T-Cell Recognition of Tumor-associated Carbohydrates: The Nature of the Glycan Moiety Plays a Decisive Role in Determining Glycopeptide Immunogenicity

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ABSTRACT

A berrant glycosylation is one of the most constant traits of the malignant cell phenotype. To study T-cell responses to tumor-associated glycans, the mouse hemoglobin-derived decapptide Hb(67—76), which binds well to the MHC class II molecule Eκ and is nonimmunogenic in CBA/J mice, was used. O- or N-glycosylation at its primary T-cell receptor contact residue, position 72, with different glycans attached to either threonine, serine, or asparagine. The carbohydrate moieties included tumor-associated mucins, i.e., the Tn and T antigens, mucin-related glycans, and mucin-unrelated glycans. The side chain of the amino acid in position 72 points away from the MHC binding site when the Hb(67—76) peptide is bound to Eκ, so the assumption was that this was also the case for glycans attached to this position. The glycosylated Hb(67—76) peptide analogues were then studied for binding to Eκ and for immunogenicity in CBA/J mice. All 16 glycopeptides bound well to Eκ, although those with the more complex carbohydrates bound more weakly than those with monosaccharides. Six of 12 O-glycosylated and 0 of 4 N-glycosylated glycopeptides were able to induce a T-cell proliferative response with a stimulation index above 3.0. Some glycophones were not immunogenic, suggesting that there may be holes in the T-cell repertoire due to a lack of T-cell receptor regions accommodating certain glycan structures. The four strongest immunogenic glycophones were all O-glycosylated, and interestingly, three of them carried the tumor-associated Tn or T antigen. On the other hand, the Hb(67—76) peptide analogue with the natural mucin Core2 structure attached did not elicit any T-cell response. T cells primed to a glycopeptide with a simple glycan structure such as Tn did not cross-respond significantly to other glycopeptides, indicating a high degree of carbohydrate specificity in T-cell recognition. T cells primed to a glycopeptide carrying the more complex T antigen showed a complicated pattern of cross-responses to glycopeptides with simpler glycan moieties. The fact that it is possible to raise MHC class II-restricted T-cell responses against tumor-associated carbohydrate structures opens new perspectives for the designing of cancer vaccines.

INTRODUCTION

A berrant glycosylation is one of the most constant traits of the malignant cell phenotype (1), and a significant part of the cancer patient’s humoral antitumor response is directed against cell membrane carbohydrates. The tumor-cell-associated carbohydrate antigens may derive from increased expression of glycans present in low amounts on the normal counterpart cell or expression of glycans not expressed by the normal counterpart cell. The Tn, T, and sialyl-Tn antigens belong to the latter category. These O-linked mucin-type glycans are highly expressed in human adenocarcinomas (2, 3) due to a blockage in mucin chain elongation (4).

The existence in cancer patients of a humoral antitumor response directed against glycan structures has recently been explored thapeutically, and promising clinical results have been obtained in patients with ovarian, breast, and colorectal cancer by immunization with either the T antigen or sialyl-Tn antigen coupled to the carrier keyhole limpet hemocyanin (5–8). Patients developed IgM and IgG antibodies specific for the glycan moieties, and improved patient survival and prolonged remission correlated with antibody production (6, 7). Vaccination of melanoma patients with the ganglioside GM2 coupled to keyhole limpet hemocyanin has yielded similar results (9, 10).

The issue of T-cell responses to tumor-associated carbohydrate antigens has received less attention. There seems to be no spontaneous development of T cell-mediated immune responses against Tn- and T-expressing tumors. It has nevertheless been demonstrated that mice immunized with the Tn- and T-rich glycoprotein epiglycanin or with the Tn and T antigens coupled to protein carriers show prolonged survival after a challenge with the high Tn- and T-expressing tumor TA3-Ha (11). T cells mediating delayed-type hypersensitivity were generated, and they could specifically recognize carbohydrate determinants on the TA3-Ha tumor-associated epiglycanin. Moreover, the T antigen conjugated to the carrier keyhole limpet hemocyanin has been shown to be a successful anticancer immunotherapeutic agent in mice (12). Its administration to mice bearing TA3-Ha tumors provided 25% long-term survival, and the survival rate was significantly increased when immunization of tumor-bearing mice with T antigen-keyhole limpet hemocyanin was preceded by treatment with cyclophosphamide. In a similar study, the role of Tn antigen as an active immunogen against the TA3-Ha tumor was examined (13). Protection against transplants of this highly invasive mouse mammary adenocarcinoma was achieved by prior immunization with neuraminidase-treated Tn-rich ovine submaxillary mucin. The immunized mice showed delayed-type hypersensitivity and T-cell proliferative responses to ovine submaxillary mucin, suggesting that the immunization had raised T helper cells that might be partially responsible for the protective effect.

