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Chemoenzymatically synthesized multimeric Tn/STn MUC1 glycopeptides elicit cancer-specific anti-MUC1 antibody responses and override tolerance

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The MUC1 mucin represents a prime target antigen for cancer immunotherapy because it is abundantly expressed and aberrantly glycosylated in carcinomas. Attempts to generate strong humoral immunity to MUC1 by immunization with peptides have generally failed partly because of tolerance. In this study, we have developed chemoenzymatic synthesis of extended MUC1 TR glycopeptides with cancer-associated O-glycosylation using a panel of recombinant human glycosyltransferases. MUC1 glycopeptides with different densities of Tn and STn glycoforms conjugated to KLH were used as immunogens to evaluate an optimal vaccine design. Glycopeptides with complete *O*-glycan occupancy (five sites per repeat) elicited the strongest antibody response reacting with MUC1 expressed in breast cancer cell lines in both Balb/c and *MUC1*.Tg mice. The elicited humoral immune response showed remarkable specificity for cancer cells suggesting that the glycopeptide design holds promise as a cancer vaccine. The elicited immune responses were directed to combined glycopeptide epitopes, and both peptide sequence and carbohydrate structures were important for the antigen. A MAb (5E5) with similar specificity as the elicited immune response was generated and shown to have the same remarkable cancer specificity. This antibody may hold promise in diagnostic and immunopreventive measures.

Key words: cancer vaccine/glycopeptides/MUC1/O-glycosylation/STn

Introduction

The human mucin MUC1 is a polymorphic transmembrane glycoprotein expressed on the apical surfaces of simple and glandular epithelia (Taylor-Papadimitriou *et al*., 1999). MUC1 is highly overexpressed and aberrantly O-glycosylated in adenocarcinomas. The extracellular domain of the mucin contains variable number of tandem repeats (TRs) (25–125) of 20 amino acid residues with five potential sites for O-glycosylation. *O*-Glycans are incompletely processed in cancer cells resulting in the expression of the pancarcinoma carbohydrate antigens Tn (GalNAcα1-*O*-Ser/Thr), STn (NeuAcα2-6GalNAcα1-*O*-Ser/Thr), and T (Galβ1-3GalNAcα1-*O*-Ser/Thr) (Springer, 1984). MUC1 expressed by breast carcinoma cells carries the short cancer-associated Tn, STn, and T antigens, as well as the mono- and disialyl core 1 structure (ST, NeuAcα2-3Galβ1-3[NeuAcα2-6]$_{+/-}$ GalNAcα1-*O*-Ser/Thr) found widely in normal cells (Miles *et al*., 1995; Lloyd *et al*., 1996; Dalziel *et al*., 2001). In contrast, MUC1 expressed in normal breast epithelial cells generally carry branched core 2 *O*-glycans (Galβ1-3[GlcNAcβ1-6]GalNAcα1-*O*-Ser/Thr) with lactosamine extensions (Burchell *et al*., 2001). The cell membrane-bound mucin MUC1 has long been considered a prime target for immunotherapeutic intervention. The existence of anti-MUC1 antibodies and circulating immune complexes containing MUC1 in breast cancer patients that correlates with improved prognosis clearly supports MUC1 as a target (Rughetti *et al*., 1993; von Mensdorff-Pouilly *et al*., 2000a,b). However, the stimulation of an effective cellular or humoral immune response to cancer-associated forms of MUC1 in patients or transgenic animals expressing the human *MUC1* gene (using defined immunogens as opposed to cell-based therapies) has not been achieved (Miles and Taylor-Papadimitriou, 1999; Vlad *et al*., 2004). Strategies for active specific immunotherapy based on peptide/protein immunogens have so far been limited to unglycosylated MUC1 TR peptides of different lengths, conjugated to different carriers, or administered with an adjuvant. These strategies have generally failed to produce effective immune responses to MUC1 expressed by cancer cells in hosts where the mucin is expressed as a self-antigen (Goydos *et al*., 1996; Karanikas *et al*., 1997; Rowse *et al*., 1998; Adluri *et al*., 1999; Acres *et al*., 2000; Gilewski *et al*., 2000; von Mensdorff-Pouilly *et al*., 2000a; Soares *et al*., 2001). Although antibodies directed to MUC1 peptides can be generated, these antibodies do not recognize MUC1 expressed by cancer cells (Karanikas *et al*., 1997).

