Biochemical Engineering of Surface α2–8 Polysialic Acid for Immunotargeting Tumor Cells*

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To target tumor cells for immunotherapy, we evaluated the feasibility of altering the epitopes on the surface polysialic acid of tumor cells. A precursor (*N*-propionylmannosamine), when incubated with leukemic cells, RBL-2H3 and RMA, resulted in substitution of the *N*-acetyl groups of surface α2–8 polysialic acid with *N*-propionyl groups. Expression of the altered α2–8 *N*-propionylpolysialic acid on the surface of tumor cells induced their susceptibility to cell death mediated by monoclonal antibody 13D9 (mAb 13D9), which specifically recognizes α2–8 *N*-propionylated polysialic acid. The expression of α2–8 *N*-propionylated polysialic acid and the lysis of tumor cells by antibody-dependent cytotoxicity depended on the time and dose of incorporation of *N*-propionylated mannosamine. *In vivo*, mAb 13D9 effectively controlled metastasis of leukemic cells RMA when mice were administered the precursor *N*-propionylated mannosamine.

Sialic acid is ubiquitous on the surface of eukaryotic cells, where as a glycoconjugate substituent, it is involved in a number of crucial biological processes (1). The permissiveness of the enzymes involved in sialic acid biosynthesis and sialoside formation (2–5) have been exploited for the bioengineering of cell surface molecules. This strategy was first reported by Reutter and co-workers (6, 7), who demonstrated that exposing mammalian cells in tissue culture and *in vivo,* to different *N*-acyl-mannosamine precursors, resulted in the expression of the unnatural *N*-acylated sialic acid residues on the cell surface glycoconjugates. This technique was used by the authors to study the effect of cell surface sialoside structural changes on viral receptors (7, 8).

More recently, Bertozzi and co-workers (9) have exploited this enzymatic permissiveness further by successfully using *N*-levulinoylmannosamine as the precursor to introduce *N*-levulinoylsialic residues on the surface of a number of human cell lines. This procedure introduces unique active keto groups on the surface of the cells, which via the use of appropriate chemical reagents, can be used for the chemotargeting of drugs.

We now report the successful application of the enzymatic permissiveness of sialic acid to the immunotargeting of cancer cells and the potential of our protocol to further the development of efficacious carbohydrate-based vaccines. Although some success has been reported (10) in creating cancer vaccines based on cell surface glycoconjugate antigens, the area remains problematic due to the fact that cancer cells fail to produce markers that distinguish them from normal cells. Population densities of cell surface carbohydrate antigens of cancer cells do differ from those of normal cells, but their individual structures are identical. Thus glycoconjugate vaccines based on these antigens are poorly immunogenic. Therefore we propose to introduce modified carbohydrate antigens on the surface of cancer cells to which a strong immunogenic response can be induced. We chose α2–8 polysialic acid (polysialic acid) as our target antigen, because although not a universal cancer antigen, it is found on a number of important cancers (11–13), and there is strong evidence that it is associated with metastasis (12, 14). In addition we have previously demonstrated that in its *N*-propionylated form (NPr polysialic acid)[1] it is an excellent immunogen (15, 16). In fact it is the basis of a potential group B meningococcal vaccine and is able, when conjugated to a protein carrier, to induce in mice high affinity NPr polysialic acid-specific antibodies (15, 16). Although NPr polysialic acid protein conjugates do induce some antibodies that cross-react with polysialic acid, the protective antibody is predominantly based on a length-dependent (helical) form of the NPr polysialic acid, which mimics a unique capsular epitope on the surface of group B meningococci (16).

EXPERIMENTAL PROCEDURES

Cell Lines—The rat leukemic cell line (RBL-3H3) (13) was obtained from the American Type Culture Collection (Manassas, VA), and the mouse leukemic cell line (RMA) was the gift of H. G. Ljunggren (Karolinska Institute, Stockholm, Sweden).