A recent study of the chemical composition of material eluted from MHC class II molecules (14, 15) revealed the occurrence of glycan groups, which presumably had been attached to some of the MHC-bound peptides. This raises the possibility that tumor-associated carbohydrates attached to peptides may become presented to T helper cells. In the past few years, a number of studies have been carried out with glycopeptides as model antigens for T cells. Experiments with synthetic glycopeptide analogues of well-characterized T-cell epitopes having O- and N-glycosylations placed centrally in the peptide (16–22) or near the terminals (18–20, 23) have shown that glycopeptides are indeed immunogenic and that the carbohydrate moiety may be part of the T-cell epitope. We investigated the consequence of linking the small mucin-type O-glycosylation α-1–GalNAc (the Tn antigen) to the MHC class II Eκ binding peptide Hb(67—76)3

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3 The abbreviations used are: Hb(67—76), peptide derived from CBA/J mouse hemoglobin β chain; Hb(66—76)Y, peptide derived from CBA/J mouse hemoglobin β chain extended with a COOH-terminal tyrosine; T72, Hb(67—76) substituted with threonine at position 72; S72, Hb(67—76) substituted with serine at position 72; T72(S72), Hb(67—76) substituted with serine (Tn) at position 72; S72(T72), Hb(67—76) substituted with serine (Tn) at position 72; TCR, T-cell receptor; LNC, lymph node cell; dTh, thymidine; SI,
derived from CBA/J mouse hemoglobin (24). In the CBA/J (H-2b) mouse, Hb(67–76) is a self-peptide of which the mouse is naturally tolerant. It was shown that substitution of solvent accessible amino acid positions in the antigen within the MHC binding core region (25) with the Tn group O-linked to serine or threonine did not prohibit the binding of the glycopeptide to the MHC molecule. Furthermore, glycopeptides with the Tn antigen positioned at the primary TCR contact residue of the Hb(67—76) peptide (26–28), were immunogenic in the CBA/J mouse (24).

In the present work, we studied the repertoire of glycan recognition by T cells. The primary TCR contact residue of the Hb(67—76) peptide was glycosylated with different glycan groups, and the effects on glycopeptide binding to MHC and glycopeptide immunogenicity and the ability of primed T cells to discriminate between the different glycan were studied. The glycan attached to position 72 were selected to represent tumor-associated carbohydrate antigens, mucin-related glycans, and mucin-unrelated glycans, and they varied with respect to sugar nature, complexity, branching, and anomeric configuration. We report that the nature, complexity, branching, and anomeric configuration of the glycan group did not substantially affect the glycopeptide binding to \\E, but only about half of the glycopeptides were immunogenic. Some T-cell cross-responses to glycopeptides with simpler structurally related glycans were also observed.

**MATERIALS AND METHODS**

**Synthesis of Hb(67–76) Glycopeptide Analogues.** The synthesis of the Hb(67–76) glycophosphate analogues was performed by multiple column techniques using Fluoro-9-yl-methoxybenzyl amino acid-pentafluorophenyl esters as described elsewhere (29). The first amino acid was linked to either kieselguhr-supported polyamide resin or polyethylene glycol dimethyl acrylate. The subsequent amino acids were coupled to the resin by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. More complex glycan structures were attached as described elsewhere (30). The glycosylated peptides were cleaved from the resin by 95% trifluoroacetic acid, and the final purification was achieved by high-performance liquid chromatography using 2-azido-2-deoxy-2,3,6-tri-O-acetyl-D-galactopyranosyltrichloroacetimidate as a glycosyl donor. More complex glycans were introduced as described elsewhere (30). The glycosylated peptides were cleaved from the resin by 95% trifluoroacetic acid, and the final deacetylation of the sugar moiety was achieved by treatment with dilute sodium methoxide. The chemical structure of the glycan groups N- and O-linked to Hb(67–76) glycopeptide analogues is shown in Fig. 1.

