

Carbohydrate and Peptide Specificity of MHC Class II-Restricted T Cell Hybridomas Raised Against an O-Glycosylated Self Peptide¹

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MHC class II E^k-restricted, IL-2 secreting T cell hybridomas were raised against the synthetic glycopeptide Hb(67–76)- α -GalNAc, (T⁷²(Tn)), in CBA/J mice (H-2^k). The fine specificity of the hybridomas against the glycan moiety was investigated by testing their response against a panel of Hb(67–76)-derived glycopeptides, all with a glycan attached to serine or threonine at the position 72 in the peptide, but with different glycans. The hybridomas showed a high degree of specificity for the α -GalNAc moiety with few and faint cross-responses to the glycopeptides having other glycans attached even though some of these were structurally very similar to α -GalNAc. The fine specificity of the hybridomas for the peptide moiety was investigated by testing their responses to a panel of Hb(67–76)- α -GalNAc glycopeptides with alanine substitutions at all positions except at the two MHC binding anchor positions, I⁶⁸ and K⁷⁶, and the T⁷² to which the α -GalNAc was attached. Glycopeptides substituted with alanine at positions where the amino acid side chain pointed toward the TCR did not stimulate the hybridomas, whereas glycopeptides substituted with alanine at positions orientated down into the MHC binding groove stimulated many of the hybridomas. These results indicate that the glycan attached to the peptide as well as solvent-accessible parts of the peptide are recognized with a high degree of specificity by the T cells, whereas the parts of the peptide buried in the MHC binding site are less important or totally ignored by the T cells. *The Journal of Immunology*, 1997, 158: 3769–3778.

Work from the last 5 years has made clear that T cells primed against certain glycopeptides give a proliferative or cytotoxic response to the immunizing glycopeptide (1–11).

As we report elsewhere,³ we have studied the sugar specificity of the T cell response to a number of glycopeptides differing in the sugar moiety but all with the glycan attached to the same residue of the mouse hemoglobin-derived peptide Hb(67–76)⁴ or VITAFNEGLK, which is nonimmunogenic in the CBA/J mouse,

but which is known to bind very well to the MHC class II molecule E^k (12, 13). This peptide is immunogenic in the CE/J mouse (also H-2^k), and the N⁷² is the primary TCR contact residue for CE/J T cells (14). Structural studies of the Hb(67–76) peptide-E^k complex have shown that the side chain of N⁷² is pointing away from the MHC molecule when the peptide lies in the binding groove of E^k (15). Guided by this information, we synthesized a number of glycopeptides with different O- or N-linked glycans at position 72 of the Hb(67–76) peptide and studied their binding to E^k and immunogenicity, and the glycan specificity of the T cell response.³ All of the glycopeptides bound to E^k and several of them were immunogenic. When T cells were primed to a glycopeptide carrying a simple glycan structure, they cross-responded only slightly to the corresponding unglycosylated peptide, and no significant cross-response was observed to other glycopeptides. This indicated that the primed T cells had a high degree of carbohydrate specificity. However, since the experiments were all performed with primed T cells taken directly from the immunized mouse, the results bear only on the polyclonal T cell proliferative response where minor cross-responses may be difficult to detect.

To perform a thorough analysis of the carbohydrate and peptide specificity of the glycopeptide-specific T cell clones, we raised a panel of T cell hybridomas against the glycopeptide 4 (T⁷²(Tn)). This glycopeptide contains the small mucin-type O-glycosylation N-acetylgalactosamine (α -D-GalNAc) frequently expressed by tumor cells and known as the Tn-antigen, attached to residue 72 of the Hb(67–76) peptide 1. With the resulting panel of hybridomas we have performed a detailed analysis of the repertoire of clones responding to immunization with T⁷²(Tn). We report here that T cell hybridomas recognizing the glycopeptide were highly dependent on the GalNAc, but some of them accepted large differences in the peptide structure.

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⁴ Abbreviations used in this paper: Hb(67–76), peptide derived from CBA/J mouse hemoglobin β -chain; F-moc, 9-fluorenylmethoxycarbonyl; α -GalNAc or Tn, N-acetyl- α -D-galactosamine; β -Gal, β -D-galactose; α -GlcNAc, N-acetyl- α -D-glucosamine; Hb(64–76)Y, peptide derived from CBA/J mouse hemoglobin β -chain extended with a carboxyl-terminal tyrosine; HSP(236–245), peptide derived from mouse heat shock protein 70; HSP-Tn, HSP(236–245) glycosylated with α -GalNAc; S⁷², Hb(67–76) substituted with serine at position 72; S⁷²(Tn), Hb(67–76) substituted with serine-Tn at position 72; T⁷², Hb(67–76) substituted with threonine at position 72; T⁷²(Tn), Hb(67–76) substituted with threonine-Tn at position 72; for abbreviations of peptides and glycopeptides see also Figure 1.

Materials and Methods

Peptides and glycopeptides

The synthesis of the Hb(67–76)-modified peptides was performed by multiple column techniques using 9-fluorenylmethoxycarbonyl (F-moc) amino acid pentafluorophenyl esters, described in detail elsewhere (16). The first amino acid was linked to MacroSorb 250 resin (Sterling Organics, Dudley, Carmington, U.K.) by an acid-labile 4-hydroxymethylphenoxycetic acid linker. Incorporation of GalNAc into F-moc/pentafluorophenyl-protected hydroxyamino acids (serine/threonine) was achieved with high stereoselectivity using the trichloroacetimidate method and 2-azido-2-deoxy-2,3,6-tri-O-acetyl-D-galactopyranosyl trichloroacetimidate as a glycosyl donor. The peptides and glycopeptides were cleaved from the resin by 95% trifluoroacetic acid and the final deacetylation of the sugar moiety of the glycopeptide was achieved by treatment with dilute sodium methoxide. The sequences, structures, and nomenclature of the peptides and glycopeptides used are shown in Figure 1 and discussed in the legend.

Preparation of T cell hybridomas

Seven- to eight-week-old female CBA/J mice (H-2^k) were immunized in the hind footpads and at the base of the tail with 0.1 ml of water in oil emulsion containing equal amounts of glycopeptide T⁷²(Tn) 4 in PBS and CFA with *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI). A total of 100 µg of the Ag was injected into each mouse. Lymph node cells from popliteal and inguinal lymph nodes of the immunized mice were prepared on day 10 after immunization and cultured in tissue culture media RPMI (Life Technologies, Roskilde, Denmark) containing 10% FCS for 72 h in cluster trays (Nunc A/S, Roskilde, Denmark) using 2 mg/ml glycopeptide for restimulation. The restimulated T cells were fused using PEG with the TCR double-negative fusion partner BWα[−]β[−] (17). To ensure clonality, all hybridomas were cloned by limiting dilution in 96-well microtiter plates containing 1, 0.3, and 0.1 cell/well and growing T cell hybridomas were tested for glycopeptide specificity as described below.