Mice—Female C57BL/6 mice were purchased from Charles Rivers (Montreal, Quebec, Canada) and maintained in our Institutional Animal Facility.

Polysialic Acids—NAc and NPr polysialic acids (11-kDa fractions) were obtained from colominic acid as described previously (16).

Monoclonal Antibodies—mAb 13D9, specific for NPr polysialic acid, has been described previously (16); mAb 735, specific for polysialic acid (17), was the gift of D. Bitter-Suermann (Medizinishe Hochschule, Hannover, Germany).

Flow Cytometry—For flow cytometry, cells were incubated with mAbs 13D9 or 735 in 50 μl of RPMI + 1% FBS on ice. After 30 min the cells were washed and incubated with fluorescein isothiocyanate anti-mouse IgG2a (obtained from Cedarlane Laboratories, Ontario, Canada) in 50 μl of RPMI + 1% FBS on ice. After another 30 min the cells were washed and fixed in 1% formaldehyde and assayed on a flow cytometer (Coulter Incorporation, Miami, FL). Fluorescence intensities are expressed in arbitrary units.

Antibody-dependent Cytotoxicity—For antibody-dependent cytotoxicity measurements, 1×10^6 cells were pretreated with ManNPr in 24-well plates. Tumor cells ($1–2 \times 10^4$), after treatment with ManNPr, were harvested, washed with PBS, and incubated with antibodies (735 or 13D9, 1 mg/ml) on ice for 1 h. Cells were washed and incubated with 10% rabbit complement (Cedarlane Laboratories, Ontario, Canada) at

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[1] The abbreviations used are: NPr polysialic acid, *N*-propionylated polysialic acid; MTT, 3-(4,5-dimethulthiazol-2-yl)2,5-diphenyltetrazolium bromide; ManNPr, *N*-propionyl-D-mannosamine; FBS, fetal bovine serum; PBS, phosphate-buffered saline.
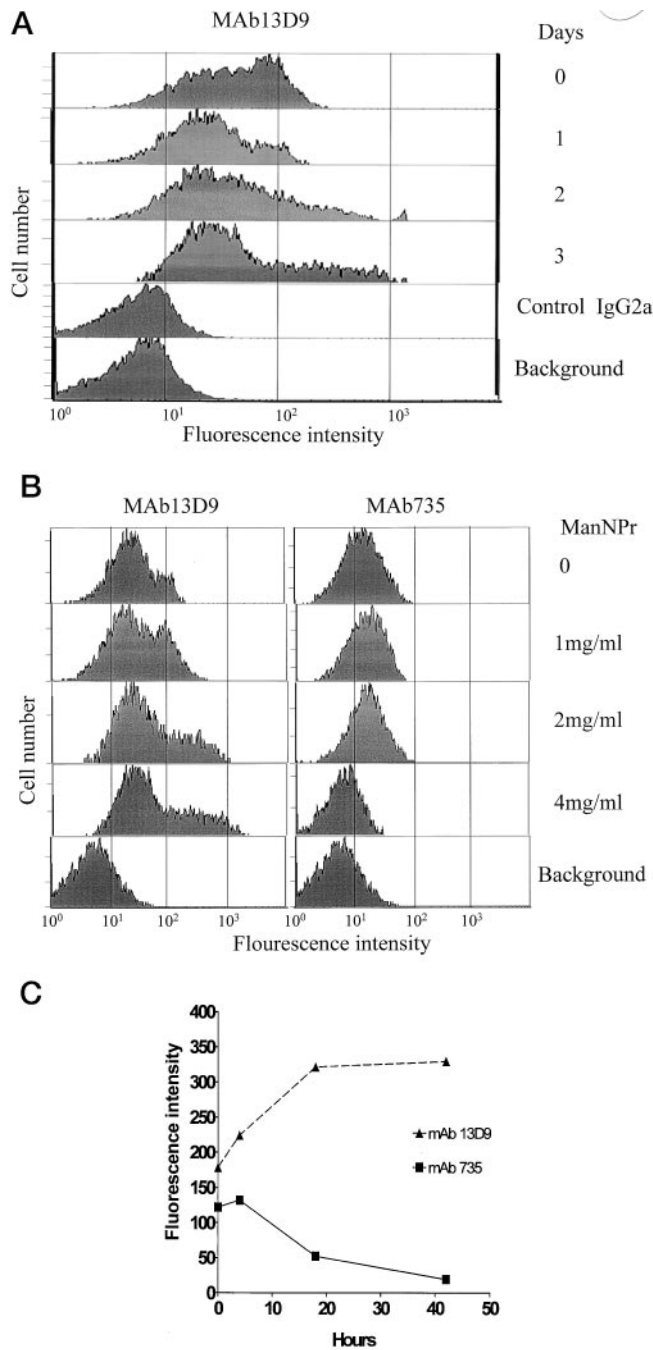
FIG. 1. **NPr polysialic acid expression on the surface of tumor cells.** *A*, rat leukemia cells (RBL-2H3) were incubated with 4 mg/ml ManNPr in RPMI medium supplemented with 8% FBS for 3 days. At daily intervals aliquots of the cells were harvested, and the expression of NPr polysialic acid was monitored by flow cytometry using mAb 13D9. *B*, RBL-2H3 cells were incubated with different concentrations of ManNPr in the same medium described in *A*. Following harvesting of the cells the expression of polysialic acid and its NPr analog were measured by flow cytometry using mAb 735 and mAb 13D9, respectively. *C*, mouse leukemic cells (RMA) were incubated with 2 mg/ml ManNPr, and the expression of polysialic and its NPr analog were measured by flow cytometry using mAb 735 and mAb 13D9, respectively.