**Peptide Radiolabeling.** Peptide Hb(67–76)Y (2 μg) was labeled with 1.0 μCi of 25I (Amersham, Little Chalfont, United Kingdom) using chloramine T as described previously (31, 32). The peptide was separated from free iodine by Sephadex G10 (Pharmacia, Uppsala, Sweden) size exclusion chromatography and kept at −20°C until use. In all experiments, the peptide specific activity was approximately 1×10^6 cpm/pmol. The radiolabeled peptide was more than 95% pure as analyzed by reverse-phase high-performance liquid chromatography using on-line γ spectrometry (Packard Instruments, Downers Grove, IL).

**MHC Class II Purification.** Murine E molecules were produced from an AKR-derived B lymphoma cell line, AKTB-1b, as described previously (31, 32), using the monoclonal antibody 14–4–45 covalently coupled to 10 mg/ml to cyanogen bromide-activated Sepharose 4B (Pharmacia). The protein content of the MHC preparations was determined using a micro-bicinchoninic acid assay. The protein content was calculated. The resulting structure is shown in Fig. 2.

**RESULTS**

**Binding of Glycopeptides to E.** First, the ability of the Hb(67–76) glycophosphate analogues to bind to E molecules was assessed. The relative binding was measured in a competitive binding assay in which each glycopeptide was added to a mixture of affinity-purified E molecules and 125I-labeled Hb(64–66)Y peptide. The capacity of the glycopeptide to inhibit the binding of the labeled peptide to E was calculated. Fig. 3 shows the binding curves for peptide Hb(67–76) and the substituted glycopeptides 4–19. The binding data for glycopeptides 4 and 5 were the same as those reported previously (24). All of the glycopeptides were able to bind to E, and their binding curves ran in parallel to that of Hb(67–76). A nonbinding peptide (TSSPATSAPE) was included as a control.

On the basis of the IC50 values, the glycopeptide binding relative to...
Fig. 1. Primary structure of peptides and glycopeptides used in the study. The threonine-substituted peptide 2 and serine-substituted peptide 3 are based on the wild-type Hb(67–76) 1. The substitution was made at the Asn72 in the wild-type peptide. In the text, peptides 2 and 3 will be called the Hb peptides T72 and S72, respectively. Likewise, glycopeptide 4 will be called T72 (Tn) and glycopeptide 5 will be called S72 (Tn), in accordance with a previous report (24). These names have been given to the compounds used most frequently. They will be used in the text concurrently with the peptide or glycopeptide number to assist the reader in identifying the chemical structure of the compound. The glycan of compounds 12 and 13 is also called the T antigen, and the glycan of compound 19 is called Core2. The chemical names of the glycans and the amino acid to which the glycan is attached are as follows: 4, T(α-α-GalNAc); 5, S(α-α-GalNAc); 6, S(β-β-GalNAc); 7, T(β-β-Gal); 8, S(α-α-Fuc); 9, S(β-β-Fuc); 10 and 11, N(β-β-GlcNAc-N); 12, T(β-β-Gal-(1→3)-α-α-GalNAc); 13, S(β-β-Gal-(1→3)-α-α-GalNAc); 14, N(α-α-Glc-(1→4)-β-β-Glc-N); 15, T(α-α-Man); 16, N(α-α-Glc-(1→4)-α-α-Glc-(1→4)-β-β-Glc-N); 17. T(α-β-GlcNAc); 18, T(α-β-GalNAc); 19, T(β-β-Gal-(1→3)-β-β-GlcNAc-(1→6)-α-α-GalNAc).
the nonmodified Hb(67–76) 1 binding was calculated as the percentage of binding. Table 1 presents the relative binding efficiencies of all 16 glycopeptides. Glycopeptides 4–10, 15, 17, and 18 carrying a monosaccharide moiety were on average at least as good as Hb(67–76) 1 in binding to Eκ. Glycopeptides 12–14, 16, and 19 carrying more complex glycan moieties were still able to bind to Eκ, although with an average binding efficiency of 78%, whereas that of the Hb(67–76) peptide is 100%. The nonglycosylated substituted peptides T72 (peptide 2) and S72 (peptide 3) bound with 300% efficiency (24). The addition of a third glucose residue to glycopeptide 14, giving rise to glycopeptide 16, reduced the binding only a little. Glycopeptides 8 and 9 with a glycan in the L-isomeric form bound with the same efficiency as the glycopeptides carrying a glycan in the D-form. The different anomeric configuration of the fucose residue in glycopeptides 8 and 9 did not affect the glycopeptide binding ability. Moreover, glycopeptide 6, in which GalNAc is O-linked to S72 in the β configuration, showed the same binding efficiency as glycopeptide analogues 4 and 5 with Tn O-linked to T72 and S72 in the α configuration (24). We observed a significant reduction in the binding efficiency for glycopeptide 11. Glycopeptides 10 and 11 carried the same saccharide (β-D-GlcNAc) N-linked to Asn72, but they differed in one amino acid residue in the peptide sequence, with glycopeptide 11 having T in position 74 instead of G. Surprisingly, the substitution of the N-acetyl group with an azido group in the α-D-GalNAc moiety yielded a superior binding glycopeptide (glycopeptide 18). However, this influence could be expected by careful inspection of Fig. 2, in which the CH3 group (visible at the bottom of the glycan) of the N-acetate is seen to be in direct contact with the MHC molecule. Glycopeptide 19 with the large, branched glycan bound well to the Eκ molecule (71%), in spite of its bulky character.

