Comblike Dendrimers Containing Tn Antigen Modulate Natural Killing and Induce the Production of Tn Specific Antibodies

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Received July 29, 2005

Comblike glycodendrimers were prepared by the chemoselective ligation of cysteine-modified glycopeptides (**1**−**7**) with a 3-maleimidopropionate-modified linear synthetic carrier (**8**). Glycodendrimers bearing mono-, di-, or tri-Tn clusters (**9**−**11**) were tested as inhibitors using plant and mammalian lectins. In the former group, the *Codium fragile* lectin showed moderate discrimination among **9**, **10**, and **11**. In the latter group, A and B isoforms of rat NKR-P1 lectin strongly discriminated between **9** and **10**. **10** caused a 4-fold increase in killing of the NK resistant tumor cell lines at concentrations as low as 10^{-8} M. Surprisingly, **11** interacted exclusively with the rat NKR-P1B isoform and inhibited efficiently natural killing in both rats and humans, even in the presence of the activating compounds **9** and **10**. Dinitrophenol haptenization or influenza virus hemagglutinin T-cell epitope conjugation increased the immunogenicity of the parent compounds and resulted in the production of Tn specific antibodies.

Introduction

Malignant transformation of cells often results in dramatic changes in the abundance and structure of their surface carbohydrates.[1,2] A number of tumor-associated surface-expressed carbohydrate antigens have been identified.[3,4] Tn (α-D-GalNAc-Ser/Thr) and T (α-D-Gal-1→3-α-D-GalNAc-Ser/Thr) are of particular interest because they are overexpressed by the largest group of malignant tumors.[5] The Tn antigen is overexpressed in about 90% of human adenocarcinomas and also in more then 70% of lung, colon, and stomach carcinomas, and the extent of its expression correlates with carcinoma differentiation and aggressiveness.[6]

Since their original discovery, the potency of carbohydrate antigens for vaccination against tumors has been evaluated using natural sources of carbohydrate antigens such as OSM,[a,7] semisynthetic conjugates of carbohydrate or glycopeptide antigens with KLH[8,9] or BSA,[10] semienzymatically produced glycosylated mucin sequences,[11] and lipidated glycopeptides.[12] To eliminate some of the disadvantages of these preparations,[13] branched, synthetic, low-molecular weight scaffolds have been introduced.[13] These molecules can generally be prepared by a solid-phase synthesis. However, with the increasing molecular weight of the target compound, incomplete sequences accumulate, and difficulties in isolation of the target compound may arise. To provide precision and chemical unambiguity, a chemoselective ligation based on assembly of unprotected building blocks under mild aqueous conditions has been introduced.[14−16] Systematic clinical trials comparing the efficacy of all these preparations have only recently been performed: trimeric Tn clusters were found to be more efficient for vaccination than Tn monosaccharide conjugates, and conjugates to KLH were more effective than conjugates to BSA.[17] Moreover, MUC1 glycosylated with Tn at three to five sites per 20 amino acids and conjugated to KLH induced the strongest antibody response against tumor cells expressing this antigen.[17]

To explore the potential of additional types of fully synthetic compounds, we prepared tetrameric comblike dendrimers with one to three copies of the serine Tn antigen (as a B-cell epitope) on each branch. To maximize yields and purity, we used a chemoselective ligation strategy, an approach based on the assembly of modified building blocks in aqueous buffered solution. We used a nucleophilic addition of cysteine-modified glycopeptides to a 3-maleimidopropionate-modified linear KG tetramer.[18] The binding capacity of the dendrimers was evaluated in lectin binding assays using four plant and two mammalian lectins. To modulate the immunogenicity of these compounds, we modified the parent compounds by N-terminal haptenization with 2,4-dinitrophenol (DNP)[19] or by C-terminal conjugation with the T-cell epitope derived from the influenza virus hemagglutinin[20] (HA306-318, PKYVKQNTLKLAT). In the present work, we show that our compounds are not only able to elicit the production of specific antibodies but also act as specific modulators of the activity of NK cells, which are essential for efficient elimination of tumors.

Synthetic Strategy. Building blocks (**1**−**7**) were synthesized following standard protocols of solid-phase synthesis using

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[a] Abbreviations: BSA, bovine serum albumin; BSM, bovine submaxillary mucin; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, *N*,*N*′-Dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DNP, 2,4-dinitrophenol; ELISA, enzyme-linked immunosorbent assay; ELLA, enzyme-linked lectin assay; HCTU, *O*-(1*H*-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOSu, *N*-hydroxysuccinimide; HRP, horseradish peroxidase; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IVH, influenza virus hemagglutinin; KLH, keyhole limpet hemocyanin; NK, natural killer; OSM, ovine submaxillary mucin; RP-HPLC, reverse phase high-performance liquid chromatography; t_R, retention time; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

10.1021/jm050741g CCC: $33.50
Published on Web 09/16/2006

Table 1. Physicochemical Data of Comblike Glycopeptide Dendrimers (**9**−**15**)

compd	sequence	purity[a] (%)	t_R (min)[b]	yield (%)	molecular weight found	molecular weight calcd[c]
9	(Ac-Ser(Tn)-Gly_3-Cys-NH_2)$_4$LC[d]	96.7	12.5	72	3896.35	3899.03
10	(Ac-[Ser(Tn)]$_2$-Gly_3-Cys-NH_2)$_4$LC	97.7	13.1	75	5057.98	5060.15
11	(Ac-[Ser(Tn)]$_3$-Gly_3-Cys-NH_2)$_4$LC	97.8	13.6	77	6218.07	6221.23
12	(Dnp-Ser(Tn)-Gly_3-Cys-NH_2)$_4$LC	98.5	14.3	60	4393.10	4395.37
13	(Dnp-[Ser(Tn)]$_2$-Gly_3-Cys-NH_2)$_4$LC	96.8	14.6	62	5554.15	5556.39
14	(Dnp-[Ser(Tn)]$_3$-Gly_3-Cys-NH_2)$_4$LC	97.5	15.1	65	6714.12	6717.47
15	(Ac-[Ser(Tn)]$_2$-Gly_3-*P*[e]-Cys-NH_2)$_4$LC	98.2	14.2	85	10999.98	11003.35

[a] Determined by RP-HPLC. [b] Gradient 0−100% in 30 min with 2 min lag after sample injection (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in MeOH; column used, Vydac 208TP53 C8 3.2 × 250 mm; flow rate = 0.75 mL/min, λ = 218 nm. [c] Monoisotopic mass. [d] LC = linear tetravalent comblike carrier (compound **8**). [e] *P* = T-cell epitope (PKYVKQNTLKLAT).