Assay for IL-2 production

T cell hybridomas were cultured in 96-well flat-bottom microtiter plates at 10⁵ cells/well together with 10⁵ mitomycin treated cells of the B cell lymphoma CH27 (H-2^k). Peptides or glycopeptides were added in the *in vitro* culture at 10, 3, 1, 0.3, 0.1, and 0.03 µg/ml peptide or glycopeptide. Background proliferation was measured in cultures to which an equivalent volume of PBS had been added. The final volume per well was 200 µl. Supernatant (100 µl) was harvested after 24 h and the contents of IL-2 in the culture medium was measured using the IL-2-dependent cell line HT-2 (4000 cells/well). [³H]Thymidine (Amersham, Copenhagen, Denmark) (1 µCi) was added to each well after 4 h. Cells were harvested on a Filtermate 196 (Packard Instruments, Downers Grove, IL) after 20 h in culture. The [³H]thymidine incorporation was determined by liquid scintillation counting (Topcount, Packard Instruments).

Peptide radiolabeling

The peptide Hb(64–76)Y (GKKVITAFNEGLKY) (2 µg) was labeled at the tyrosine with 0.5 mCi ¹²⁵I (Amersham) using chloramine T as previously described (18, 19). The labeled 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 10⁵ cpm/pmol. The radiolabeled peptide was more than 95% pure as analyzed by reverse phase HPLC using on-line gamma spectrometry (Packard Instruments).

MHC class II purification

Murine E^k molecules were produced from the AKR-derived B lymphoma cell line, AKTB-1b, as previously described (18, 19) using the mAb 14-4-4S covalently coupled at 10 mg/ml to cyanogen bromide-activated Sepharose 4B (Pharmacia). The protein content of the MHC preparations was determined using a micro-bicinchoninic acid (BCA) assay (Pierce, Rockford, IL) and the purity was confirmed by SDS-PAGE and silver staining.

Peptide-MHC class II binding assay

Binding of peptide and glycopeptide to E^k was measured by incubating a mixture of 10⁵ cpm ¹²⁵I-labeled Hb(64–76)Y peptide with 5 µM of affinity-purified E^k molecules together with different concentrations of peptide or glycopeptide at room temperature for 3 days. The reaction buffer contained 8 mM citrate, 17 mM phosphate, and 0.05% Nonidet-P40 (pH 5.0). Peptide-E^k complexes were separated from free peptide by gel filtration using Sephadex G25 (Pharmacia) spun columns (18, 19) and binding was

determined by measuring the radioactivity of the excluded void volume as well as the included volume using a gamma counter (Cobra Autogamma, Packard Instruments). The fraction of peptide bound to MHC relative to the total amount of offered peptide was calculated. Based on these results, IC₅₀ values were determined for each peptide analogue. The relative bindings of peptide analogues 2 to 19 were subsequently calculated as percent of the binding of the nonmodified Hb(67–76) peptide 1 using the formula: binding of peptide analogue = (IC₅₀ of nonmodified peptide)/(IC₅₀ of peptide analogue) × 100. The binding experiments were performed at least twice with identical results. Nonspecific binding was between 0 and 1%.

FACS analysis

T cell hybridomas were tested for CD4 and CD8 expression. Cell staining was performed by using rat phycoerythrin-conjugated rat anti-mouse CD4 (H129.19) and rat anti-mouse CD8 (53–6.7) Abs (PharMingen, San Diego, CA). Cells (10⁶) were added to each FACS tube. The cells were incubated with primary Ab diluted in FACS-buffer (PBS containing 0.1% NaN₃ and 5% FCS) for 30 min at 4°C in the dark. After incubation the cells were washed three times in FACS buffer and fixed in FACS buffer containing 1% paraformaldehyde before the FACS analysis. The samples were analyzed with a FACScan from Becton Dickinson, Mountain View, CA.

MHC restriction of hybridomas

Two L cell lines, RT 4.15HP transfected with A^k and DCEK Hi7 transfected with E^k, kindly supplied by Ronald Germain (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), were used to map the MHC restriction of the hybridomas. The hybridomas were cultured with glycopeptide and the transfected cell lines. The MHC restriction to E^k was furthermore confirmed by the ability of the monoclonal anti-E^k Ab 14-4-4S to block the Ag presentation of mitomycin C-treated CH27. The CH27 lymphoma was cultured with glycopeptide and graded doses of 14-4-4S before addition of the glycopeptide-specific hybridoma. The supernatant was assayed for IL-2 as described above.

Test for effect of soluble GalNAc on hybridoma recognition

Soluble α-D-GalNAc was used in attempts to stimulate an IL-2 response of the glycopeptide specific T cell hybridomas. Test for inhibition of the hybridoma response was performed with mitomycin C-treated B cell lymphoma CH27 pulsed with a suboptimal dose of glycopeptide T⁷²(Tn) 4 for 2 h. The peptide-pulsed cells were washed and cultured in a 96-well microtiter plate (10⁵ cells/well). The hybridoma was added together with a 10-fold dilution of the *p*-nitrophenyl glycoside of α-D-GalNAc (Sigma Chemical Co., St. Louis, MO) starting from 10 µg/ml. The supernatant was harvested after 24 h and the contents of IL-2 was measured as described above.