Proliferative Response of Glycopeptide-primed T Cells. Once their capacity for binding to Eκ had been assessed, the 16 glycopeptides and wild-type peptide Hb(67–76) 1 were tested for their immunogenicity in CBA/J mice. Table 2 shows the proliferative responses of T cells from mice immunized with either 1 of 16 glycopeptides or the nonmodified Hb(67–76) peptide to the antigen used for the immunization. The proliferative responses for glycopeptides 4 and 5 have been reported previously (24). They are included to allow comparisons. For practical purposes, we chose to regard as significant a proliferation with an experimental/control index (SI) higher than 3.0. Glycopeptides 4, 5, 6, 7, 13, and 15 met this criterion, but glycopeptides 9, 10, 11, 12, 14, 16, 17, and 18 also elicited a statistically significant response. The nonglycosylated substituted peptides T72 (peptide 2) and S72 (peptide 3) have both previously been found to be immunogenic, with SIs of 4.6 and 3.6, respectively (24). The results in Table 2 were used to calculate the SIs depicted in Fig. 4.

Cross-Responses of Glycopeptide-primed T Cells to Other Glycopeptides. The four most immunogenic glycopeptides were glycopeptides 4, 5, 7, and 13. To investigate whether T-cell recognition is saccharide specific, T cells from mice immunized with one of these glycopeptides were tested for proliferative response to the immunogenic O-glycosylated glycopeptides 4, 5, 6, 7, 13, and 15 and to the two nonglycosylated substituted peptides 2 and 3. The T-cell proliferative responses varied, depending on the glycopeptide used for immunization (Fig. 5). T cells primed to glycopeptide 4 (Fig. 5A) responded only to the glycopeptide used for the immunization, although some cross-reactivity (SI = 2.9) toward peptide 2 was observed, in agreement with previous observations (24). T cells primed to glycopeptide 5 (Fig. 5B) also responded only to the glycopeptide...
used for the immunization, although they showed some cross-reactivity to the corresponding nonglycosylated peptide (SI = 2.5) and to glycopeptide 6 (SI = 2.9). Fig. 5C shows that T cells primed to glycopeptide 7, which contains β-α-Gal attached to T72, did not cross-respond to any peptide or glycopeptide. Finally, Fig. 5D presents the cross-response of T cells primed to glycopeptide 13 with the T antigen linked to S72. These T cells cross-responded to glycopeptides 5 and 6 and, to a lesser extent, to S72-containing nonglycosylated peptide 3. The cross-responses are also illustrated in Table 3, which shows the SIs calculated from the data obtained in the experiments presented in Fig. 5. Responses and cross-responses with a SI of 3.0 or more are shown in bold in Table 3.

Table 4 summarizes all of the results obtained with Hb(67–76) analogues 2–19 concerning their ability to bind to Eα, immunogenicity, and cross-reactivity.