Fmoc/tBu chemistry. Amino acids were coupled in a 5-fold molar excess using TBTU/DIEA activation with the exception of Fmoc-Ser(3,4,6-tri-*O*-Ac-α-D-GalNAc)-OH, which was coupled in 1.3-fold molar excess in order to save this valuable glycosylated amino acid. In all cases the coupling reaction proceeded to completeness in 2 h. *O*-Acetyl groups protecting the hydroxy groups of the GalNAc residues were removed by treatment of the glycopeptide resin with a 15% solution of hydrazine hydrate in methanol (2 × 40 min). Cleavages from the resin and side chain deprotections were achieved simultaneously using cleavage cocktails that depended on the amino acid composition.

Building block **8** was prepared according to the following procedure. First, a linear KG peptide tetramer was synthesized using Boc/Bn chemistry. Amino acids were coupled in 5-fold molar excess using TBTU/DIEA activation. Cleavage from the resin and side-chain deprotection were achieved simultaneously by 1 h treatment with HF:anisole 9:1 (v/v). Second, the peptide was then coupled in a solution of DMF and 10% $NaHCO_3$ 1:1 (v/v) with freshly prepared succinimido 3-maleimidopropanoate in 1.1-fold molar excess.

Glycopeptide dendrimers (**9**−**15**) were prepared by a chemoselective ligation strategy. This approach offers efficient and effective preparation of high molecular weight compounds of high homogeneity and is based on the assembly of purified unprotected building blocks in buffered aqueous solution. For the preparation of our comblike dendrimers we used a nucleophilic addition of the thiol group of the cysteine-modified antigens (compounds **1**−**7**) to an activated double bond of the 3-maleimidopropionate-modified linear tetrameric KG carrier (compound **8**). This procedure well-known from protein chemistry, which is compatible with the stability of the *O*-glycosidic bond, worked well in our hands and provided target compounds in good purity and yields (60−85%, Table 1). Structures of prepared comblike dendrimers **9**−**11** are given in Figure 1. During dialysis of the crude products we noticed that a portion of compounds **12**−**14** remained irreversibly bound to the membrane (probably due to the strong electrostatic interactions of DNP groups with the material of the membrane), resulting in lower yields.

Biochemical Assessment. The binding properties of compounds **9**−**11** were first evaluated using four commercially available plant lectins with specificity for the D-GalNAc residue: *Bandeiraea simplicifolia*, *Codium* fragile, *Dolichos biflorus*, and *Vicia villosa* B4.[21] The results of binding assays are presented in Figure 2. We observed that the binding activities of the comblike dendrimers were superior to those of the standard carbohydrate inhibitor, D-GalNAc. Moreover, the individual lectins differed in their ability to discriminate between the tested dendrimers. *B. simplicifolia* and *D. biflorus* lectins (Figure 2A,C) showed no or negligible discrimination. Some ability to discriminate was found for the *V. villosa* lectin (Figure 2D). This lectin discriminated between **9** and **10**; compound **11** showed the same binding properties as compound **10.** The *C. fragile* lectin (Figure 2B) differentiated among all three tested dendrimers and also showed the strongest binding affinity of all lectins tested. The ability of the latter two lectins to discriminate between the tested compounds was related to the density of the carbohydrate coating, an effect that was most evident for compounds **9** and **10** (Figure 2B,D). Altogether, the *C. fragile* lectin displayed the best ability to discriminate between the tested compounds and showed the highest difference between the binding of the monosaccharide and the dendrimers (**11** was 10^5 times more effective than D-GalNAc monosaccharide).

To provide a preliminary biochemical assessment of the ability of compounds **9**−**11** to raise the production of anti-Tn antibodies, we tested their abilities to inhibit the binding of the anti-Tn monoclonal antibody 83D4 to plates coated with asialo-OSM. We were able to prove the dependence of the inhibitory potencies of these glycodendrimers on the degree of sugar substitution; while there has been very little inhibition by the monosaccharides in this experimental system, the dendrimers turned out to be efficient inhibitors with IC_{50} values ranging from 10^{-5} M for compound **9** up to 10^{-8} M for compound **11** (Figure 3A).

The endogenous mammalian lectins are proteins that are very relevant to the potential in vivo application of compounds **9**−**11**. These receptors may be responsible for the therapeutic efficacy of glycodendrimers due to their ability to activate the cells of the immune system[22] and may influence their biodistribution in the organism.[22] Therefore, a major receptor of natural killer cells critical for the activation of cytotoxic tumor killing lymphocytes, NKR-P1,[22,23] has been selected as the relevant test system. Compounds **9**−**11** were tested as inhibitors of binding of two isoforms of rat NKR-P1, NKR-P1A and NKR-P1B, to their high affinity ligand β-D-$GlcNAc_{23}$BSA neoglycoprotein.[24] In assays with rat NKR-P1A, compound **9** had inhibitory activities comparable with the standard monosaccharide ligand, D-GlcNAc (Figure 3B). However, compound **10** was a much better inhibitor with IC_{50} of 10^{-10} M, ranking this compound itself among the high-affinity ligands for NKR-P1A. Notably, the compound **11** was a very poor inhibitor for NKR-P1A with an inhibitory activity even weaker than the negative control, D-mannose (Figure 3B). When similar tests were performed with rat NKR-P1B receptor, the results for compounds **9** and **10** were very similar to those obtained with the A isoform: compound **9** had activity comparable to that of D-GlcNAc monosaccharide, while compound **10** had a very high inhibitory activity (IC_{50} of 3×10^{-10} M). However, the binding of compound **11** to NKR-P1B was entirely different: not only was there specific binding, but the IC_{50} was about 10 times lower