Results

Response pattern of the T cell hybridomas

To investigate the fine specificity of the T cell response toward the glycopeptide 4 (T⁷²(Tn)) (10), a panel of T cell hybridomas directed against the glycopeptide was prepared. CBA/J female mice were immunized with glycopeptide 4 and the lymph node cells were cultured and fused with the TCR negative cell line BWα[−]β[−] (17). The T cell fusion generated approximately 120 hybridomas that were initially tested for response to T⁷²(Tn) glycopeptide 4, against which the hybridomas had been raised, and for response to the T⁷² peptide 2, and glycopeptide 5, S⁷²(Tn). Forty-four of the generated hybridomas responded to 1 or 2 of these Ags. From these, 22 were selected for detailed study, which showed stable growth and gave a vigorous response to at least one of the three Ags. All of the 22 hybridomas showed one of the four response patterns illustrated in Figure 2. One pattern was shown by 5 hybridomas that recognized only the T⁷²(Tn) 4 glycopeptide against which they were raised (Fig. 2A). Some of the T⁷²(Tn) glycopeptide-specific hybridomas had a small cross-response toward the S⁷²(Tn) glycopeptide 5 (not shown). By repeated clonings of these hybridomas no changes in cross-response were obtained, indicating that the cross-responding hybridomas were not mixtures of 2 or more hybridomas. A second pattern was shown by 2 hybridomas

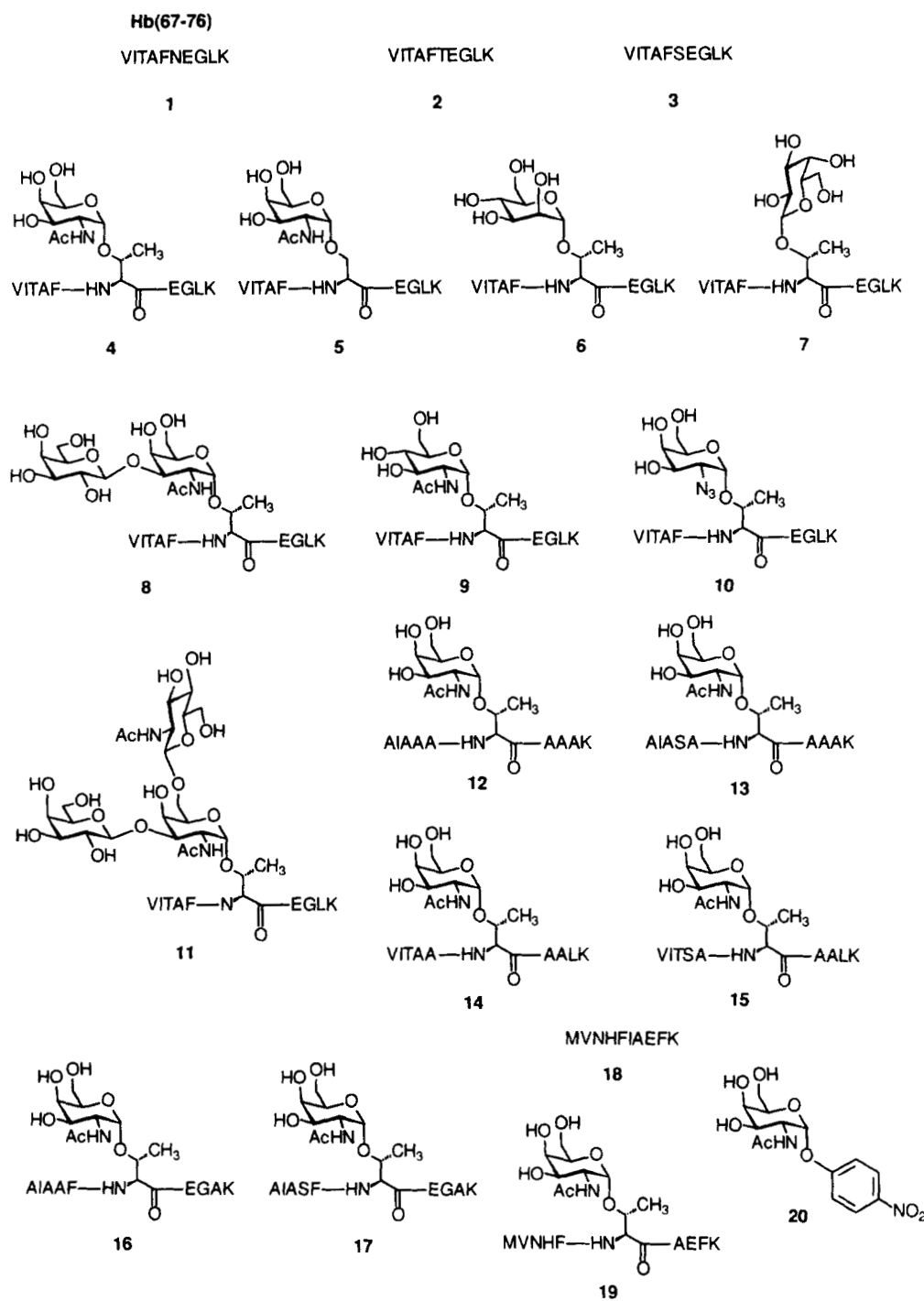


FIGURE 1. Primary structure of the peptides and glycopeptides used in the study. The threonine-substituted peptide 2 and serine-substituted peptides 3 are based on the wild-type Hb(67-76) peptide 1. The substitution was made at the asparagine 72 in the wild-type peptide. In the text, peptides 2 and 3 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). 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 names of the glycosylation used and the amino acid to which the glycan is attached are as follows: 4, T(α -D-GalNAc); 5, S(α -D-GalNAc); 6, T(α -D-Man); 7, T(β -D-Gal); 8, S(β -D-Gal-(1-3) α -D-GalNAc); 9, T(α -D-GlcNAc); 10, T(α -D-GalN₃); 11, S(β -D-Gal-(1-3) β -D-(GlcNAc-(1-6)) α -D-GalNAc). The glycopeptides 12 to 17 all have the T(α -D-GalNAc) glycan, but have substitutions in the amino acid sequence. The peptide 18 is HSP(236-245) derived from mouse heat shock protein 70, and the glycopeptide 19 is HSP(236-245) substituted at I²⁴¹ with T(α -D-GalNAc). 20 is the *p*-nitrophenyl glycoside of α -D-GalNAc.

that did not recognize the T⁷²(Tn) glycopeptide 4, but instead recognized the S⁷²(Tn) glycopeptide 5 (Fig. 2B). A third pattern was displayed by 12 hybridomas that responded approximately equally

well to Tn-glycopeptide, irrespective of whether it had serine or threonine in position 72, but clearly responded only when the Tn group was on the peptide (Fig. 2C). Three hybridomas of the 44