DISCUSSION

The aim of the present study was to assess whether different peptide-attached glycans can be specifically recognized by T cells. A set of glycopeptides was synthesized, all of which had essentially the same Hb(67–76) 1 peptide derived from mouse hemoglobin and carried a saccharide attached to position 72 of the peptide. Each glycopeptide had a distinct carbohydrate attached, representing the
Table 1 Relative binding to MHC class II Eα molecules of Hb(67–76) glycopeptide analogues

<table>
<thead>
<tr>
<th>Peptide/glycopeptide used for immunization</th>
<th>Mean cpm [3H]dTh incorporation/culture ± SD</th>
<th>Glycopeptide/Chemical name</th>
<th>Carbohydrate type</th>
<th>% bindinga</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, Hb(67–76)</td>
<td>3216 ± 425</td>
<td>T72(α-D-Gal)</td>
<td>Mucin</td>
<td>87b</td>
</tr>
<tr>
<td>4, T72 (Tn)</td>
<td>2004 ± 204a</td>
<td>T72(α-D-GalN)</td>
<td>Mucin-unrelated</td>
<td>87</td>
</tr>
<tr>
<td>5, S72 (Tn)</td>
<td>3076 ± 628b</td>
<td>S72(β-D-GalN)</td>
<td>Mucin-unrelated</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>1904 ± 269b</td>
<td>N72(β-D-GalN-α-GlcNAc)</td>
<td>Mucin-related</td>
<td>93</td>
</tr>
<tr>
<td>7</td>
<td>3707 ± 579</td>
<td>T72(β-D-Gal-1-3)-α-GalN</td>
<td>Mucin-related</td>
<td>118</td>
</tr>
<tr>
<td>8</td>
<td>7591 ± 729</td>
<td>T72(β-D-Gal-1-3)-α-GalN</td>
<td>Mucin-unrelated</td>
<td>87</td>
</tr>
<tr>
<td>9</td>
<td>6621 ± 784</td>
<td>T72(β-D-Gal-1-4)-β-D-GalN</td>
<td>Mucin-related</td>
<td>43</td>
</tr>
<tr>
<td>10</td>
<td>5854 ± 1191</td>
<td>T72(β-D-Gal-1-4)-β-D-GalN</td>
<td>Mucin-related</td>
<td>76</td>
</tr>
<tr>
<td>11</td>
<td>2073 ± 660</td>
<td>T72(α-GalN3)</td>
<td>Mucin-related</td>
<td>68</td>
</tr>
<tr>
<td>12</td>
<td>1301 ± 638</td>
<td>T72(α-GalN3)</td>
<td>Mucin-unrelated</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>2194 ± 469</td>
<td>T72(α-D-GalN)</td>
<td>Mucin-related</td>
<td>93</td>
</tr>
<tr>
<td>10</td>
<td>5854 ± 1191</td>
<td>T72(α-D-GalN)</td>
<td>Mucin-related</td>
<td>76</td>
</tr>
<tr>
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<td>2073 ± 660</td>
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<td>68</td>
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<tr>
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<td>1301 ± 638</td>
<td>T72(α-D-GalN)</td>
<td>Mucin-unrelated</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>1904 ± 269b</td>
<td>N72(β-D-GalN-α-GlcNAc)</td>
<td>Mucin-related</td>
<td>139</td>
</tr>
<tr>
<td>14</td>
<td>3707 ± 579</td>
<td>T72(β-D-Gal-1-3)-α-GalN</td>
<td>Mucin-related</td>
<td>192</td>
</tr>
<tr>
<td>15</td>
<td>7591 ± 729</td>
<td>T72(β-D-Gal-1-3)-α-GalN</td>
<td>Mucin-unrelated</td>
<td>71</td>
</tr>
</tbody>
</table>

Most common tumor-associated mucins, mucin-related glycans, or mucin-unrelated glycans. Fourteen Hb(67–76) glycopeptide analogues with N, S, or T at position 72 were synthesized, and the carbohydrate moieties N- or O-linked to this position varied with respect to the nature, complexity, branching, and anomeric configuration of the carbohydrate. The two Tn-glycosylated glycopeptides (peptides 4 and 5), also designated here as T72 (Tn) and S72 (Tn) and already studied in a preceding report (24), were also included (see Fig. 1). The Hb(67–76) peptide is nonimmunogenic in CBA/J mice, but it is known to bind well to the MHC class II molecule Eα, using Ieα and Lysb6 as the major anchor residues (26, 27, 36). Position 72, to which the glycan was attached in the present panel of glycopeptides, is an asparaginyl in CBA/J mouse hemoglobin. From structural studies (25), it is known that the asparagine side chain projects away from the Eα molecule and up toward the TCR when the Hb(67–76) peptide is lying in the Eα molecule binding groove. For the present investigation, the assumption was that the glycan attached to position 72 would also project away from the MHC molecule and toward the TCR. This was confirmed by molecular modeling (Fig. 2).