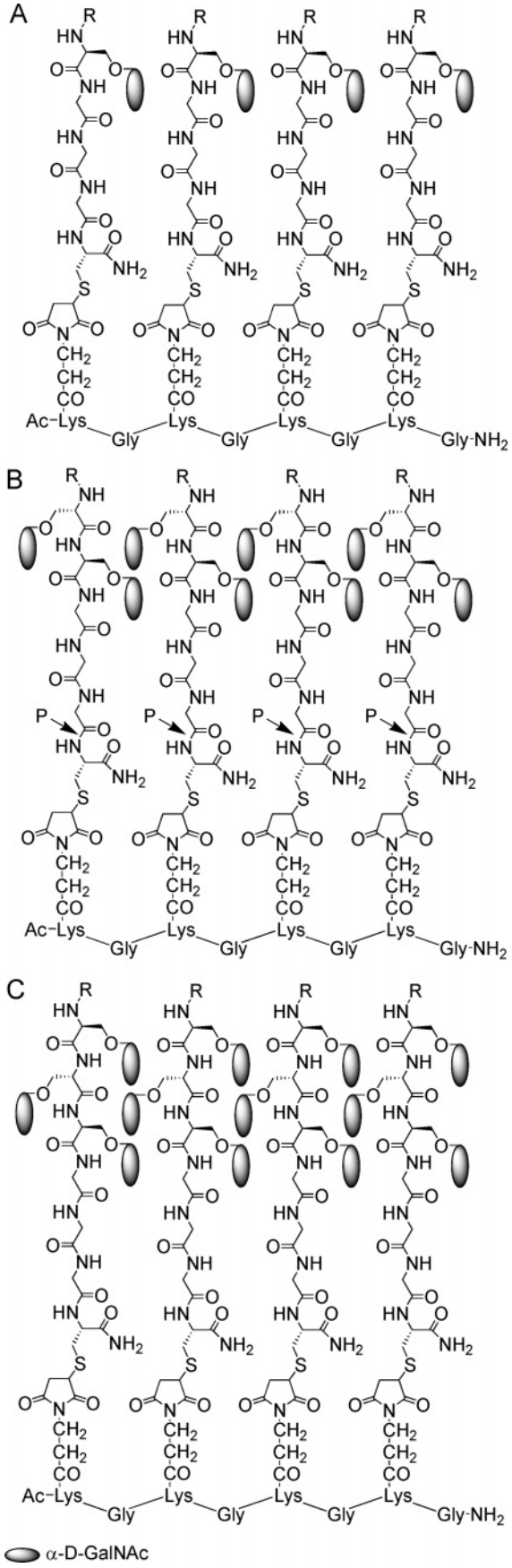

Figure 1. Structure of comblike dendrimers: **9** (R = acetyl) and **12** (R = 2,4-dinitrophenyl) (A); **10** (R = acetyl), **13** (R = 2,4-dinitrophenyl), and **15** (R = acetyl, *P* = PKYVKQNTLKLAT peptide sequence) (B); **11** (R = acetyl) and **14** (R = 2,4-dinitrophenyl) (C) prepared by chemoselective ligation.

than measured for compound **10** (IC_{50} of **11** = 3×10^{-11} M; see Figure 3C).

Immunological Evaluation. Tn-antigen-containing structures **9**−**15** were tested for their ability to elicit an immune response in Balb/c mice (Table 2). The results indicate that Tn antigen conjugated with a small and defined immunodominant molecule (DNP) or a T-cell peptide epitope (HA306-318) induces a humoral immune response. Interestingly, the highest titers of anti-Tn IgG antibodies were detected in the serum of IVH presensitized animals treated with compound **15**. Presensitization of these animals with IVH did not substantially increase anti-Tn IgG antibody levels but rather increased the production of anti-Tn IgM and IgA antibodies. Even more interestingly, presensitization of animals with DNP resulted in a dominant increase of anti-Tn IgA antibodies, which has not been previously reported. Furthermore, anti-Tn IgG and IgM antibodies were tested for their ability to bind to Tn-positive Jurkat cells at equivalent concentrations (50% of their maximum antibody titer as determined by ELISA). The results are presented in Table 3. Cell-staining capacity was generally higher for IgM anti-Tn antibodies and further increased in the animals presensitized with DNP/IVH and vaccinated with DNP/IVH-conjugated Tn antigen.

Since the dendrimers **9** and **10** both had a high affinity for NKR-P1A (activating) and NKR-P1B (inhibitory) receptors, it was necessary to perform a test of cytotoxicity to evaluate the possible in vivo activities. For this purpose, two rodent leukemic cell lines, an NK-sensitive line (YAC-1) and NK-resistant line (P815), were employed. When compared to the control, compound **9** stimulated the natural killing of both YAC-1 and P815 targets (Figure 4,parts A and D, respectively) in a dose-dependent manner, but the enhancement of natural killing was more profound in the case of the NK-resistant P815 cell line (nearly 3-fold increase in natural killing using 10^{-6}−10^{-8} M of compound **9** compared to control, Figure 4D). The effects of compound **10** on natural killing were very similar, except that the activation potential was even higher (Figure 4B,E) and there was a nearly 4-fold increase in natural killing in the case of the NK-resistant cell line P815 (Figure 4E). However, the effect of compound **11** on natural killing was exactly the opposite; this compound efficiently inhibited natural killing in both tested cell lines (Figure 4C,F).