It may be predicted that the most promising glycoforms of mucin-based cancer vaccines are MUC1 glycoproteins or glycopeptides carrying the simple mucin-type *O*-glycans Tn, STn, and T, which are widely expressed in adenocarcinomas (including breast and ovarian cancers), and show limited distribution in normal adult tissues (Springer, 1984). The expression of these *O*-glycans in cancer correlates with

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poor prognosis and natural antibodies to these carbohydrate haptens increases in cancer patients (Miles *et al*., 1995; Soares *et al*., 1996; Werther *et al*., 1996). Analysis of immune responses to these *O*-glycans has been approached by immunizing with animal mucins expressing the Tn and sialosyl-Tn glycoforms or with sialosyl-Tn disaccharides linked through a spacer to keyhole limpet hemocyanin (KLH). Immunization with animal mucins expressing the Tn and sialosyl-Tn glycoforms increases antibody titers of primarily IgM to Tn and IgG to sialosyl-Tn (O'Boyle *et al*., 1992) (unpublished data). Immunization with the sialosyl-Tn disaccharides coupled to KLH induces IgM and IgG antibodies reactive with the sialosyl-Tn hapten (Longenecker *et al*., 1994; Ragupathi *et al*., 1999). There are compelling reasons for combining these cancer-associated carbohydrate haptens with the natural mucin peptide backbone on which they are expressed in cancer cells in a vaccine design: (1) antibodies to carbohydrate haptens are generally of low affinities compared with antibodies to peptides; (2) the natural mucin backbone will present the sugar haptens in a specific pattern with clustering of epitopes resembling the immune target; (3) combined glycopeptide epitopes involving binding to both the carbohydrate and the peptide or conformational peptide epitopes, which are dependent on glycosylation, may be included in the response (Schuman *et al*., 2003); (4) glycopeptides with high density of immature *O*-glycans may better stimulate the innate immune system through interaction with, for example, human macrophage C-type lectin (Iida *et al*., 1999; Denda-Nagai *et al*., 2002); and (5) the natural anti-MUC1 antibodies in cancer patients appear to preferentially react with MUC1 glycopeptides compared with unglycosylated peptides (von Mensdorff-Pouilly *et al*., 2000a).

Developments in chemical synthesis of glycopeptides in the last two decades have made glycopeptides with defined structures available (Paulsen and Brockhausen, 2001). However, the synthesis of large glycopeptides with multiple *O*-glycans such as multiple TR MUC1 glycopeptides is complex, time consuming, and costly. Enzymatic glycosylation of peptides is rapidly becoming an alternative with increasing availability of glycosyltransferases in recombinant form. Furthermore, availability of isoenzymes of many glycosyltransferases with distinct differences in substrate specificities and kinetic properties allow for a high degree of control of the enzymatic synthesis of glycopeptides (Wandall *et al*., 1997; Bennett *et al*., 1998). In this study, chemoenzymatic synthesis of multiple-repeat MUC1 glycopeptides with different *O*-glycan density and Tn (GalNAcα1-*O*-Ser/Thr) and STn (NeuAcα2-6GalNAcα1-*O*-Ser/Thr) glycoforms was developed using recombinant glycosyltransferases. Different polypeptide GalNAc-transferase isoforms were used to direct sites of *O*-glycan occupancy (Bennett *et al*., 1998), and the sialyltransferase ST6GalNAc-I was used to produce STn glycoforms (Kurosawa *et al*., 2000). The optimal vaccine design was found to be Tn and STn glycoforms with high *O*-glycan density, and glycopeptides conjugated to KLH were found to overcome tolerance in human *MUC1* transgenic (MUC1.Tg) mice. In both wild-type Balb/c mice and *MUC1*.Tg mice, the glycopeptides with complete *O*-glycan occupancy elicited the strongest antibody response reacting with MUC1 expressed in breast cancer cell lines, thus representing the most effective vaccine design. The elicited humoral immune response showed remarkable specificity for cancer cells suggesting that the glycopeptide design holds promise as a cancer vaccine. The cancer-associated T structure (Galβ1-3GalNAcα1-*O*-Ser/Thr), sialylated versions hereof as well as the core 3 structure were also synthesized demonstrating that capability for the synthesis of more complex structures.

