyl-D-mannosamine; mAbs, monoclonal antibodies; Neu5Ac, *N*-acetyl-D-neuraminic acid; Neu5PAc, *N*-phenylacetyl-D-neuraminic acid; Neu5R, unnatural *N*-acyl-D-neuraminic acid; NMS, normal mouse serum; PAcGM3, *N*-phenylacetyl GM3; PBS, phosphate-buffered saline; PrGM3, *N*-propionyl GM3; TACAs, tumor-associated carbohydrate antigens.

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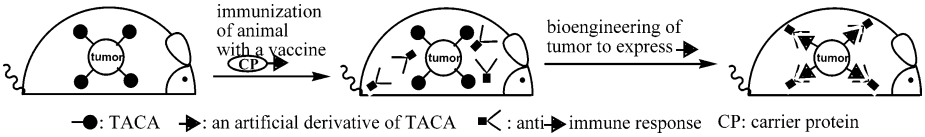

FIGURE 1: New strategy for cancer immunotherapy based on metabolic engineering of tumor cells.

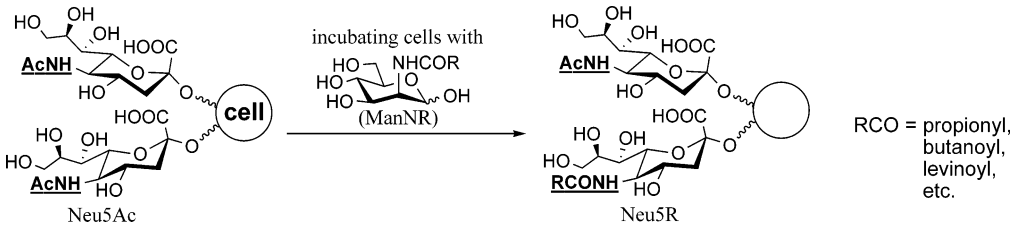

FIGURE 2: Metabolic engineering of cell surface Neu5Ac with *N*-acyl-D-mannosamine derivatives as biosynthetic precursors.

of an artificial derivative of a natural TACA. After a specific immune response to the artificial antigen is established, the patients are treated with the correspondingly modified monosaccharide that can serve as a biosynthetic precursor of the artificial oligosaccharide TACA. This treatment will initiate the expression of the artificial antigen in place of the natural one on tumor cells that will then be recognized by antibodies specific to the artificial antigen.

For the strategy to work, it has to meet two conditions. First, there must be a synthetic vaccine that can induce a specific immune response in cancer patients. Second, there must be an effective method for bioengineering of cancer cells to achieve expression of the artificial antigen.

The first condition may be addressed by chemically modifying carbohydrate antigens to increase their immunogenicity (*35*−*40*). The second condition may be addressed by a unique and powerful glycoengineering technique, namely, metabolic engineering of cell surface Neu5Ac, pioneered by Reutter and co-workers (*41*, *42*) and significantly expanded and generalized by the brilliant work of Bertozzi and co-workers (*43*−*47*). This technique takes advantage of the remarkable substrate permissibility of carbohydrate biosynthetic enzymes. The natural biosynthetic precursor of Neu5Ac is *N*-acetyl-D-mannosamine (ManNAc). By providing cells with an *N*-acyl derivative of D-mannosamine (ManNR), which can compete with ManNAc, we can force cells to express oligosaccharides that contain the unnatural sialic acid (Neu5R) (Figure 2). A number of artificial sialic acids have been delivered onto a cell surface by this technique (*41*, *42*, *46*−*54*).

Antigenicity of the artificial TACA derivatives may be enhanced by differences between their structures and the structure of the natural TACA, but for use in metabolic engineering, the N-modified mannosamine must be an acceptable substrate for enzymes involved in the biosynthesis of Neu5Ac and the relevant sialo-TACA. In our first proof-of-concept experiment (*34*), we modified the mannosamine precursor and vaccine with a propionyl group, an acyl group that is only one carbon longer than the acetyl group of the natural ManNAc and Neu5Ac. Jennings et al. (*55*) adopted the same design in their recent report targeting GD3. Despite promising results of these studies, modification with a propionyl group may not be the ideal approach, especially for vaccine design. To identify an optimized structure for both metabolic engineering and vaccine development, it is necessary to systematically study and compare the immunogenicity of an array of artificially modified sialo-TACAs as well as the efficiencies that correspondingly modified D-mannosamine derivatives achieve for bioengineering of tumor cells.