37 °C for 2 h. The cytotoxic assay was performed as described previously (18) in 96-well plates, and cell viability was measured by the MTT colorimetric method. MTT was dissolved at a concentration of 5 mg/ml in PBS, and the solution was sterilized by filtration. After adding 10 μl of MTT solution into each well, cells were incubated for 4 h. 150 μl of 1.5 M HCl and 500 μl of isopropyl alcohol were used to rupture the cells. A standard curve was established by measuring MTT incorporation ($A_{570\,nm}$) of a known number of tumor cells, and the percent cytotoxicity
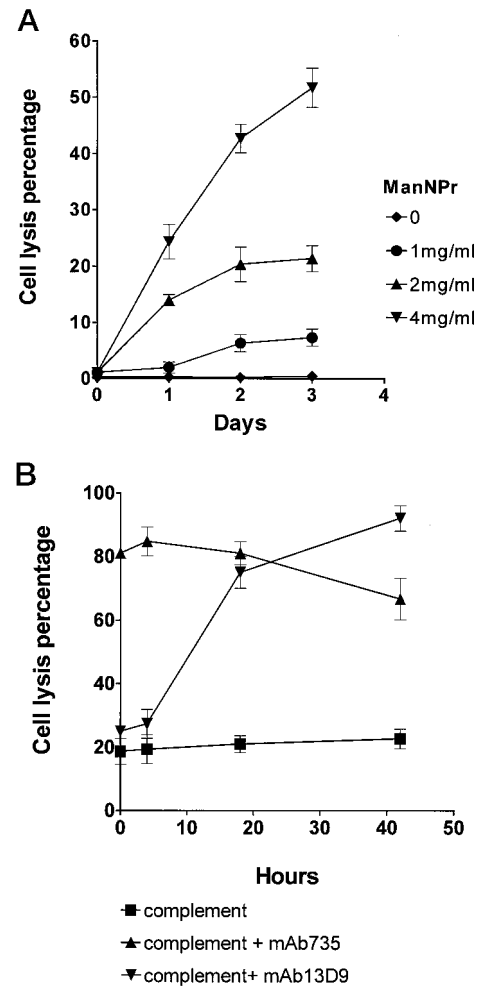