Because of the different impacts our compounds had on natural killing, we investigated their combined effects. We therefore tested the influence of the individual compounds and their combinations on natural killing of the NK-resistant rat cell line P815 at an effector:target cell ratio of 30:1 and 10^{-8} M concentrations. It is evident that the inhibitory effect of compound **11** is predominant (Figure 4G). This dendrimer inhibited natural killing efficiently in the presence of equimolar concentration of activating dendrimer **9** or **10** and even in the presence of a combination of **9** and **10** at equimolar concentrations. Furthermore, to evaluate the immunological effects of the dendrimers from the standpoint of their potential use in human therapies, the latter test was repeated using the human NK-resistant cell line RAJI, and very similar results were obtained for this human cell line (Figure 4H).

Discussion

We have shown previously that *β*-*N*-acetyl-D-hexosamine-coated dendrimers have the potential to be efficient tools in experimental tumor therapies.[23] These compounds are targeted to the tumor neovascular epithelial linings and efficiently recruit the killer lymphocytes into the tumor sites.[23] We have also

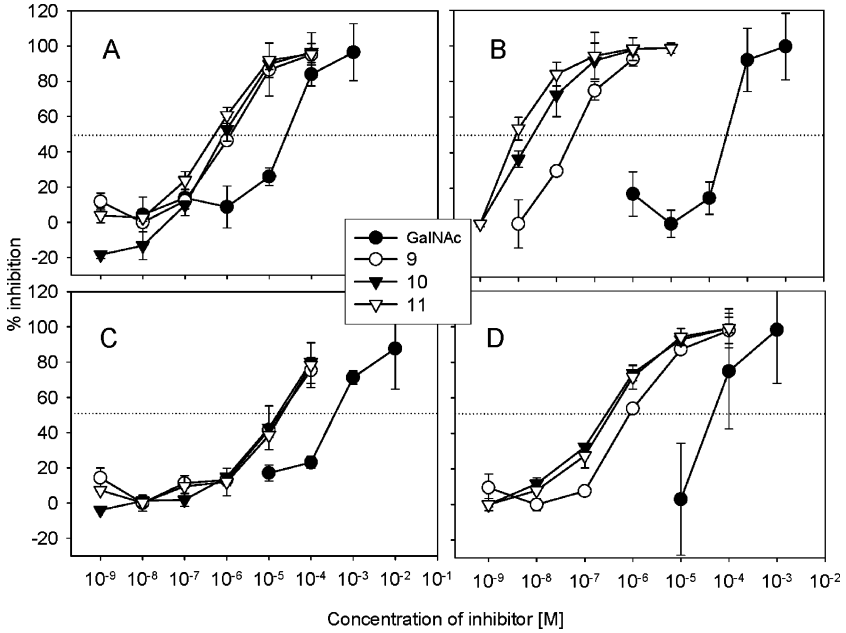

Figure 2. Inhibitory activities of the comblike dendrimers using model plant lectins. Dendrimers **9**−**11** were used together with a standard carbohydrate (D-GalNAc) as inhibitors of binding of *B. simplicifolia* (A), *C. fragile* (B), *D. biflorus* (C), and *V. villosa* (D) lectins to microtiter plates coated with desialylated bovine submaxillary mucin. Results are presented as average values ± range from duplicate experiments.

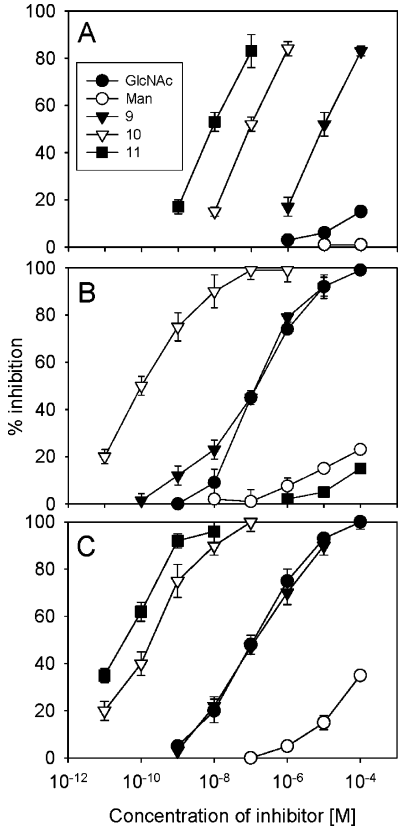

Figure 3. Inhibitory activities of the comblike dendrimers using monoclonal anti-Tn antibody 83D4 (A) and two isoforms of the recombinant soluble rat NK cell receptors, NKR-P1A (B) and NKR-P1B (C). GlcNAc and Man were used as a positive and a negative control, respectively. Results are presented as average values ± range from duplicate experiments.

proven the ability of these compounds to significantly activate NK cells and natural killing through their high-affinity binding to the NKR-P1A (activating) receptors at the surface of NK cells. In vivo tumor treatment experiments using mouse melanoma and rat colorectal carcinoma models proved the

Table 2. Production of Serum Anti-Tn Antibodies Evaluated Using ELISA

vaccination antigen	sensitization[a]	$\log_2$ of antibody titer of anti-Tn antibodies (±SD) IgG	IgM	IgA
−	−	5.83 ± 0.43	4.87 ± 0.33	3.27 ± 0.29
−	DNP	5.52 ± 0.57	4.99 ± 0.51	3.64 ± 0.35
−	IVH	6.08 ± 0.61	4.38 ± 0.36	3.84 ± 0.40
9	−	8.22 ± 0.73	5.30 ± 0.59	4.17 ± 0.39
10	−	9.57 ± 1.04	5.74 ± 0.62	4.49 ± 0.48
11	−	10.12 ± 1.13	5.90 ± 0.65	4.63 ± 0.41
12	−	7.83 ± 0.81	5.41 ± 0.58	5.01 ± 0.43
13	−	8.17 ± 0.85	5.96±0.53	5.20 ± 0.46
14	−	10.30 ± 0.74	6.29 ± 0.64	5.89 ± 0.53
12	DNP	12.68 ± 1.08	6.03 ± 0.59	12.27 ± 1.34
13	DNP	14.16 ± 1.17	6.61 ± 0.72	13.84 ± 1.30
14	DNP	15.67 ± 1.37	7.32 ± 0.71	17.62 ± 1.85
15	−	16.83 ± 1.52	5.74 ± 0.61	5.30 ± 0.57
15	IVH	18.26 ± 1.93	15.27 ± 1.63	8.16 ± 0.83

[a] Prior to the immunization, animals were injected with five doses of 0.15% DNP at 1-week intervals or two doses of 50 μL of a commercial influenza virus at 2-week intervals.

critical role of the density and mode of presentation of the carbohydrate ligand in polyamidoamine-type dendrimers.[23] In the light of these results, it appeared interesting to evaluate alternative ways of presentation of the active carbohydrate epitopes, and comblike dendrimers seemed an attractive possibility.