Since we have already demonstrated that a number of *N*-acylsialic acids and sialo-TACAs are more immunogenic than the *N*-acetyl and *N*-propionyl counterparts (*39*, *40*), this research investigates the efficiency of correspondingly modified D-mannosamines in metabolically engineering sialo-TACAs on tumor cells. For this purpose, several *N*-acyl derivatives of D-mannosamine were synthesized and used to treat different tumor cell lines. Expression of artificially modified forms of GM3, a prominent sialo-TACA of many tumors (*27*), was assessed on tumor cells by flow cytometry to test the results of metabolic engineering.

MATERIALS AND METHODS

Materials. Various *N*-acyl-D-mannosamines, including ManNPr, ManNBu, ManNiBu, and ManNPAc, were synthesized by a chemical synthesis method described previously (*56*). Tumor cell lines were purchased from American Type Tissue Culture (Manassas, VA).

Cell Culture. Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) supplemented with 1 mM sodium pyruvate, 10 mM HEPES, and 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) for K562 and B16-F0 cells or 20% FCS for SKMEL-28 cells.

Antiserum Preparation. Six C57BL/6 mice were immunized with KLH conjugates of PrGM3, BuGM3, ^{i}BuGM3, or PhAcGM3, containing 2 μg of carbohydrate (loading rate of 11−14%) in TDM+MPL (Ribi) adjuvant (Sigma, St. Louis, MO) (*40*). Mice were immunized by intraperitoneal injection on day 0, 14, 21, and 28. Mice were bled from the post-ocular venus plexus on day 35, and blood samples were clotted to obtain antisera. Sera from all six mice in a group were pooled and frozen at −80 °C. Control serum (termed NMS) was obtained from the same mice in a preimmunization bleed or was obtained from Jackson Laboratories (Bar Harbor, ME); similar results were obtained using NMS from either source.

Metabolic Labeling and Flow Cytometry Analysis. Cells were incubated with various concentrations (0, 10, 20, and 40 μM) of *N*-acyl derivatives of D-mannosamine, harvested at different time points (0−96 h), washed in FACS buffer [phosphate-buffered saline (PBS) containing 1% FCS and

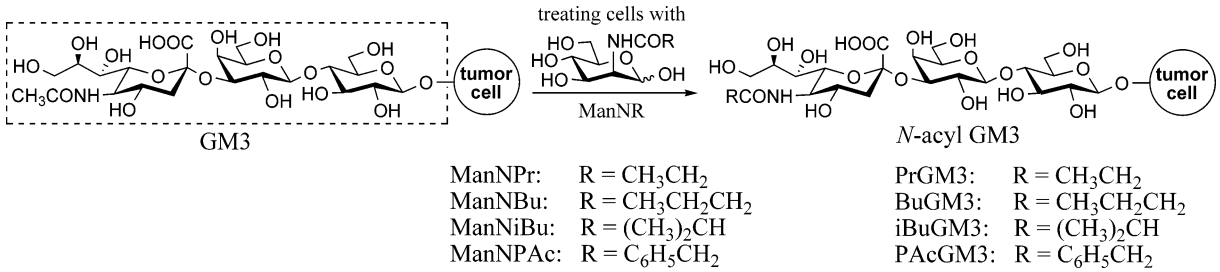

FIGURE 3: Metabolic engineering of GM3 on tumor cells.