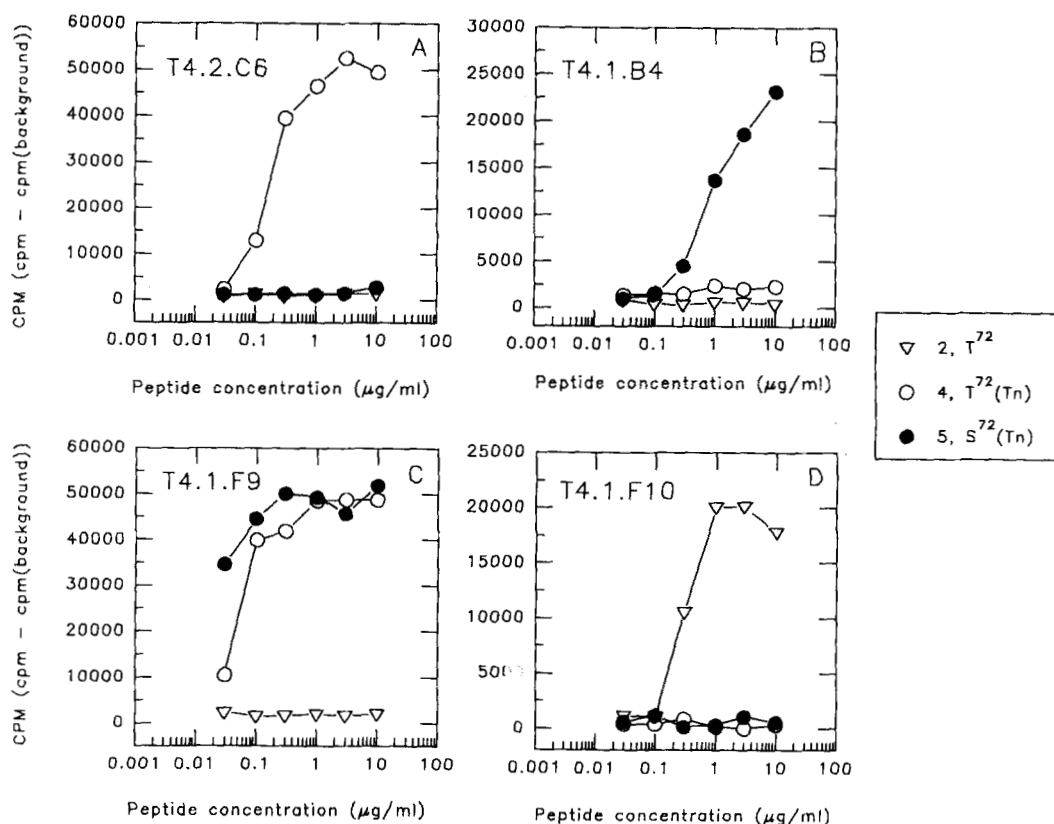


FIGURE 2. The response patterns of four representative T cell hybridomas raised against the glycopeptide $T^{72}(Tn)$. The T cell hybridomas were incubated for 24 h with the B cell lymphoma CH27 and graded doses of glycopeptide 4 (○), 5 (●), and nonglycosylated 2 (△). The IL-2 content of culture supernatant was measured by proliferation of the IL-2 dependent cell-line HT-2. HT-2 proliferation was measured by [^3H]thymidine incorporation. Abscissa: log of the peptide or glycopeptide concentration ($\mu g/ml$). Ordinate: HT-2 (^3H]thymidine incorporation in supernatant with Ag - [^3H]thymidine incorporation in supernatant without Ag) = $\Delta(E - C)$ cpm. Backgrounds were between 500 and 1300 cpm.

Hybridoma	T4.1.B4	T4.1.B11	T4.1.C11	T4.1.D5	T4.1.D7	T4.1.F9	T4.1.F10	T4.1.G2	T4.1.G3	T4.1.G4	T4.1.G5	T4.1.G11	T4.2.B5	T4.2.C6	T4.2.D3	T4.2.D5	T4.2.F3	T4.2.F5	T4.2.F7	T4.2.G6	T4.2.G10	T4.2.G11
Peptide																						
4, $T^{72}(Tn)$	a)	b)																				
5, $S^{72}(Tn)$																						
2, T^{72}																						

FIGURE 3. Summary of the responses for 22 selected T cell hybridomas to the immunizing glycopeptide 4 ($T^{72}(Tn)$) to the peptide 2 (T^{72}) and to glycopeptide 5 ($S^{72}(Tn)$). Empty box indicates no response (a). Filled box indicates full response (b). Filled triangle indicates partial response (c). A hybridoma gives a partial response to a glycopeptide when the dose of glycopeptide required to elicit the hybridoma's 50% maximal response is ≥ 50 times higher than that of $T^{72}(Tn)$.

had a fourth response pattern. They did not recognize any of the glycopeptides but only the unglycosylated peptide 2, T^{72} (Fig. 2D). Figure 3 gives an overview of the responses of all 22 hybridomas to the two glycopeptides and the unglycosylated peptide.

MHC restriction and CD4/CD8 expression of T cell hybridomas

Since the hybridomas were generated by immunizing CBA/J mice ($H-2^k$) with $T^{72}(Tn)$, the possibility that some of the hybridomas were restricted to A^k still existed even though the native Hb(67-76) peptide 1 has shown high affinity binding to the E^k molecule (14, 20, 21). With the aid of the two L cell lines

RT 4.15HP and DCEK Hi7 that were transfected with A^k and E^k , respectively, the MHC-restriction was mapped. All 22 hybridomas gave an IL-2 response toward $T^{72}(Tn)$ 4 when incubated with Ag and DCEK Hi7 (E^k), but were nonresponsive when incubated with the glycopeptide and RT 4.15HP (A^k), confirming the restriction to E^k (not shown). In addition, the proliferative response against the glycopeptide was inhibited by the anti- E^k mAb 14-4-4S, confirming the E^k restriction of the hybridomas (not shown). Fluorometric analysis of the 22 hybridomas stained with Abs against CD4 and CD8 revealed that half of the hybridomas had surface expression of CD4 and none expressed CD8 (not shown). However, the absence of CD4 expression did not seem to influence the affinity of the hybridomas for the