For initial evaluation of the biological activities of glycodendrimers, binding assays with plant lectins of the appropriate specificity are often used. The technique employed is usually a plate binding or plate inhibition assay. The latter assay is preferred because it allows testing of a wide range of chemical compounds independently of their molecular size and polydispersity. We employed an inexpensive assay in which the plates were coated with asialo-OSM to provide the binding surface for selected plant lectins. The synthesized comblike dendrimers served as the inhibitors in this assay. To the best of our knowledge, this is the first study in which binding activities of carbohydrate-coated dendrimers have been extensively evaluated. Even though the inhibitory activities of our compounds were higher than those of the standard monosaccharide inhibitors for all four plant lectins, only two of the lectins had the ability

Table 3. Flow Cytometric Analysis of Anti-Tn Antibodies Bound to the Surface of Tn-Positive Jurkat Cells

vaccination antigen	sensitization[a]	Anti-Tn antibodies FACS (% of gated cells) (±SD)			
		IgG		IgM	
		pretreatment	post-treatment	pretreatment	post-treatment
−	−	10.2 ± 3.21	9.7 ± 2.43	9.2 ± 1.81	10.1 ± 3.48
−	DNP	9.9 ± 2.84	11.4 ± 3.08	10.9 ± 2.40	9.2 ± 3.52
−	IVH	10.6 ± 2.93	8.9 ± 3.87	10.5 ± 4.12	11.7 ± 3.35
9	−	11.8 ± 4.27	17.9 ± 4.10	10.33 ± 2.4	23.1 ± 6.19
10	−	9.8 ± 3.57	29.3 ± 7.38	10.4 ± 2.15	28.9 ± 8.33
11	−	10.4 ± 2.11	25.8 ± 6.87	9.63 ± 2.19	27.1 ± 7.73
12	−	10.7 ± 3.80	22.4 ± 8.41	11.0 ± 1.97	26.0 ± 6.58
13	−	10.1 ± 2.21	27.7 ± 7.74	9.7 ± 3.15	31.8 ± 9.83
14	−	9.3 ± 2.04	28.6 ± 7.12	10.5 ± 3.25	33.9 ± 10.22
12	DNP	10.0 ± 3.77	35.2 ± 7.83	11.4 ± 2.32	41.3 ± 9.44
13	DNP	10.1 ± 2.07	38.4 ± 12.25	10.7 ± 2.95	44.8 ± 11.75
14	DNP	9.75 ± 2.02	33.6 ± 8.96	11.0 ± 1.94	43.4 ± 11.55
15	−	10.5 ± 2.67	35.8 ± 9.62	10.7 ± 1.88	25.3 ± 7.20
15	IVH	9.2 ± 2.93	38.9 ± 9.36	10.6 ± 3.06	30.8 ± 6.88

[a] Prior to the immunization animals were injected with five doses of 0.15% DNP at 1-week intervals or two doses of 50 μL of a commercial influenza virus at 2-week intervals.

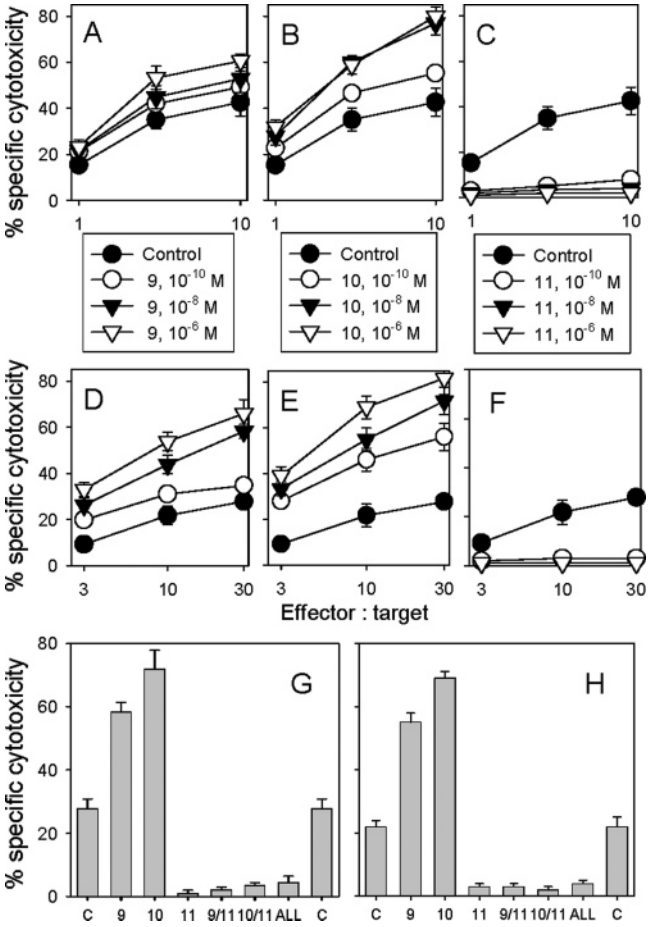

Figure 4. Comblike dendrimers **9** (panels A and D) and **10** (panels B and E) enhance and **11** (panels C and F) inhibits natural killing of NK-sensitive tumor cell line YAC-1 (A−C) and NK-resistant tumor cell line P815 (D−F) by rat NK cells. Compounds were dissolved in PBS (pH 7.4) and tested at the indicated concentrations together with the control sample (PBS only) at an effector:target cell ratio given in each panel. The combined effects of the dendrimers were also tested using NK-resistant mouse tumor cell line P815 (G) and human tumor cell line RAJI (H) at an E:T ratio of 30:1. Compounds were tested at 10^{-8} M concentrations.