0.1% bovine serum albumin], and incubated on ice for 15 min in normal mouse serum (NMS) diluted 1:10 in FACS buffer. Cells were pelleted, resuspended, and incubated for 60 min on ice in NMS or antiserum diluted 1:10 in FACS buffer. Antiserum was pooled from mice immunized with the corresponding N-modified GM3 conjugated to keyhole limpet hemocyanin (KLH). Cells were washed two times with FACS buffer and incubated for 30 min on ice with 7 μg/mL FITC-conjugated goat anti-mouse IgG heavy and light chain antibody. Cells were washed and fixed, and cytometry was performed on a Becton Dickenson FACScan flow cytometer. Data are presented as raw histograms or geometric mean fluorescence values (MFVs) derived from 12 000 events.

Cytotoxicity Assay. SKMEL-28, K562, and B16-F0 cells were cultured for 72 h in the presence of 40 μM ManNPAc. Cells were washed, incubated for 1 h at 4 °C with a 1:10 dilution of anti-NPAcGM3 immune serum, washed, and incubated for 1 h at 37 °C with 1:5 to 1:40 dilutions of rabbit serum (Cedarlane, Hornby, ON) as a source of complement. The cells were diluted 1:1 with 0.4% trypan blue (Sigma). Trypan blue positive and total cell counts were determined by microscopic examination, and the percent cytotoxicity was calculated as (trypan blue positive cells)/(total cells).

RESULTS AND DISCUSSION

GM3 is a trisaccharide TACA, Neu5Acα(2→3)Gal*p*β-(1→4)Glc*p*, with a Neu5Ac residue attached to the nonreducing end (*9*, *10*). It exists in gangliosides or glycoproteins with the lipid tails or peptide chains inserting into cell membranes, and the trisaccharide is displayed on the cell surface, where it can be recognized by antibodies. GM3 is abundantly expressed on a number of tumors (*27*), especially malignant melanoma. Therefore, GM3 represents a potential target for immunotherapy of cancers, but immune tolerance to native GM3 is a problem. According to the strategy proposed above to deal with the problem (*34*), we intend to metabolically engineer GM3 by use of artificial *N*-acyl derivatives of D-mannosamine to induce expression of artificial *N*-acyl derivatives of GM3 on tumor cells (Figure 3). The studies reported here were designed to test the efficiency with which GM3-bearing tumor cells incorporate different *N*-acyl derivatives of D-mannosamine and form GM3 derivatives having new engineered antigenic epitopes that may potentially serve as molecular targets for cancer immunotherapy.

Metabolic engineering of cancer cells was tested with several artificial *N*-acyl analogues of ManNAc, including *N*-propionyl-D-mannosamine (ManNPr), *N*-butanoyl-D-mannosamine (ManNBu), *N*-isobutanoyl-D-mannosamine (ManNiBu), and *N*-phenylacetyl-D-mannosamine (ManNPAc). The correspondingly N-modified GM3 antigens were thus *N*-propionyl GM3 (PrGM3), *N*-butanoyl GM3 (BuGM3), *N*-isobutanoyl GM3 (^{i}BuGM3), and *N*-phenylacetyl GM3 (PAcGM3) (Figure 3). These *N*-acyl derivatives were selected because we recently demonstrated that GM3 trisaccharides modified with these *N*-acyl groups are immunogenic when coupled to a protein carrier, making them promising candidates for development as cancer vaccines (*40*). In addition, ManNPr, ManNBu, ManNiBu, and ManNPAc were also proven to be more efficient than some other D-mannosamine derivatives as substrates of sialic acid aldolase (*56*), an enzyme that may bypass the biosynthetic bottleneck in the metabolic engineering of Neu5Ac on cell surfaces (*57*).