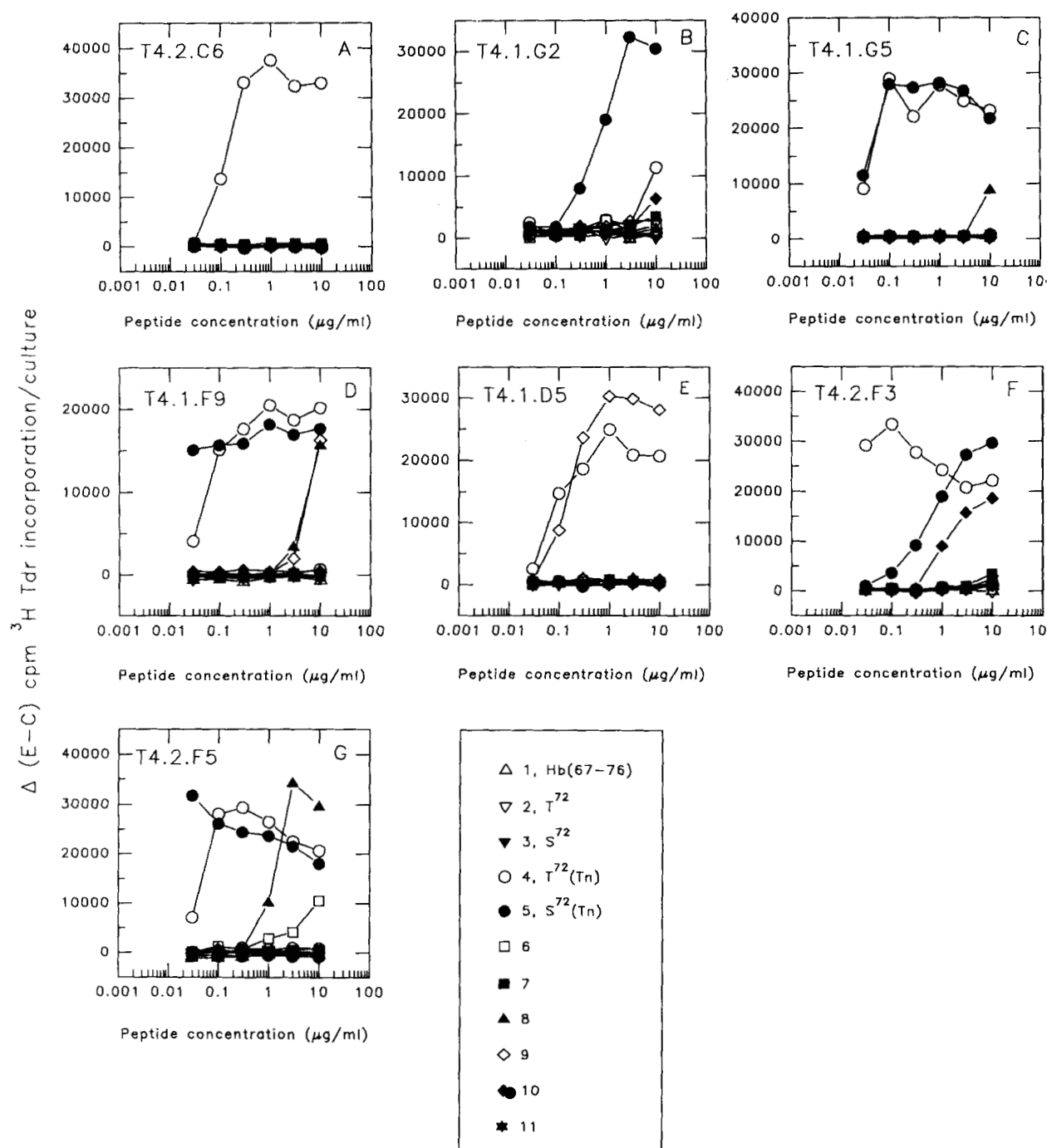


FIGURE 4. The glycan specificity for seven glycopeptide specific T cell hybridomas. The hybridomas were incubated for 24 h with the B cell lymphoma CH27 and graded doses of various peptides and glycopeptides. The IL-2 content was determined with the aid of the IL-2-dependent cell line HT-2 as in the experiment shown in Figure 2. Proliferation was measured by [^3H]thymidine incorporation. Abscissa: log of the peptide or glycopeptide concentration ($\mu\text{g/ml}$). Ordinate: HT-2 [^3H] thymidine incorporation in supernatant with Ag - [^3H] thymidine incorporation in supernatant without Ag = $\Delta(E - C)$. Backgrounds were between 500 and 2100 cpm.

epitope, since the hybridomas expressing the CD4 molecule had the same dose-response pattern as the hybridomas lacking CD4 expression (not shown).

Glycan specificity of the hybridomas

Because the hybridomas clearly differed in their responses to position 72 substituted peptides or glycopeptides, the fine specificity of the hybridomas against the glycan in position 72 was investigated with the aid of a number of glycopeptides differing in the

glycan attached to the threonine or serine in position 72. In addition to the $\text{T}^{72}(\text{Tn})$ and $\text{S}^{72}(\text{Tn})$ glycopeptides 4 and 5 and the T^{72} peptide 2, the hybridomas were tested for response to the panel of peptides and glycopeptides 6 to 11 depicted in Figure 1. Used in these experiments were only the 19 T cell hybridomas responding to the $\text{T}^{72}(\text{Tn})$ and/or $\text{S}^{72}(\text{Tn})$ glycopeptide. Even though some of the glycans attached to threonine 72 only differed slightly from Tn in structure, most of the hybridomas showed a strict selectivity for $\text{T}^{72}(\text{Tn})$, or $\text{S}^{72}(\text{Tn})$, or both (Fig. 4, A-C). Interestingly, some of

Hybridoma Peptide / Glycopeptide	T4.1.B4	T4.1.B11	T4.1.C11	T4.1.D5	T4.1.D7	T4.1.F9	T4.1.G2	T4.1.G3	T4.1.G5	T4.1.G11	T4.2.B5	T4.2.C6	T4.2.D5	T4.2.F3	T4.2.F5	T4.2.F7	T4.2.G6	T4.2.G10	T4.2.G11
4, T ⁷² (Tn)	a)	b)																	
5, S ⁷² (Tn)																			
2, T ⁷²																			
3, S ⁷²																			
1, Hb(67-76)																			
6																			
7																			
8																			
9																			
10																			
11																			

FIGURE 5. Response patterns of 19 T cell hybridomas raised against glycopeptide T⁷²(Tn) to glycopeptides with different glycans attached to threonine or serine at position 72. Empty box indicates no response (a). Filled box indicates full response (b). Filled triangle indicates partial response (c). A hybridoma gives a partial response to a glycopeptide when the dose of glycopeptide required to elicit the hybridoma's 50% maximal response is ≥ 50 times higher than that of T⁷²(Tn).

the hybridomas that recognized both T⁷²(Tn) and S⁷²(Tn) had a cross-reaction against the more complicated glycopeptide 8 at high concentration (Fig. 4, D and G). Two hybridomas recognized glycopeptide 9, which differs minimally from T⁷²(Tn) 4, having α -D-GlcNAc instead of α -D-GalNAc. The hybridoma T4.1.F9 required glycopeptide 9 in high concentration (Fig. 4D), and T4.1.D5 recognized it in low concentration (Fig. 4E). The hybridoma T4.2.F3 (Fig. 4F) responded to glycopeptide 10 with the *N*-acetyl group substituted with a azido group. The hybridoma T4.2.F5 recognized the complicated glycopeptide 8 and also had a slight recognition of the mannose-containing glycopeptide 6 (Fig. 4G). Both hybridomas recognized T⁷²(Tn) 4 and S⁷²(Tn) 5. Figure 5 gives an overview of the response patterns of all 19 hybridomas to these peptides and glycopeptides. It may be worth noticing that none of the 19 glycopeptide recognizing hybridomas recognized the wild-type Hb(67-76) peptide 1. None of the hybridomas recognized the mucin core structure 11 that contains a trisaccharide.