Results

Chemoenzymatic synthesis of multimeric Tn and STn MUC1 glycopeptides

Synthetic 60-mer MUC1 TR peptides were glycosylated using site-selective recombinant polypeptide GalNAc transferases (GalNAc-T2, -T4, and -T11) (Wandall *et al*., 1997; Bennett *et al*., 1998; Schwientek *et al*., 2002). The sites of GalNAc attachments in MUC1 TR sequences were strictly controlled as indicated by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of MUC1 60-mer TR peptides glycosylated *in vitro* with recombinant GalNAc transferases. GalNAc-T11 was used to add two GalNAc residues per TR, GalNAc-T2 to add three residues, and sequential use of GalNAc-T2 and -T4 to add all five residues (Figure 1). Sites of attachments were confirmed by mass spectrometry, as previously described (Wandall *et al*., 1997; Bennett *et al*., 1998; Schwientek *et al*., 2002). Glycosylation with GalNAc-T4 to achieve five GalNAc residues per repeat only allowed 14 in total because of the design of the peptide with the NH_2 terminal being too truncated. Further glycosylation of GalNAc residues with sialic acid to form STn was achieved with recombinant murine ST6GalNAc-I. Evaluation of number of sialic acid residues attached by MALDI-TOF may be underestimated because of the labile nature of this sugar linkage. The sialylation is considered complete as evaluated by immunoreactivity pattern with anti-STn (positive) and anti-Tn (negative) monoclonal antibodies (MAbs). The core 1 T structure was produced using a recombinant β3Gal transferase (Ju *et al*., 2002). Glycopeptides formed are depicted on top of each MALDI-TOF profile in Figure 1.

MUC1 glycopeptides with complete O-glycan attachment are most immunogenic and Tn and STn glycopeptides elicit strong antibody responses in MUC1 transgenic mice

In initial studies, MUC1 Tn glycoforms with two, three, and five *O*-glycans per repeat were tested as immunogens, and the glycopeptide with three and five *O*-glycans yielded the strongest immune response to the respective immunogens by enzyme-linked immunosorbent assays (ELISA) and, more importantly, induced antibodies reactive with MUC1-expressing cancer cells (not shown). For the further studies, MUC1 with complete *O*-glycan occupancy was chosen, and as shown in Figure 2 sera from wild-type Balb/c mice (Figure 2a and c) and *MUC1*.Tg mice (Figure 2b and d) immunized with either the complete Tn glycosylated MUC1 ($MUC1_{60}Tn_{15}$) or the complete STn glycosylated