Flow cytometry was used to detect the metabolic engineering of GM3 on tumor cell surfaces. Antisera specific for each N-modified GM3 were prepared as described previously (*40*). Briefly, PrGM3, BuGM3, ^{i}BuGM3, and PAcGM3 were covalently coupled with KLH, and the resultant glycoconjugates were utilized to immunize groups of six C57BL/6 mice. Blood samples were pooled for each group and used to prepare antisera. Antigen-specific antibody titers were analyzed by an ELISA with GM3 derivatives conjugated to human serum albumin (HSA) as capture antigens. Antigen-specific antibodies were mainly of the IgG isotype (*40*). The specificity of these antisera reflects overall immune responses to GM3 trisaccharide derivatives, analogous to in vivo responses that would be achieved by active immunotherapy. Therefore, this approach may provide insights into future clinical bioengineering of GM3 combined with active immunotherapy (Figure 1). A future alternative would be development of monoclonal antibodies (mAbs) with specificity for appropriate target antigens; these mAbs could be used for passive immunotherapy approaches. In addition, mAbs would provide greater definition of specificity and control of cross-reactivity than is achieved with antisera. Nevertheless, the antisera used in these studies provided specificity to distinguish the different N-modified GM3 trisaccharides.

Among the four *N*-acyl derivatives of mannosamine mentioned above, ManNPAc was our first focus. ManNPAc is the best substrate for sialic acid aldolase (*56*), and the PAcGM3 conjugate vaccine produced strong and specific antibody responses (*40*). Incorporation of ManNPAc into GM3 biosynthesis was studied using SKMEL-28 human melanoma cells as a tumor model, since GM3 is a prominent antigen on melanoma cells (*22*). SKMEL-28 cells were incubated in medium containing 10, 20, or 40 μM ManNPAc, harvested at various time points, and assessed for PAcGM3 expression by flow cytometry with antiserum generated against PAcGM3-KLH and FITC-labeled goat anti-mouse secondary antibody. Incubation of SKMEL-28 cells with 40

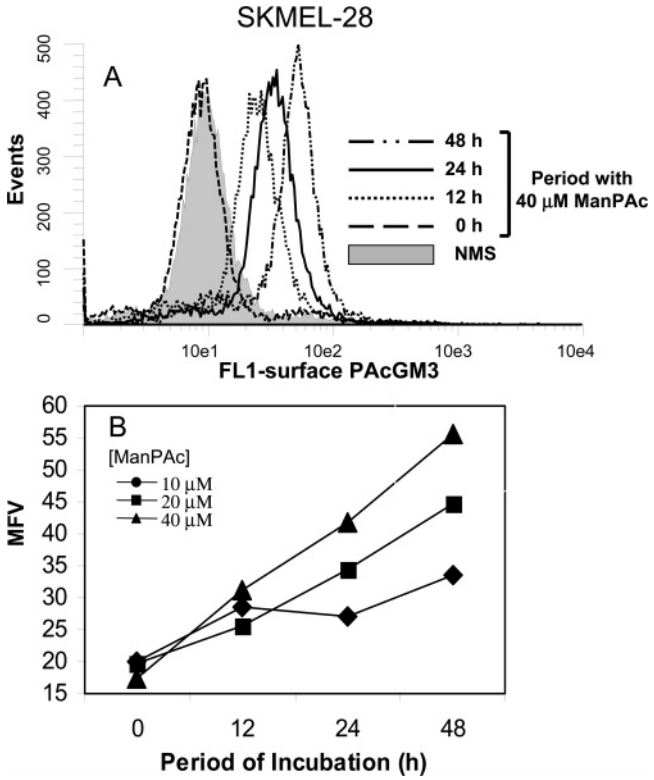

FIGURE 4: Metabolic engineering of SKMEL-28 cells using ManNPAc induces expression of PAcGM3 on the cell surface. SKMEL-28 cells were cultured with 10, 20, or 40 μM ManNPAc for various periods, stained with anti-serum to PAcGM3, labeled with FITC-labeled goat anti-mouse antibody, and analyzed by flow cytometry. (A) Expression of PAcGM3 after incubation with 40 μM ManNPAc for various periods. (B) MFVs after incubation of SKMEL-28 cells with 10, 20, or 40 μM ManNPAc. Nonspecific background (with NMS instead of specific antiserum, as in panel A) is not subtracted in panel B but was similar to the signal at 0 h (no incubation with mannosamine) as in panel A. PAcGM3-specific labeling is indicated by the increase in the MFV with mannosamine incubation (relative to 0 h).