Peptide dependency of the hybridomas

In the Hb(67-76) peptide 1 the side chain of asparagine in position 72 is known to be orientated toward the TCR when the peptide is in the binding groove of the E^k molecule and it functions as the dominant contact residue to the TCR (14). In other studies it was found that the side chains of T⁶⁹, A⁷⁰, and L⁷⁵ within the Hb(67-76) peptide were orientated more or less toward the TCR (15). It was of interest to know whether the specificity of the hybridoma response to the glycopeptide 4 was directed solely to the GalNAc moiety, a distinct possibility because the hybridomas were not recognizing the unglycosylated peptides 2 T⁷² and 3 S⁷² (Fig. 5), or whether parts of the peptide were also recognized. A new series of peptides were prepared for the purpose. To retain the binding to

Table I. Primary structure of the alanine-substituted glycopeptides and HSP glycopeptide and their relative binding to E^k

Code	Peptide Sequence	% Binding
4, T ⁷² (Tn)	VITAF(-GalNAc)EGLK	87
5, S ⁷² (Tn)	VITAFS(-GalNAc)EGLK	89
12	AIAAAT(-GalNAc)AAAK	124
13	AIASAT(-GalNAc)AAAK	110
14	VITAAAT(-GalNAc)AALK	140
15	VITSAT(-GalNAc)AALK	126
16	AIAAFT(-GalNAc)EGAK	44
17	AIASFT(-GalNAc)EGAK	47
18	MVNHFAIEFK	162
19	MVNHFT(-GalNAc)AEFK	55

^a Percentage of binding was calculated from the formula: $(IC_{50} \text{ Hb(64-76)} / IC_{50} \text{ Hb analog}) \times 100$.

E^k, the primary anchor residues I⁶⁸ and K⁷⁶ were retained in all of the new glycopeptides. The significance of exposed amino acids as well as the secondary anchor residues, F⁷¹ and E⁷³, was evaluated by substituting them with alanine. Since the Hb(67-76) peptide 1 naturally contains an alanine in position 70, a position known to influence T cell recognition by acting as a secondary T cell contact residue (22, 23), that alanine was substituted with serine. All of the alanine-substituted glycopeptides are illustrated in Table I together with their percentage binding to affinity purified E^k molecules. An overview of the hybridomas' response patterns is shown in Figure 6. The details for some of the hybridomas are shown in Figure 7. None of the T cell hybridomas recognized glycopeptide 12 that had alanine substitutions at all positions except at I⁶⁸, K⁷⁶, and the

Hybridoma Peptide/ Glycopeptide	T4.1.B4	T4.1.B11	T4.1.C11	T4.1.D5	T4.1.D7	T4.1.F9	T4.1.G2	T4.1.G3	T4.1.G5	T4.1.G11	T4.2.B5	T4.2.C6	T4.2.D5	T4.2.F3	T4.2.F5	T4.2.F7	T4.2.G6	T4.2.G10	T4.2.G11
4, T ⁷² (Tn)	a)	b)																	
5, S ⁷² (Tn)																c)			
12																			
13																			
14																			
15																			
16																			
17																			
18																			
19																			

FIGURE 6. Response patterns of 19 T cell hybridomas raised against glycopeptide 4, T⁷²(Tn) to alanine-scan glycopeptides containing the primary anchors and the GalNAc attached to threonine at position 72. Empty box indicates no response (a). Filled box indicates full response (b). Filled triangle indicates partial response (c). A hybridoma gives a partial response to a glycopeptide when the dose of glycopeptide required to elicit the hybridoma's 50% maximal response is ≥ 50 times higher than that of T⁷²(Tn).

glycosylated threonine in position 72. With exception of the hybridoma T4.2.F3, which showed a partial response, none of the other hybridomas recognized glycopeptide 13, in which the wild-type A⁷⁰ was substituted with serine. Renewed assays for peptide binding demonstrated that these two glycopeptides were even better binders to E^k than the T⁷²(Tn) (Table I), as shown previously (10).

Hb(67–76) uses positions I⁶⁸ and K⁷⁶ as primary anchors when the peptide binds to E^k. In addition, F⁷¹ and E⁷³ seem to be secondary anchors (14, 20, 21, 24). The effect on T cell recognition was investigated by substituting them both with alanine, and also substituting G⁷⁴ with alanine to create glycopeptide 14, in which all the amino acids in the central part of the peptide were alanines except for the glycan carrying T⁷². Furthermore, the A⁷⁰ was substituted with serine, yielding glycopeptide 15. These two glycopeptides were also found to be better binders to E^k than the unmodified peptide (Table I). Interestingly, more than one-third of the selected hybridomas gave a full response to both of these modified glycopeptides despite such major changes (Figs. 6 and 7).

These results indicated that amino acids in the peptide pointing out of the binding groove toward the TCR contribute to the T cell response against the glycopeptide (15). This was confirmed by substituting the amino acids in the Hb peptide with potential for TCR contact. Substitution of V⁶⁷, T⁶⁹, and L⁷⁵ with alanine (glycopeptide 16), abolished the response of all except three clones. An alanine to serine substitution at position 70, i.e., glycopeptide 17, extinguished the response for one hybridoma but allowed two other hybridomas to respond (Fig. 6 and Fig. 7, C, E, and H).

The peptide dependency was further tested by using a completely different E^k binding peptide, HSP(236–245) derived from the murine 70-kDa heat-shock protein (HSP70). Called peptide 18, this contains the E^k binding motif V²³⁷ and K²⁴⁵, but differs from Hb(67–76) at all other positions. Furthermore, it is known to bind

to E^k in almost the same conformation as the Hb(67–76) (15, 21). None of the hybridomas responded to the native HSP peptide. However, the glycopeptide 19 with threonine-*O*-GalNAc substituting for I²⁴¹, stimulated four hybridomas to cross-respond (Fig. 6 and Fig. 7, C and F).