FIG. 2. **Antibody-mediated cytotoxicity is dependent on the expression of NPr polysialic on tumor cells.** *A*, RBL-2H3 cells were incubated with increasing concentrations of ManNPr for 3 days. At daily intervals the cells were harvested, washed with PBS, and incubated with mAb 13D9 as described previously. The cells were then subjected to a cytotoxicity assay (18). *B*, RMA cells were incubated with ManNPr (4 mg/ml), and aliquots were harvested at different time intervals. They were then washed with PBS and incubated without antibody, with mAb 735 and mAb 13D9, and subjected to the cytotoxicity assay as described previously.

of the unknown samples was calculated using the formula: % cytotoxicity = (1 − number of live cells/total number of cells) × 100%.

Inhibition of Antibody-dependent Toxicity—For inhibition of antibody-dependent cytoxicity, RMA cells were preincubated with ManNPr (2 mg/ml) for 24 h, and the washed cells ($1–2 \times 10^4$ in 35μl of PBS) were distributed into wells of a 96-well plate. 25 μl of mAb 13D9 (20 μg/ml) was then added to each well. This was followed by 40 μl of NAc or NPr polysialic acids (1 mg/ml) into the first well with the 2-fold serial dilutions of the inhibitor solution in subsequent wells. The cells were washed and incubated with rabbit complement at 37 °C for 2 h, and the cytotoxic assay was performed as described above.

RESULTS AND DISCUSSION

To examine the feasibility of our strategy for targeting cancer cells, we first synthesized the required precursor ManNPr, essentially using a previously described method (7). We then performed a series of experiments to demonstrate that both a rat leukemic cell line (RBL-2H3) (13) and a mouse leukemic cell line (RMA) (19) can incorporate ManNPr into the cell surface polysialic acid (Fig. 1). RBL-2H3 cells were treated with ManNPr at the same concentration for different times (Fig. 1*A*) and for the same time at difference concentrations (Fig. 1*B*). The pretreated cells were stained with mAb 13D9, specific for NPr polysialic acid (16). Flow cytometric analysis indicated that the
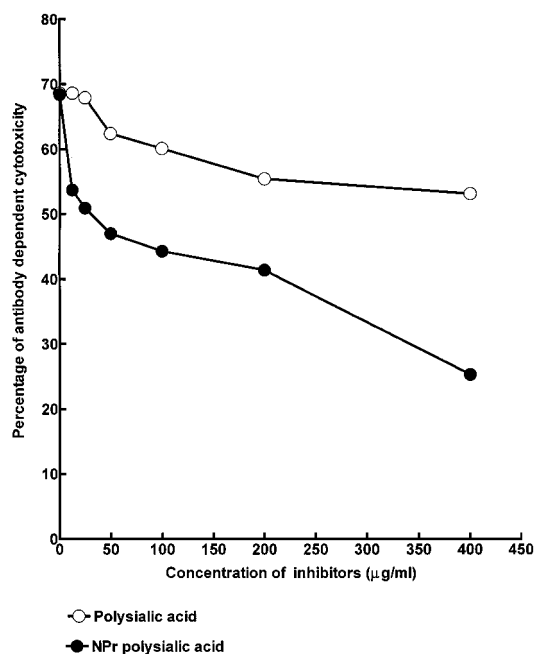
FIG. 3. **Inhibition of antibody (mAb 13D9)-dependent cytotoxicity of RMA cells by NAc and NPr polysialic acids.**