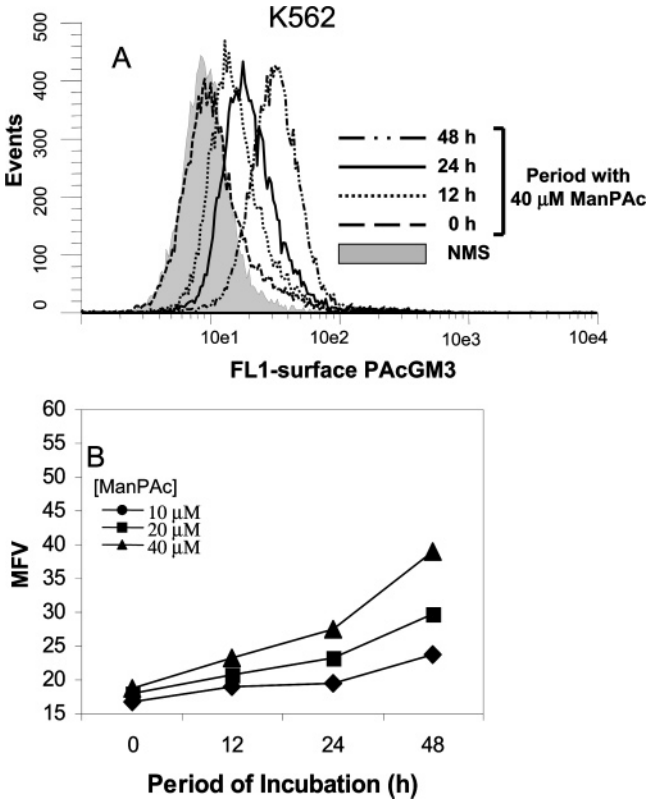

FIGURE 5: Expression of PAcGM3 on K562 cells. K562 cells were cultured with 10, 20, or 40 μM ManNPAc and analyzed for PAcGM3 expression by flow cytometry. (A) Expression of PAcGM3 after incubation of K562 cells with 40 μM ManNPAc for different time periods. (B) MFVs for PAcGM3 expression after incubation of K562 cells with different concentrations of ManNPAc. Nonspecific background (with NMS instead of specific antiserum, as in panel A) is not subtracted in panel B but was similar to the signal at 0 h (no incubation with mannosamine) as in panel A. PAcGM3-specific labeling is indicated by the increase in the MFV with mannosamine incubation (relative to 0 h).

μM ManNPAc resulted in expression of PAcGM3 that was detected by 12 h and increased after 24−48 h (Figure 4A). Longer incubation for up to 96 h further increased the MFV of PAcGM3 expression to a plateau level (data not shown), but substantial labeling was already observed at 48 h with >83% of SKMEL-28 cells showing labeling for PAcGM3 above the negative control (SKMEL-28 cells with no ManPAc incubation). Incubation with 10−20 μM ManNPAc or for shorter time periods produced a lower level of labeling (Figure 4B). These studies established conditions for effective metabolic engineering of SKMEL-28 cells for expression of PAcGM3 (incubation with 40 μM ManPAc for 48−96 h). Overall, these results demonstrate that SKMEL-28 cells efficiently use ManNPAc to biosynthesize *N*-phenylacetyl-D-neuraminic acid (Neu5PAc) and incorporate Neu5PAc into GM3, resulting in cell surface expression of PAcGM3.

Results of studies with SKMEL-28 demonstrated metabolic engineering of GM3 in a melanoma cell line, but we wished to test the general applicability of this approach in GM3-expressing cells from a different type of tumor. Accordingly, we tested the capacity of ManNPAc for metabolic engineering of K562 cells, a human leukemia cell line that also expresses GM3 (*58*). As with SKMEL-28 cells, incubation with ManNPAc induced cell surface expression of PAcGM3 by K562 cells (Figure 5). The level of PAcGM3 expression was dependent upon incubation time and ManNPAc concentration. Substantial labeling was observed after incubation for 48 h in 40 μM ManNPAc, which resulted in positive staining for PAcGM3 on >83% of the K562 cells. Thus, results with K562 and SKMEL-28 cells were similar, although the MFV for PAcGM3 expression was lower for K562 cells. This difference may relate to differences in GM3 expression levels, rates of GM3 synthesis and turnover, rates of cell proliferation, or the efficiency of ManNPAc uptake and incorporation into GM3.