to discriminate between our compounds with varying degrees of carbohydrate substitution. The results obtained for assays with the *C. fragile* lectin correlate with previous findings about the ability of this lectin to bind an α-D-GalNAc residue positioned on the reducing or nonreducing end of the saccharide moiety.[25] We assume that this is due to the structural complementarity of the Tn dimer and lectin binding groove.[26] Similarly, our results obtained with the *V. villosa* lectin seem to agree with the affinities of Tn1−Tn3 glycopeptides measured previously using surface plasmon resonance; the affinity increased with increasing degree of substitution, but the increase was more dramatic from the monosubstituted to the disubstituted compound than from the disubstituted to the trisubstituted compound.[27]

In addition to plant lectins, the comblike dendrimers were also tested using a mammalian lectin system, namely the NKR-P1 receptor of natural killer cells. In rodent natural killer cells, these receptors occur as several isoforms: NKR-P1A, -B, and -D. These isoforms are functionally interesting, since NKR-P1A is the major activating receptor of rodent (rat and mouse) NK cells, while NKR-P1B has inhibitory effects.[28] We have shown previously that these isoforms have distinct binding specificities toward both simple and complex oligosaccharide structures.[28] According to our findings, we did not expect that these receptors would interact with the α-D-GalNAc-containing dendrimers. However, we observed surprising binding affinities of these compounds toward both A and B isoforms of NKR-P1. While compounds **9** and **10** both bound strongly to both NKR-P1 isoforms, compound **11** displayed a unique and selective affinity for NKR-P1B, making it one of the best ligands identified for this NKR-P1 isoform to date. These results are in sharp contrast to data obtained previously with polyamidoamide-based dendrimers that reacted only with the A isoform.[28] Thus, while compounds **9** and **10** cannot selectively target any specific isoform of NKR-P1, compound **11** is the best reagent available so far for the selective targeting of NKR-P1B. The exact manner in which the comblike dendrimers described here interact with the carbohydrate-binding groove of NKR-P1 receptors will require further investigation, but the α-anomeric configuration of D-GalNAc is quite possibly an important component of this recognition mechanism. We speculate that while the extended oligosaccharide sequences require β-linked sugar units in order to fit horizontally into the binding grooves of these receptors, the α-linked D-GalNAc units of the tested dendrimers may interact with the groove vertically.[23] This mode of interaction would be less sensitive to amino acids interrupting the integrity of the groove, and as shown in Figure 3, may produce interesting selectivity.[23]

Vaccines designed to induce an optimal antibody response have several components, all of which must be optimized. The

first component is the antigen itself, which must closely resemble its native form on the target cell, in this case, Tn expression on tumor mucins. While the Tn antigen is a monosaccharide covalently linked to serine or threonine, monoclonal antibodies and sera selected for preferential reactivity with cancer cells usually interact with clusters of such antigens.[29] The second component is the carrier molecule, usually an immunogenic protein, like KLH or BSA. In our study this part of the vaccine was substituted by linking one to three copies of Tn antigen to a comblike synthetic peptide backbone using a completely synthetic approach, which resulted in the preparation of fully immunocompetent antigens. As anticipated, the best immune response was obtained using constructs with a cluster of three Tn antigens, while vaccine immunogenicity was lower when one or two Tn molecules were used (Tables 2 and 3). The third component of the vaccine is an immunologic adjuvant molecule. A synthetic vaccine containing Tn antigen linked to a T-cell epitope peptide derived from ovalbumin and able to activate T cells has only recently been described.[30] For this reason, we developed an alternative approach that combines a tumor-associated Tn antigen with the hapten DNP (**12**−**14**) or with an immunodominant T cell epitope from IVH designated HA306-318 (**15**). Conjugation of the Tn antigen with HA306-318 itself resulted in production of substantially higher antibody titers compared to unconjugated Tn vaccines. Interestingly, IVH sensitization further increased the anti-Tn antibody titer, particularly in the IgA and IgM classes (Table 2).

Our rationale for the haptenization approach was based on positive results of DNP-modified tumor vaccines in human melanoma clinical trials.[31] These trials indicated that DNP modification is a clinically feasible, safe, and effective procedure that induces significant patient antitumor response. This approach relies on the availability of the autologous tumor cells as a source of tumor rejection antigens and the ability of haptenization to render immunogenic proteins to which the host is otherwise unresponsive. To the best of our knowledge, our results provide the first example of DNP conjugation to a Tn antigen. Consistent with the published data, DNP sensitization followed by application of haptenized Tn vaccine significantly increased anti-Tn antibody production, especially within the IgG and IgA classes (Table 2).

The ability of our carbohydrate dendrimers to elicit antibody production is promising for the development of a therapeutic anticancer vaccine. However, activation of cellular immune responses, especially by T cells, NK/T cells, and NK cells, is of importance for permanent protection against cancer. Therefore, we tested the potency of our compounds to activate the killer lymphocytes. In the short-time cytotoxicity assays that measure mostly the activities of NK cells, both compounds **9** and **10** exerted significant activation effects (Figure 4). The ability of these compounds to activate natural killing of the resistant tumor cell lines in both rodents and humans (P815 and RAJI, parts G and H of Figure 4, respectively) is of particular importance. While the molecular mechanism for this activation in rodents and in humans may differ (in humans, the NKR-P1 is not an activating NK cell receptor, and the comblike dendrimers tested here did not react with human NKR-P1; data not shown), these results point to the potential of the newly synthesized compounds not only for use in animal experimental therapy models but also for use in human cancer therapies. These findings open new doors for biological testing of this compound in vivo, which is interesting in the light of the previously reported significant antitumor properties of GlcNAc-coated polyamidoamide-type dendrimers.[23] We also expect that combination of the effective humoral immune response with induction of NK cell activity, as demonstrated for compound **10**, should translate into an effective antitumor immune response in further in vivo experiments.