The effect of free Tn on hybridoma recognition of glycopeptides

To further characterize the presentation of glycopeptide to the hybridomas, we attempted to inhibit the response by incubating the B cell lymphoma CH27 with free Tn in the form of the nitrophenyl glycoside of α -D-GalNAc (peptide 20, see Fig. 1). For these experiments we used one T cell hybridoma with high and one with intermediate affinity to the T⁷²(Tn) glycopeptide 4 against which the hybridomas had been raised. The APC were pulsed with suboptimal doses of T⁷²(Tn) and incubated with hybridoma and 10 μ g/ml glycoside 20. The compound 20 failed to interfere with the activation of the hybridoma by T⁷²(Tn) (not shown).

Discussion

In this paper we report upon the specificities of 22 mouse T cell hybridomas raised against the synthetic glycopeptide 4, VI-TAFT(α -D-GalNAc)EGLK. The peptide is derived from mouse hemoglobin except for the threonine substitution to which the glycan group α -GalNAc was attached. In the parent peptide 1, Hb(67–76) an asparagine is found in this position. This peptide contains an E^k binding motif (P1, isoleucine and P9, lysine) and functions as a T cell epitope in E^k expressing mouse strains with a hemoglobin that differs in amino acid sequence in the Hb(67–76) sequence (12, 13). The T⁷²(Tn) glycopeptide 4 has been shown to bind as well to E^k as the Hb(67–76) peptide 1 (10), and the glycopeptide was used here to raise T cell hybridomas that secreted IL-2 upon activation.

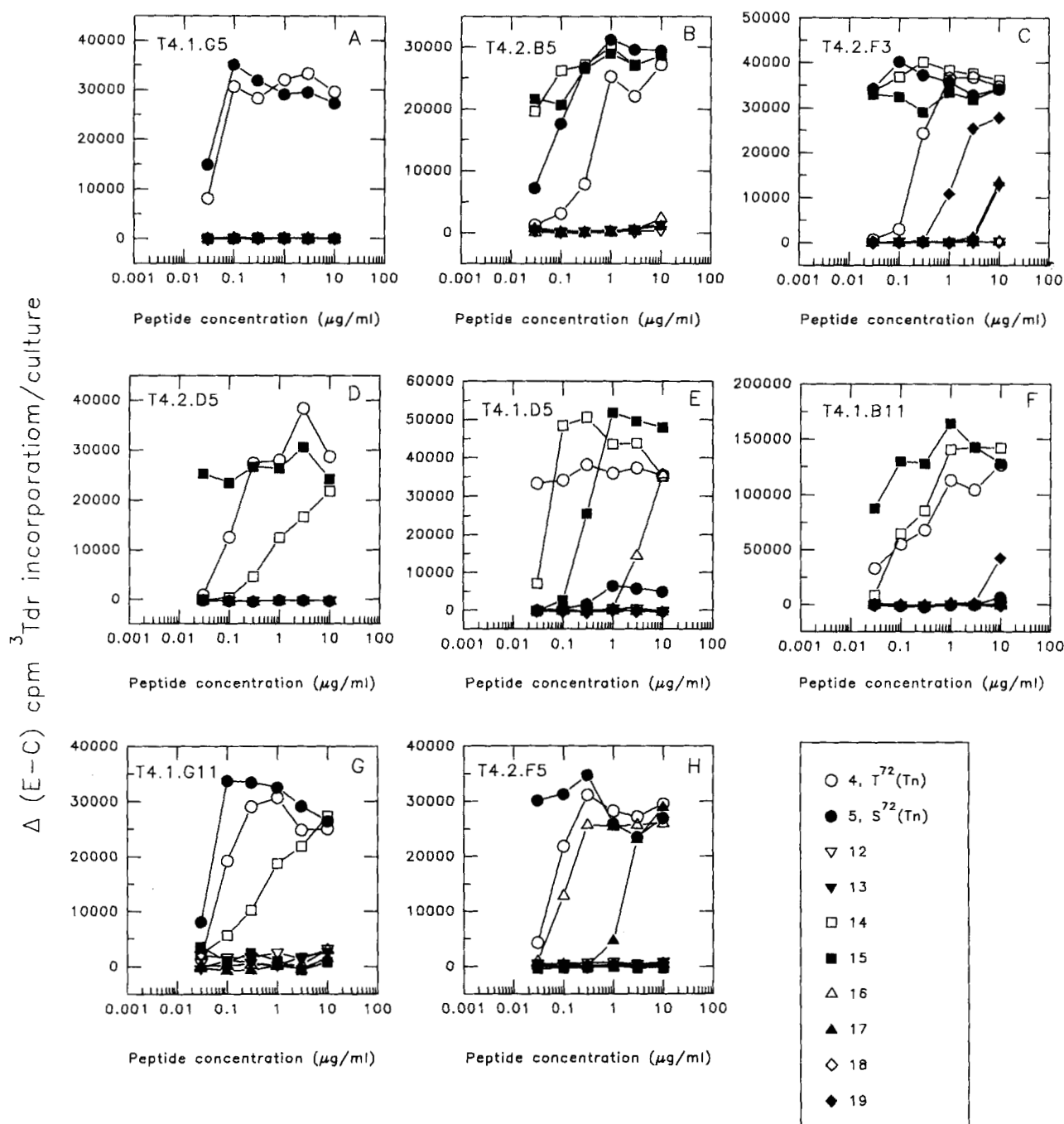


FIGURE 7. The peptide dependency of the glycopeptide-specific T cell hybridomas. The hybridomas were incubated for 24 h with the B cell lymphoma CH27 and graded doses of various peptides and glycopeptides. The IL-2 content was determined with the aid of the IL-2 dependent cell-line HT-2 as in the experiment shown in Figure 2. Abscissa: log of the peptide or glycopeptide concentration ($\mu\text{g/ml}$). Ordinate: HT-2 (^3H)thymidine incorporation in supernatant with Ag - [^3H]thymidine incorporation in supernatant without Ag = $\Delta(E - C)$. Backgrounds were between 500 and 1500 cpm.