We studied several other cell lines to further generalize our findings and provide important controls. These cell lines included another GM3-expressing cell line, B16-F0 (*22*) (a murine melanoma cell line), and three tumor cell lines that have not been reported to express GM3, RMA (a murine lymphoma cell line) and CCL 53.1 and CRL 6322 (both murine melanoma cell lines). Like the findings described above, but to a lesser extent, following incubation with ManNPAc for various times and concentrations, PAcGM3 was expressed by B16-F0 cells (Figure 6). This was not true for RMA, CCL 53.1, or CRL 6322 cells incubated with 40 μM mannosamine precursor for 96 h (data not shown). The lack of PAcGM3 expression on RMA, CCL 53.1, and CRL 6322 cells may be due to insufficient expression of GM3 for metabolic engineering. We conclude that ManNPAc can

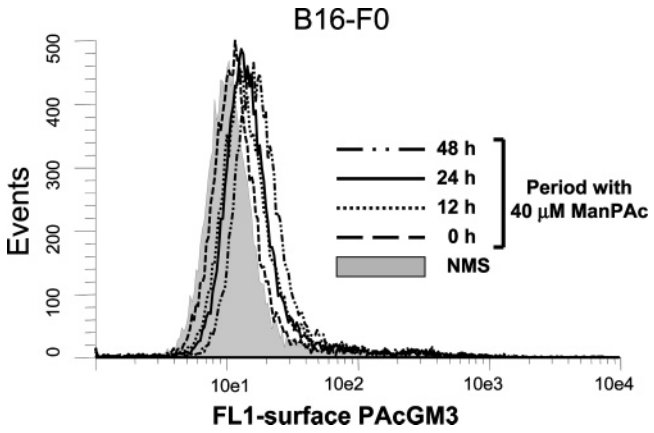

FIGURE 6: Metabolic engineering of surface PAcGM3 on B16-F0 cells. B16-F0 cells were cultured with 40 μM ManNPAc and analyzed for PAcGM3 expression by flow cytometry for different time periods.

be used for efficient metabolic engineering of GM3 on multiple types of GM3-positive tumor cells.

The negative results with some cell lines also indicate the specificity of staining with the PAcGM3 antiserum. RMA cells are known to express a high level of Neu5Ac and can take up and incorporate modified forms of mannosamine to express artificial sialic acids on the cell surface (*34*). If the PAcGM3 antiserum detected Neu5PAc generated by incorporation of ManNPAc, we would have observed an increased level of staining of ManNPAc-treated RMA cells with this antiserum. The lack of staining under these conditions indicates that the PAcGM3 antiserum specifically recognized the full PAcGM3 epitope but not its partial structures, such as PAc-modified sialic acid by itself.

Additional studies compared the efficiency of GM3 metabolic engineering by other derivatives of D-mannosamine. SKMEL-28 or K562 cells were incubated for 0−96 h in medium containing 40 μM ManNPr, ManNBu, ManNiBn, or ManNPAc. The cells were then stained with different antisera specific for each of the corresponding modified GM3 trisaccharides and analyzed by flow cytometry. In contrast to the results with ManNPAc, the other mannosamine precursors (ManNPr, ManNBu, and ManNiBn) did not induce significant expression of modified GM3 (Figure 7). We conclude that ManNPr, ManNBu, and ManNiBn are not efficient candidates for metabolic engineering of tumor cells in our strategy for cancer immunotherapy. These studies examined metabolic engineering with a maximum substrate concentration of 40 μM, and it is possible that higher levels of ManNPr, ManNBu, or ManNiBn could produce detectable levels of modified GM3. Other in vitro studies have used precursor concentrations of up to 3−20 mM for metabolic engineering, and these conditions result in incorporation of ManNPr and ManNBu by tumor cells (*34*, *53*, *55*). However, such high concentrations may be difficult to achieve in vivo. Since our goal was to investigate bioengineering of cancer cells under conditions closer to those that may be achieved in vivo, we used lower substrate concentrations, at which ManNPr, ManNBu, and ManNiBn were not effective. We conclude that ManNPAc is the most effective of the four D-mannosamine derivatives for metabolic engineering of cell surface GM3.