uptake of ManNPr, as determined from the relative surface expression of NPr polysialic acid, was both time- (Fig. 1A) and dose (Fig. 1B)-dependent. The RBL-2H3 cells above were, in addition to mAb 13D9, also stained with mAb 735, specific for polysialic acid (17). The predominant specificities of these mAbs allowed for the successful monitoring of the transformation of the cell surface polysialic acid to its *N*-propionylated analog. Flow cytometric analysis showed that as the expression of polysialic acid on the cell surface declined with exposure of the cells to increasing amounts of ManNPr, the expression of NPr polysialic acid on the cell surface increased (Fig. 1B). RMA cells gave similar flow cytometric profiles when subjected to the above experiments (data not shown), and from these data curves depicting the time dependence of the transformation of the polysialic acid on the surface this cell line to NPr polysialic acid were constructed. (Fig. 1C). The curves indicate that as the density of NPr polysialic acid on the cell surface increases with time and eventually plateaus, the density of polysialic acid decreases and plateaus concomitantly.

To determine whether NPr polysialic acid is a useful marker to target and kill tumor cells, assays of antibody-dependent cytotoxicity were carried out, and the results are shown in Fig. 2, *A* and *B*. Following preculture with the precursor (ManNPr), RBL-2H3 cells were further treated with mAb 13D9 and incubated with rabbit complement at 37 °C. The resultant cell counts demonstrated that lysis of tumor cells was dependent only on the time and dose of their exposure to ManNPr, because mAb 13D9 alone failed to lyse the cells. Thus, the more NPr polysialic acid was expressed on the cell surface, the more cells were killed (Fig. 2A). Previous studies (16) demonstrated that although mAb 13D9 did not cross-react with polysialic acid, its antigenic specificity has some similarities, being based on an epitope located on an extended helical segment ($n > 10$) of NPr polysialic acid (20). Thus our results show that ManNPr can be incorporated into the cells in sufficient quantities to form this complex epitope, which has a requirement for many contiguous *N*-propionylated sialic acid residues. To confirm this result further, RMA cells were subjected to the same assay except that mAb 735 was used as the antibody. mAb 735 exhibited strong binding to the native cell surface polysialic acid and also
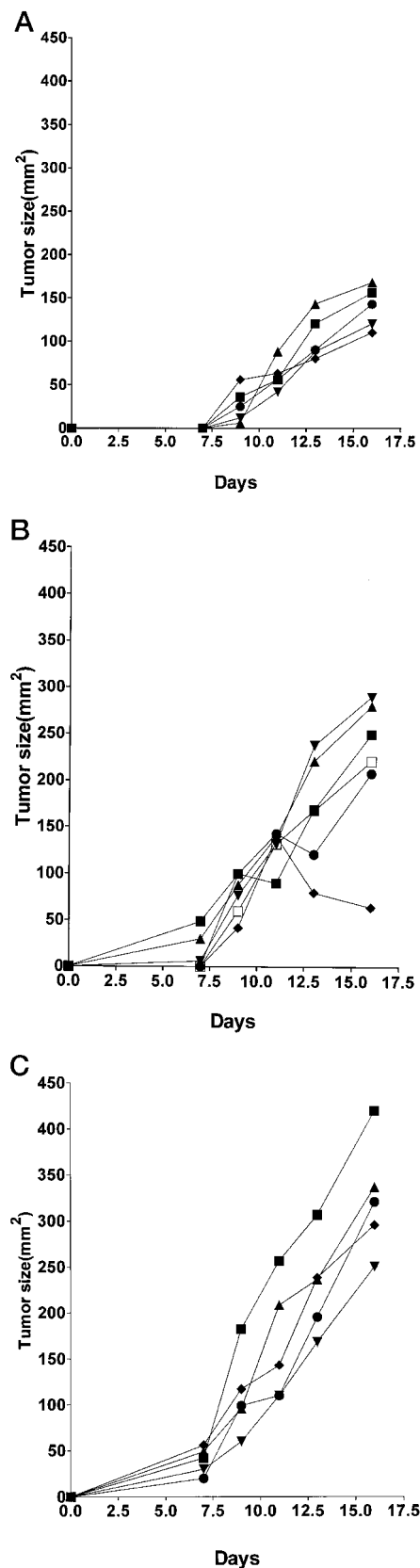