Twenty-two stable and vigorously responding hybridomas were selected for further study. They responded by IL-2 secretion to either $\text{T}^{72}(\text{Tn})$ 4, or to the unglycosylated threonine substituted peptide T^{72} 2 or the serine-Tn substituted glycopeptide $\text{S}^{72}(\text{Tn})$ 5 by IL-2 secretion. Two hybridomas failed to respond to the glycopeptide against which they had been raised. Rather baffling, they responded to glycopeptide 5 instead. Most of the hybridomas gave a response to $\text{S}^{72}(\text{Tn})$ of the same magnitude as to glycopeptide 4. All of the hybridomas were found to be restricted to E^k . This confirms that the glycopeptide indeed is bound to E^k when it is being recognized, and

thus places the present T cell hybridomas in a different category from that of the T cell clones described to recognize glycan epitopes in an MHC-unrestricted manner (25, 9). Finn et al. have described the existence of T cells recognizing the highly O-glycosylated mucin glycoproteins in a MHC unrestricted manner (25), and recently Abdel-Motal et al. showed that immunization with glycopeptides could generate MHC-unrestricted CTL that recognized the carbohydrate present in a glycolipid (9).

The T cells' specificity toward the glycan is a question of major interest. Recent experiments from Deck et al. (8) showed that CD4

positive T cells generated against one glycoform did not respond to other glycopeptides with different glycan structures. However, the glycans used in that study were different anomeric forms making the carbohydrate structures very different and their influence on peptide structure unpredictable. In the present study the fine specificity toward the glycan moiety was investigated for those 19 hybridomas, which recognized only the glycosylated peptide. They were tested against a panel of different glycopeptides all having the same peptide sequence, but different glycans attached to threonine or serine at position 72. It was particularly important to determine whether the hybridomas cross-responded to the α -D-GlcNAc-containing glycopeptide 9 because the only difference between α -D-GlcNAc and α -D-GalNAc is the orientation of the hydroxyl group located at the fourth carbon atom in the carbohydrate ring structure. In the α -D-GalNAc-containing glycopeptide 4 that was used to raise the hybridomas, the hydroxyl group is parallel to the extended peptide chain, but in the α -D-GlcNAc containing glycopeptide 9, it points away from the peptide. The fact that 17 of the 19 hybridomas responded only to glycopeptide 4 and thus were able to distinguish between these two glycans constitutes strong evidence that the glycan is indeed recognized by the TCR. The cross-response of the 2 remaining hybridomas, T4.1.D5 and T4.1.F9, to glycopeptide 9 indicates that they had specificity for other parts of the glycan ring structure together with the *N*-acetyl group.

Three hybridomas gave a cross-response to glycopeptide 8, having a disaccharide attached to S⁷², but only when the glycopeptide was used in high concentration. Whether this was due to a partial degalactosylation of the Ag and consequent formation of small amounts of S⁷²(Tn) 5, or whether the hybridomas were indeed recognizing the disaccharide, is not known at present. The evidence for deglycosylation during Ag processing is discussed further below. One hybridoma gave a weak cross-response to the α -D-mannose-conjugated glycopeptide 6. One hybridoma, T4.2.F3, cross-responded to glycopeptide 10 in which the *N*-acetyl group of Tn is substituted with an azido group. This indicates that the attachment of a small group at this carbon atom in the carbohydrate ring structure had only a minor effect on the recognition of the glycopeptide. The hybridoma responding to the azide-containing glycopeptide will be used in future work to study the physical contact between TCR and glycan during the hybridoma's recognition of the glycopeptide.

We next investigated the specificity of the hybridomas toward the peptide moiety of the glycopeptide Ag. This was performed with the aid of a panel of alanine-substituted glycopeptides in which the I⁶⁸ and K⁷⁶ anchors as well as the threonine with the attached Tn-group were kept constant. As expected, assays for binding confirmed that all of them were indeed good binders to E^k. Only the alanine-substituted glycopeptides 16 and 17 were found to bind with less affinity. By substituting the E in position 73 with S (10) or A as in the alanine-substituted glycopeptides 13 and 14, the binding to E^k increased as expected, evidently because the E^k binding motif favors A, S, or N at this secondary anchor position. None of the hybridomas responded to the most completely A-substituted glycopeptide 12 with alanine in all positions except for I⁶⁸, K⁷⁶, and the Tn-carrying T⁷². The less completely A-substituted glycopeptide in which the two main anchors were retained, but the three amino acids with side chains pointing downwards in the E^k binding groove were substituted with A, were able to activate approximately one-third of the hybridomas. On the other hand, alanine substitutions of the amino acids with side chains pointing upwards from the binding groove yielded glycopeptides that were unable to stimulate any of the hybridomas, with a few exceptions. This indicates that the glycopeptide-specific T cell hybridomas are

also specific for the solvent-exposed parts of the glycan-carrying peptide.

The cross-responses of four hybridomas to the glycosylated HSP(236–245) peptide was quite a surprise, since only one of them gave a response to the most completely A-substituted glycopeptide. This may mean that the Tn group in the HSP-Tn peptide 19 has the same orientation as in T⁷²(Tn) 4, while it has a different orientation in the alanine-substituted glycopeptides. Alternatively, the partial response may be explained by a fortuitous molecular mimicry (26–29). Even though the amino acids differ between peptides 4 and 19, two additional factors may have contributed to the response. First, the HSP contains the ideal anchors, as does T⁷²(Tn), which facilitate the interaction in the MHC:peptide complex, and second, the anticipated solvent-accessible portions of the peptides belong to the same group of conserved amino acids, providing the required additional contacts with the TCR.

In further attempts at mapping the sugar specificity of the hybridomas we investigated whether their activation could be blocked by adding large amounts of “free” Tn in the form of α -D-GalNAc glycoside 20 to the assay cultures, but no effect was observed.

In preceding work (10) we observed that T cells primed with the Tn-glycosylated peptide gave, in addition to a full response to the immunogen, a cross-response to the unglycosylated peptide *in vitro*. It was not clear whether the clones giving this cross-response were directed against the glycosylated peptide and having a lower affinity for the unglycosylated peptide, or whether they were directed against the peptide itself and had no specificity against the glycan at all. The specificity pattern of the present hybridomas with 41 responding to glycosylated peptide only and 3 responding to unglycosylated peptide only, but a total lack of hybridomas reacting with both glycosylated and unglycosylated peptide is indeed striking. It indicates that T cell clones directed against unglycosylated peptide are activated during the natural polyclonal response against the glycosylated peptide. The most straightforward explanation for this is that a small amount of the glycopeptide Ag becomes deglycosylated during the 10-day priming period, and that the animal, in addition to being primed with glycosylated peptide, is being primed with small amounts of nonglycosylated peptide.

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