Different results with ManNPr and ManNBu also may be explained by differences in detection methodology. In our study, antisera were used to reflect the overall immune reactions, but a previous study used mAbs specific for modified TACAs (*34*). We used antisera with four different specificities, each of which had a potential to cross-react with the natural form of GM3, which would increase the level of background binding. A small quantity of modified GM3 expressed on tumor cells might not be detectable against this background. Indeed, we noticed that the level of background binding to SKMEL-28 and K562 cells was higher with PrGM3, BuGM3, and ^{i}BuGM3 antisera than with the PAcGM3 antiserum, consistent with our immunological studies of the corresponding glycoconjugate vaccines (*40*). There we reported cross-reactions of natural GM3 with PrGM3, BuGM3, and ^{i}BuGM3 antisera but not the PAcGM3 antiserum. This may also explain why we consistently observed a small decrease in the level of surface binding of the PrGM3, BuGM3, and ^{i}BuGM3 antisera following mannosamine precursor treatment of SKMEL-28 and K562 cells (Figure 7). We hypothesize that the mannosamine precursors may be less efficient substrates for biosynthetic enzymes or may even act as inhibitors to suppress the overall production of GM3 as previously observed in the case of polysiaclic acid biosynthesis (*59*), reducing the level of background staining that reflects the cross-reactivity of the antisera with

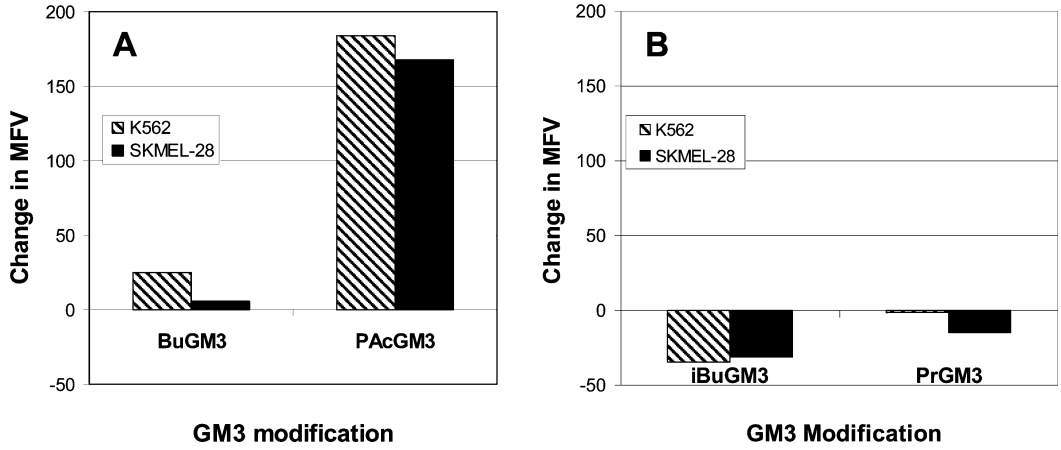

FIGURE 7: Metabolic engineering of GM3 antigens on SKMEL-28 and K562 cells by ManNPr, ManNBu, ManNiBn, and ManNPAc. Tumor cells were cultured for 96 (A) or 72 h (B) with 40 μM ManNPr, ManNBu, ManNiBn, or ManNPAc. Cells were stained with antisera specific for PrGM3, BuGM3, ^{i}BuGM3, and PAcGM3, respectively, and analyzed by flow cytometry.