FIG. 4. **Administration of ManNPr and mAb 13D9 resulted in reduced tumor growth *in vivo*.** *A*, C57BL/6 mice were injected subcutaneously with 1×10^6 RMA tumor cells in the shaven area of the rear flank. After 5 days, groups of five or six mice were injected daily with mAb 13D9 (200 μg, intraperitoneally) and ManNPr (5 mg, intraperitoneally) with mAb 13D9 alone (200 μg, intraperitoneally) (*B*) or with PBS (*C*). Tumor growth was monitored routinely by measuring the tumor size. A control IgG2a mAb did not inhibit tumor growth (data not shown).

mediated strong killing of the RMA cells. However, this killing was reduced in a time-dependent manner as ManNPr was incorporated into the cells (Fig. 2*B*). The killing of tumor cells by rabbit complement alone was not significant, thus indicating that the cytoxicity of the above cells is controlled by the specificity of the antibody used.

Confirmatory evidence that the cytotoxicity of RMA cells is mediated by surface NPr polysialic acid was obtained by showing that cytotoxicity could be inhibited by NPr polysialic acid (Fig. 3). Although we have also demonstrated previously that mAb 13D9 does not bind to short NPr sialooligosaccharides (16), we cannot, however, eliminate the possibility that nonspecific binding to these antigens occurs when they are situated on the surface of RMA cells. If this did occur it could also possibly result in them making a contribution to the total cytotoxic effect.

To determine whether the above bioengineering procedure could control tumor growth *in vivo*, we established a mouse solid tumor model. Mice were inoculated with RMA cells (10^6 cells/mouse), and 5 days after inoculation the mice were treated daily with mAb 13D9 (200 μg/mouse) and precursor ManNPr (5 mg/mouse) for a period of 8 days. Tumor growth was routinely monitored by measurement of tumor size. The data showed that in combination with ManNPr, mAb 13D9 had a greater effect on tumor size than mAb 13D9 alone, although mAb 13D9 alone was also able to reduce tumor size when compared with a control group of mice (Figs. 4, *A–C*). These results indicate what although this bioengineering procedure is able to curtail tumor growth, it is not able to completely eradicate tumor cells from the mice. This can be explained by the fact that the original inoculum was a mixture of RMA cells, some of which were not polysialylated (Figs. 1, *A* and *B*), and were therefore unable to express the helical epitope of NPr polysialic acid on which the cytotoxicity of mAb 13D9, in the presence of ManNPr, depends (16). Failure of the solid tumor cells to express polysialic acid was confirmed when mAb 735 failed to bind to tumor cells extracted from the mice (data not shown).

Despite our failure to eradicate solid tumors, we carried out experiments to determine whether our bioengineering strategy could be applied to the elimination of metastatic cancer cells.

We have shown that leukemic cells (RMA and RBL-2H3) already express polysialic acid on their surfaces, and it is likely, on the basis of our results (see later), that in their metastatic forms they still express a high density of this surface antigen (12). This would be to their advantage, because polysialic acid, in addition to its poor immunogenicity (15), is also a powerful inhibitor of alternative complement pathway activation (21). This accounts for the fact that polysialic acid is the major virulence factor in both pathogenic group B meningococci and *Escherichia coli* K1 (22). The experiments in mice were carried out as described for the solid tumor using RMA cells, except that in this case the spleens of the mice were analyzed for the presence of metastatic cells. One-fifth of a cell suspension of the whole spleen of the mice was used to initiate the tumor cell limiting dilution experiment. Following cell cultures of the spleen cells the metastasized tumor cells were easily distinguished from the normal spleen cells by microscopic examination. Our data in Tables I and II show that there were no tumor cells in the spleen of the mice treated with a combination of mAb 13D9 and ManNPr, indicating that all transported metastasized tumor cells were polysialylated and therefore were completely eliminated from the mice.