Our unique findings with regard to compound **11**, which not only reacted selectively and strongly with the inhibitory receptor NKR-P1B but also efficiently inhibited natural killing in both rodent and humans, open new avenues in the development of effective inhibitors of NK and NK/T cells in pathological situations connected with overactivation of these cells. In particular, recent findings demonstrate that iVα14 NK/T cells are responsible for allergen-induced airway inflammation,[32] a cardinal feature of allergic asthma.[33] Thus, the effective inhibition of NK cells by compound **11** may be exploited under these, and potentially other, pathological conditions.

Conclusions

The compounds described in this report display surprising reactivities with rat NKR-P1A and NKR-P1B receptors. These findings were corroborated by the unique effects of these compounds on natural killing. The major factors that influenced the effective immune response against these compounds include: the number of Tn repetitive sequences, spatial arrangement of Tn antigen in comblike dendrimers, and conjugation of Tn antigen with immunoadjuvant molecules. The chemical syntheses of complex glycopeptide conjugates described here make these intriguing compounds available for the development of vaccines against tumors and as potential antiallergic compounds.

Experimental Section

Chemicals and Materials. Acetonitrile, dichloromethane, *N*,*N*-dimethylformamide, methyl *tert*-butyl ether, trifluoroacetic acid, H-β-Ala-OH, *N*-hydroxysuccinimide, maleic anhydride, and 2,4-dinitrophenylfluoride were purchased from Fluka. Fmoc-Ala-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, Fmoc-γAbu-OH, and Fmoc-Lys(Boc)-OH were obtained from IRIS Biotech GmbH (Marktredwitz, Germany). Boc-Gly-OH, Boc-Lys(2-Cl-Bzl)-OH, and Rink amide MBHA resin (0.57 mmol of NH_2/g) were purchased from Calbiochem-Novabiochem. *B. simplicifolia*, *C. fragile* subspecies *tomentosides*, *D. biflorus*, *V. villosa* B4, OSM, and BSA were purchased from Sigma-Aldrich. Iodine-125 (IMS30, 10 mCi, 370 MBq) was purchased from Amersham Biosciences (UK, London). Anti-Tn monoclonal antibody 83D4 (IgM, κ)[34] was a gift from Dr. Luca Vannucci of the Institute of Microbiology, Prague, Czech Republic.

Amino acid analyses were performed on a BIOCHROM-20 PHARMACIA amino acid analyzer (Biotech). Samples were hydrolyzed in sealed glass tubes under nitrogen using 6 M HCl at 110 °C for 20 h. Analytical RP-HPLC was performed on a Waters Alliance HPLC system (PDA 996 detector, software Millenium Chromatography Manager) using a Vydac C_8 (208TP53, 3.2 × 250 mm) column. Conditions were as follows: gradient 0−100% in 30 min with 2 min lag after sample injection (solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in MeOH) flow rate = 0.75 mL/min, λ = 218 nm. The structures were confirmed by ESI-MS [LCQ ion-trap mass spectrometer (Finnigan Corp.), 4000 controlled unit (Thermo Separation Product, Inc.), Xcalibur software (Finnigan Corp.); positive mode] and MALDI-TOF MS (Reflex IV, Bruker Daltonics; positive mode, α-cyano-4-hydroxycinnamic acid used as a matrix).

Asialo-OSM was prepared by 1 h treatment of OSM (100 mg in 1 mL of distilled water with 1% TFA). The reaction mixture was then lyophilized from water three times.

Fmoc-Ser(3,4,6-tri-*O*-Ac-α-D-GalNAc)-OH was prepared according to the described procedure to yield 1.1 g (7%) of white

lyophilizate.[35] ^{1}H NMR (DMSO-d_6): δ 1.80 (s, 3H), 1.89 (s, 3H), 1.94 (s, 3H), 2.10 (s, 3H), 3.76 (dd, J = 10.8, 5.2 Hz, 1H), 3.82 (dd, J = 10.8, 4.0 Hz, 1H), 3.96 (dd, J = 11.6, 7.2 Hz, 1H), 4.04 (dd, J = 11.2, 6.0 Hz, 1H), 4.15−4.38 (m, 6H), 4.84 (d, J = 3.7 Hz, 1H), 5.04 (dd, J = 11.6, 3.2 Hz, 1H), 5.29 (d, J = 2.4 Hz, 1H), 7.30−7.92 (m, 10 H). Anal. Calcd for $C_{32}H_{36}N_2O_{13}$: C, 58.53, H, 5.53, N, 4.27. Found C 58.28, H 5.56, N 4.11.

Synthesis of Building Blocks 1−8. Compounds **1−7** were synthesized on RINK amide MHBA resin using the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/tBu) strategy, and the compound **8** was prepared on MHBA resin using the *tert*-butyloxycarbonyl/benzyl (Boc/Bn) strategy. Protected amino acids were coupled in 5-fold molar excess using TBTU/DIEA activation. Fmoc-Ser(3,4,6-tri-*O*-Ac-α-D-GalNAc)-OH was coupled in 1.3-fold molar excess using TBTU/DIEA activation. The *O*-acetyl groups protecting the hydroxy groups of the GalNAc residues were removed by treatment of the glycopeptide resin with a 15% solution of hydrazine hydrate in methanol (2 × 40 min). Cleavage from the resin and side chain deprotection were achieved simultaneously using cleavage cocktails that depended on the amino acid composition. Pure compounds were obtained after RP-HPLC in yields ranging from 28% to 48%. Identity and purity of the compounds were confirmed by amino acid analysis, RP-HPLC, and ESI mass spectrometry. For more details see the Supporting Information.