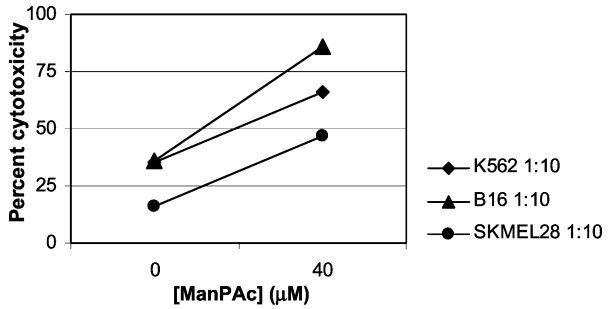

FIGURE 8: Incubation of tumor cells with ManPAc induces sensitivity to cytotoxicity mediated by anti-GM3PAc immune serum and complement. K562, B16-F0, and SKMEL 28 cells were cultured for 72 h with 40 μM ManNPAc. Cells were incubated with anti-PAcGM3 immune serum, washed, and incubated for 1 h with rabbit serum (1:10) as a source of complement. Cytotoxicity was assessed by microscopy to determine the level of trypan blue exclusion. The percent cytotoxicity was defined as (number of trypan blue-positive cells)/(total number of cells).

native GM3, but this hypothesis remains to be verified. In summary, we did not detect metabolic engineering of GM3 on K562 or SKMEL-28 cells with ManNPr, ManNBu, or ManNiBn, although these substrates might be incorporated with low efficiency, possibly detectable at higher substrate concentrations or with more sensitive detection systems. Our results with ManNPAc, however, demonstrate efficient metabolic engineering of GM3. This result, the immunogenicity of PAcGM3, and the ability of PAcGM3 to induce antibodies that do not cross-react with native GM3 all establish ManNPAc as an excellent candidate for metabolic engineering of tumor cells.

Since the goal of metabolic engineering of cell surface TACAs is to create immune recognition with the potential to kill tumor cells, we then investigated the ability of ManPAc to sensitize cells to cytotoxicity mediated by anti-PAcGM3 immune serum and complement. SKMEL28, K562, and B16-F0 cells were incubated with or without ManPAc (40 μM). All three tumor cell lines exhibited ManPAc-dependent sensitivity to cytotoxicity mediated by anti-PAcGM3 serum and complement (Figure 8). These results indicate that engineered PAcGM3 structures on the surface of tumor cells can provide for specific immune recognition by antibodies and result in complement-mediated lysis of tumor cells, which is under further detailed investigation in our lab.

In conclusion, this work has demonstrated that tumor cells take up ManNPAc, use it to biosynthesize Neu5PAc, and incorporate Neu5PAc into sialo-TACAs, such as GM3. Therefore, ManNPAc can be utilized to metabolically engineer tumor cells, including K562, SKMEL-28, and B16-F0 cells. In contrast, at the tested concentrations, ManNPr, ManNBu, and ManNiBn failed to induce the level of modified GM3 that could be detected with the antigen-specific antisera used in these studies, the reason for which is not certain and is now under further investigation. Despite the steric bulk of a phenylacetyl group, ManNPAc was permissible by enzymes involved in the biosynthesis of Neu5Ac and sialoglycoconjugates, as well as sialic acid aldolase (*56*). This suggests that the enzymes may have a cavity adjacent to the catalytic reaction center to accommodate the large phenyl group. Meanwhile, we have demonstrated that PAcGM3−protein conjugate vaccines are more immunogenic and induce more specific immune responses than conjugate vaccines with other *N*-acyl derivatives of GM3 investigated so far (*40*). These results suggest that immunotherapy based on the combination of metabolic engineering with ManNPAc and immunization with an NPAcGM3-based vaccine has promise to be developed into a useful therapy for melanoma and other GM3-bearing tumors. Moreover, because Neu5Ac is significantly overexpressed on a number of tumors and numerous TACAs contain Neu5Ac at their nonreducing ends (*11*), ManNPAc may be also applicable to the immunotherapy of other tumors with sialooligosaccharide TACAs as metabolic engineering targets.

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