The data also revealed that mAb 13D9 alone could also partially reduce the metastasis of tumor cells to a certain extent in comparison with a control group of mice (Tables I and II). One plausible explanation for this phenomenon is that the cytotoxicity of mAb 13D9 can be attributed to its ability to recognize a unique polysialic acid-associated epitope found only on the surface of *in vivo* RMA cells. This hypothesis has some credence, because a similar cytotoxic epitope is expressed on group B meningococci and *E. coli* K1. The epitope is composite in nature and is thought to be formed on the surface of the bacteria by the interaction of extended helical segments of their $\alpha 2$–8 polysialic acid capsules with another, probably lipid, surface component (16, 23). Why the expression of this type of epitope did not result in the complete cytotoxicity of all the metastatic cells is not known.

In summation we have demonstrated in mice that the metastasis of tumor cells can be controlled by bioengineering their surface polysialic acid glycoconjugates to their *N*-propionylated analogs and then by applying immunotherapy based on antibodies specific for the modified antigen. These antibodies could be either passively administered as described herein or induced *in situ* by direct immunization using an appropriate NPr polysialic acid-protein conjugate vaccine. Although this new immunotherapeutic strategy was only partially able to inhibit the growth of tumor cells, its significance cannot be underestimated because of the importance of being able to successfully control metastasis in the treatment of cancer.

A serious problem with the implementation of this strategy for the immunotargeting of cancer cells, which applies equally to their chemotargeting (9), is that in all likelihood any precursor, including ManNPr, will be taken up by both normal and cancer cells alike. Therefore, the successful application of both the above protocols will depend on a means of achieving specificity. By using polysialic acid as our target antigen we can achieve specificity mediated by the immune response, because

TABLE I
Antibodies against NPr polysialic acid control tumor metastasis in vivo

Injection of tumor cells, mAb 13D9, and ManNPr into mice was carried out as described in the legend to Fig. 3. On day 25, spleens were excised and cell suspensions prepared in medium RPMI = 8% FBS. One-fifth of the aliquots from the individual mice were used to initiate serial 2-fold dilution in 24-well plates in 1 ml of RPMI, 8% FBS. Cultures were fed regularly and monitored over a period of 1 month to score positive wells containing tumors. Spleen samples that had tumor cells were scored positive, and the samples that had no tumor cells at all dilutions were scored negative.

Group	Tumors in spleen[a]	Percentage metastasis
		%
13D9 + precursor	0/5	0.0
13D9	2/6	33.3
	4/5	80.0

[a] Number per mouse.

TABLE II
Semiquantification of metastasis by limiting dilution of spleen cells from individual mice

One-fifth of total spleen cell suspension was used to initiate a 2-fold serial limiting dilution. The samples that had no tumor cells were scored negative. The samples that had tumor cells were scored positive and were subjected to semiquantification by serial dilution.

	RMA cells treated with:															
	ManNPr + mAb 13D9					mAb 13D9						Control				
	−	−	−	−	−	+	+	−	−	−	−	+	+	+	+	−
Dilution of spleen cells	1:5	1:5	1:5	1:5	1:5	1:640	1:160	1:5	1:5	1:5	1:5	1:640	1:160	1:160	1:80	1:5

although polysialic acid is ubiquitous on fetal tissue, it is only found in a few discrete adult tissues (12, 24, 25). In addition NPr polysialic acid conjugates have been successfully used as experimental human vaccines against group B meningococcal in a number of animal species without deleterious consequences (15, 26). Although the application of the above strategy to other sialylated glycoconjugates on cancer cells is also theoretically possible, it will be more difficult, because the former are also found on adult tissues. Therefore, it will require the introduction of different methods of achieving specificity to preferentially target cancer cells. Perhaps specificity could be generated in these cases by exploiting the differing densities of some of these glycoconjugates on normal and cancer of cells or by the introduction of new technologies whereby the precursor can be preferentially delivered to cancer cells.

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