All of the 16 glycopeptides bound to the Eα molecule, confirming the results obtained in a previous paper (24). Glycosylated Hb(67–76) analogues carrying di- and trisaccharide groups bound slightly less well than did the glycopeptides carrying monosaccharides (average percentage of binding, 78 versus 103%). However, the nonglycosylated substituted Hb(67–76) peptides 2 and 3 bound with an efficiency

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### Fig. 4.

Slts obtained in proliferation assay from T cells primed with glycopeptide. Slts were calculated from the formula SI = Δcpm [3H]dTh incorporation in response to optimal glycopeptide dose/Δcpm of [3H]dTh incorporation in response to PBS using the data in Table 2. Column with bars, the SI mean with SE calculated from the results of two, three, or four immunization experiments. Columns without bars, the SI obtained in one immunization experiment. The Slts for glycopeptides 4 and 5 have been taken from Jensen et al. (24).
of 300% compared to that of unmodified peptide 1 (24), and the influence of the glycan on binding, probably through the modification of peptide structure and dynamics, can therefore be considered to be significant.

The anomeric configuration of the sugar moiety did not seem to affect the glycopeptide binding efficiency. Glycopeptide 6, which carried the Tn antigen O-linked to S in the β configuration, had a binding efficiency identical to that of glycopeptides 4 and 5, in which Tn O-linked to T and S, respectively, was in the α configuration (24). Moreover, fucose-conjugated glycopeptides 8 and 9 showed almost the same binding ability, despite the fact that the fucose group was in the α configuration in the former and in the β configuration in the latter. The two glycopeptides carrying L-fucose bound to Eβ, as did the other glycopeptides carrying a monosaccharide in the D-form. The lack of specific influence of the sugar on the binding confirms the results of the molecular modeling, indicating that the glycosylated position is directed well away from the binding cleft.

The N-glycosylated glycopeptides bound to Eβ, as did the O-glycosylated ones with the exception of glycopeptide 11. This glycosylated Hb(67–76) analogue carried β-D-GlcNAc N-linked to Asn72 as glycopeptide 10, but it contained T in position 74 instead of G to introduce an N-glycosylation site in the peptide. The relatively poor binding of this compound, due most probably to the substitution with T, was not unexpected, because the same result was found in a previous study (24). Glycopeptide 18, in which an azido group instead of an acetamido group is attached to the 2 position of the carbohydrate, was a better binder to Eβ than any of the other glycosylated Hb(67–76) analogues, yet it was not immunogenic. The pronounced effect of the azido group on binding can be rationalized by inspection of the structures of peptides 17 and 18 and Fig. 2 derived by MD simulation. The CH₃ group of the N-acetate, visible below the oligosaccharide, is pointing down toward the MHC binding cleft to give direct MHC contact. The modification to azide may therefore influence glycopeptide binding by direct interaction.

Although all of the glycopeptides bound to Eβ and thus fulfilled the basic requirement for being immunogenic, not all of them were

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**Table 3** SI of glycopeptide-primed T-cell responses and cross-responses to O-glycosylated Hb(67–76)-based glycopeptides with various glycan groups