Preparation of Comblike Dendrimers 9−15. Compounds **1−7** were dissolved in 500 μL of a 50% solution of 15 mM phosphate buffer (pH 6.7) in DMF. Linear carrier **8** was dissolved in 250 μL of DMF and added in three aliquots to the solution of corresponding building block **1−7** (effective molar ratio 4.05:1 of the respective compound to the linear KG carrier **8**). Progress of the ligation reaction was monitored by RP-HPLC (**12−14**) or by Ellman's test[36] (**9−11** and **15**). The reaction was typically completed within 12−24 h. The reaction mixture was then poured into 5 volumes of water and lyophilized. Crude products were dissolved in 5% acetic acid, dialyzed using SpectraPor tubes 1,000 MWCO ("molecular weight cutoff"; Spectrum Laboratories, Inc. Rancho Dominguez, CA) for 2 × 12 h, and lyophilized. Identity and purity were confirmed by amino acid analysis, RP-HPLC, and MALDI-TOF mass spectrometry (see the Supporting Information).

Binding Assays. Plant lectins and soluble recombinant NKR-P1A and -B[24] were radiolabeled with Na^{125}I using Iodogen (Pierce, Rockville, IL). Binding assays were performed as described previously.[24] Briefly, 96-well poly(vinyl chloride) microplates (Titertek Immuno Assay-Plate, ICN Flow, Irvine, Scotland) were coated overnight at 4 °C with 50 μL of ligand (100 μg/mL) in 10 mM Tris-HCl, 150 mM NaCl, 1mM $CaCl_2$, and 1 mM NaN_3. The plates were blocked with 1% BSA in the above buffer for 2 h at 4 °C, incubated with the radiolabeled protein at a concentration corresponding to half of the saturating amount and various dilutions of the inhibitors (total reaction volume of 100 μL), washed three times with Tris buffer (see above), and drained. A scintillation solution (100 μL) was added, and the radioactivity in the individual wells was assessed using a β-counter (Microbeta, Wallac, Turku, Finland). All experiments were performed in duplicate and the degree of inhibition was calculated with respect to the control wells (wells containing no inhibitor). Binding assays with the monoclonal antibody 83D4 were performed using the same methodology.

Animals and Immunization Procedures. Eight week old female Balb/c mice (AnLab) were used for immunization. Animals were bred in a pathogen-free animal facility with food and water ad libitum and treated with two doses of synthetic vaccines (100 μg/dose at 2-week intervals) adsorbed on 1 mg of aluminum hydroxide (Sigma-Aldrich) per dose as an adjuvant.[37] To investigate the effect of DNP haptenization of the Tn vaccines, mice were sensitized to DNP by five weekly administrations of a 0.15% DNP solution in acetone and olive oil (3:1) applied to the skin of both ears. The majority of animals responded to the last dose of DNP with a local inflammatory reaction. The significance of Tn antigen conjugation with the IVH T-cell epitope (HA306-318) was also evaluated on IVH-sensitized animals. For this purpose, mice were immunized with two doses (50 μL per dose) of a commercial influenza virus vaccine (Begrivac, Boehringer Mannheim) at 2-week intervals. Vaccination with synthetic Tn antigens was performed 1 week after the last dose of DNP or IVH. Control groups received the vehicle and aluminum hydroxide (control) or were presensitized with IVH/DNP as described above. Serum was obtained from the treated mice 2 weeks after the second dose of the synthetic Tn vaccine.

Analysis of Serum Anti-Tn Antibodies. Antibody levels were determined by ELISA as described elsewhere.[38,39] Briefly, individual animal sera were diluted with phosphate-buffered saline (PBS, pH 7.4) and incubated with BSM-coated plates (Sigma-Aldrich). Specific classes of immunoglobulins were identified using the different goat anti-mouse IgG/IgM/IgA antibodies conjugated to HRP (Sigma-Aldrich). Titers of specific antibodies were determined as the last serum dilution where the absorbance was higher than 2 × SD (standard deviation) relative to control wells.

Cytotoxicity Assays. The standard short-term (4 h) cytotoxic assays were performed and evaluated essentially as previously described.[40]

Flow Cytometric Analysis. Fluorescence-activated cell sorting (FACS) was performed using a modification of the method of Slovin et al.[41] to demonstrate antibody binding to the cell surface of Jurkat cells, a human T lymphoid leukemic cell line known to express Tn.[42] Pre- and postimmunization sera were diluted to 50% of their maximum antibody titer as determined by ELISA and incubated with 10^6 Jurkat cells for 1 h on ice. The cells were washed, treated with rat monoclonal anti-mouse IgG/IgM antibody labeled with fluorescein isothiocyanate (BD Bioscience), and analyzed by flow cytometry. Pretreatment sera-treated samples were gated to the reactivity of 10% positive cells and compared with post-treatment positivity.

Acknowledgment. Dedicated to Prof. Jan Kocourek on the occasion of his 80th birthday. We are grateful to H. Hoffmann (Institute of Organic Chemistry and Biochemistry, Czech Republic) and J. Neuzil (Griffith University, Australia) and to the reviewers for their critical reading of the manuscript and stimulating suggestions. We thank Anna Janošt'áková for technical assistance in the analysis of serum Tn-antibodies. The work on this project was supported by the Ministry of Education of the Czech Republic (MSM 6198959216, MSM 113100001, MSM 21620808), by the Grant Agency of the Academy of Sciences of the Czech Republic (A4020213 and A5020403), Grant Agency of the Czech Republic (304/06/1691), and by an Institutional Research Concept for the Institute of Organic Chemistry and Biochemistry (Z4 055 0506) and the Institute of Microbiology (AVOZ50200510).

Supporting Information Available: Synthetic procedures for the preparation of building blocks (**1−8**) and analytical data of the prepared glycopeptides and glycodendrimers (**1−15**). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM050744G