<table>
<thead>
<tr>
<th>Glycopeptide used for priming</th>
<th>4, T⁷²(Tn)</th>
<th>5, S⁷²(Tn)</th>
<th>6</th>
<th>7</th>
<th>13</th>
<th>15</th>
<th>2, T⁷²</th>
<th>3, S⁷²</th>
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<tbody>
<tr>
<td>4, T⁷²(Tn)</td>
<td>3.6</td>
<td>2.3</td>
<td>2.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.5</td>
<td>2.9</td>
<td>1.8</td>
</tr>
<tr>
<td>5, S⁷²(Tn)</td>
<td>1.8</td>
<td>4.2</td>
<td>2.9</td>
<td>1.2</td>
<td>1.8</td>
<td>0.8</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>7</td>
<td>1.8</td>
<td>1.1</td>
<td>1.6</td>
<td>5.3</td>
<td>1.0</td>
<td>1.3</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>13</td>
<td>2.6</td>
<td>5.3</td>
<td>5.4</td>
<td>4.5</td>
<td>5.5</td>
<td>1.7</td>
<td>2.1</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*SI* values were calculated from the formula SI = Δcpm ([3H]dTh incorporation in response to glycopeptide)/Δcpm ([3H]dTh incorporation in response to PBS) using the data from the experiments in Fig. 5. The highest [3H]dTh incorporation cpm value obtained from the three antigen doses was used for calculation of the SI of each response.

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T-CELL RECOGNITION OF TUMOR-ASSOCIATED CARBOHYDRATES
actually immunogenic. The most immunogenic glycopeptides, i.e., those eliciting SIs higher than 3.0 from primed T cells, were glycopeptides 4, 5, 6, 7, 13, and 15. They are all O-glycosylated. The immunogenic glycopeptides had no distinct structural features compared to the nonimmunogenic ones. However, it seems that small glycan structures of the mucin glycosylation pathway present on tumor cells with aberrant glycosylation or closely related structures are more readily recognized by the TCR. Immunogenic glycopeptides 5 and 6 carried the same carbohydrate moiety, and they differed only in the anomeric configuration of the glycan (GalNAc in the α configuration in the former and in the β configuration in the latter). In glycopeptide 7, the carbohydrate group was a β-D-Gal O-linked to T72. Although the difference between this monosaccharide and theglycan size or structure; (b) some glycopeptides may mimic naturally to a lack of TCR regions accommodating certain glycans because ofglycopeptides, in spite of their binding to MHC class II molecules, islion, was not immunogenic. The nonimmunogenicity of these two
tide 8, which had L-fucose O-glycosylated to S72 in the α configura
tion and the substitution of an N-acetyl group withanomeric configuration of the glycan (GalNAc in the α con
structure remains whether the MHC-bound peptide secondary structure is different when it has a glycan attached. This is unlikely, as indicated by previous work (24) that demonstrated that glycosylation without loss of binding is only allowed at the Val67 and Asn72 positions, which are the only well-exposed residues of the bound peptide ligand in its extended conformation.

To study whether T cells recognize the glycopeptide glycan moiety specifically, we performed a series of experiments with six immunogenic O-glycosylated Hb(67–76) analogues. The four most immunogenic ones were used to immunize mice, and their primed T cells were tested for proliferative response against the glycopeptide used for the immunization, the five other O-glycosylated immunogens and the two nonglycosylated T72- and S72-substituted Hb(67–76) peptides. T cells primed with the simple glycopeptides 4, 5, and 7 did not give significant cross-responses, indicating that the glycan group was recognized with high specificity. T cells primed with the more complex glycopeptide 13 displayed a complicated pattern of cross-responses directed against the corresponding naked peptide 3 and the Tn-glycosylated glycopeptide 5. It must be stressed that T cells primed to peptide 2 or 3 completely fail to respond to the corresponding Tn glycopeptide (24). We therefore believe that the cross-responses of glycopeptide-primed T cells to naked peptide may reflect a small extent of deglycosyla
tion of the glycopeptide antigen during the priming phase of the immune response, as discussed in Refs. 24 and 45.

Enhancement of the natural B-cell response directed against glycan structures in cancer patients using tumor-associated carbohydrate antigens coupled to appropriate carriers has proved to be a promising therapeutical tool, at least in some carcinomas and in melanoma. In the present study, we observed a specific MHC class II-restricted T-cell response against well-characterized tumor-associated carbohydrate antigens. Our results, therefore, raise the possibility of designing new glycopeptide-based vaccines against tumor antigens, with the aim of eliciting glycan-specific T helper cells that in turn, through the Th2 pathway, may lead to the production of high affinity glycan-specific antibodies.

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T-Cell Recognition of Tumor-associated Carbohydrates: The Nature of the Glycan Moiety Plays a Decisive Role in Determining Glycopeptide Immunogenicity

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