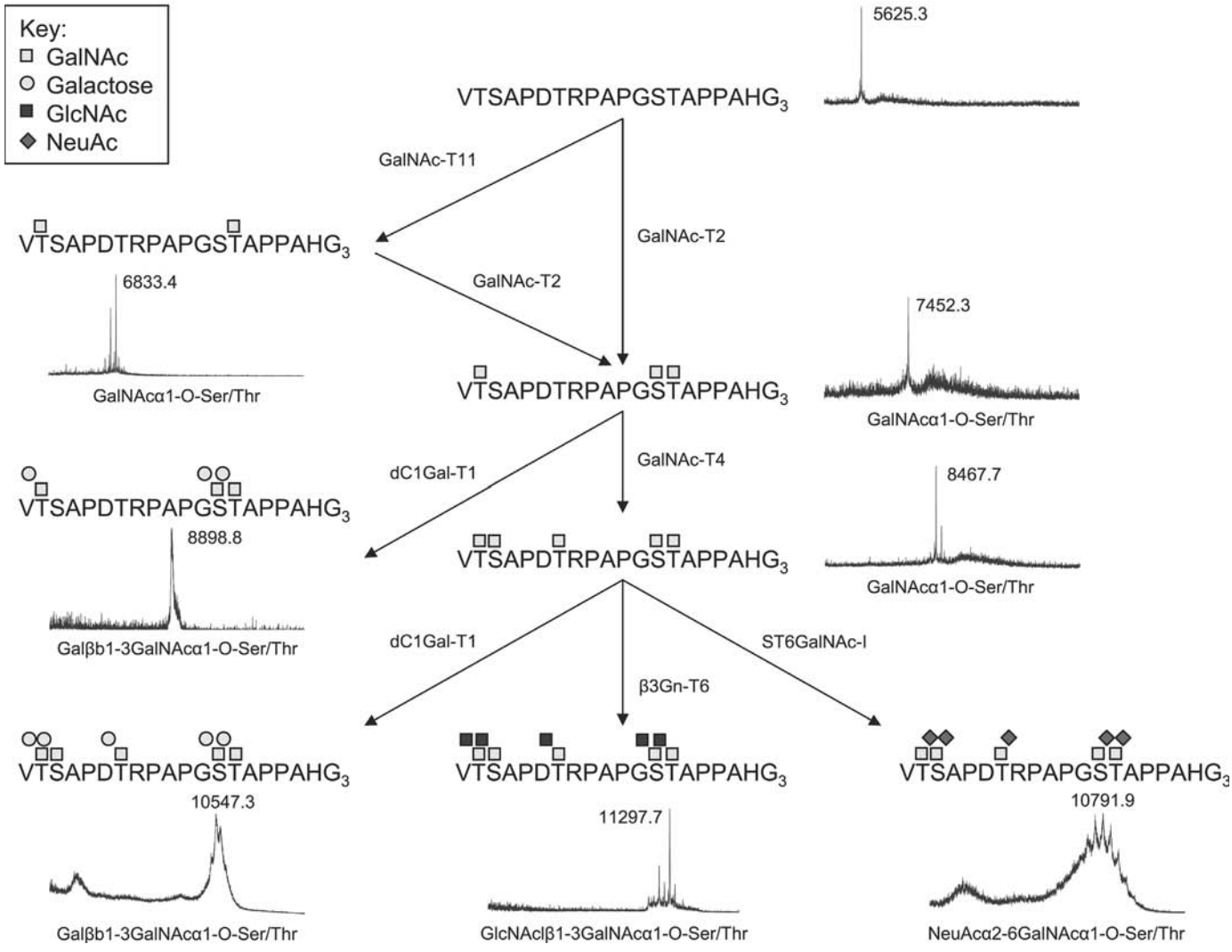

Fig. 1. Chemoenzymatic synthesis of multimeric Tn and STn MUC1 glycopeptides. Synthetic 60-mer MUC1 TR peptides were glycosylated using site-selective recombinant polypeptide GalNAc transferases (GalNAc-T2, -T4, and -T11). The sites of GalNAc attachments in MUC1 TR sequences were strictly controlled as indicated by MALDI-TOF mass spectrometry analysis of MUC1 60-mer TR peptides glycosylated *in vitro* with recombinant GalNAc transferases. GalNAc-T11 was used to add two GalNAc residues per TR, GalNAc-T2 to add three residues, and sequential use of GalNAc-T2 and -T4 to add all five residues. Sites of attachments were confirmed by mass spectrometry, as previously described (Wandall *et al*., 1997; Bennett *et al*., 1998; Schwientek *et al*., 2002). Glycosylation with GalNAc-T4 to achieve five GalNAc residues per repeat only allowed 14 in total because of the design of the peptide with the NH_2 terminal being too truncated. Further glycosylation of GalNAc residues with sialic acid to form STn was achieved with recombinant murine ST6GalNAc-I. Evaluation of number of sialic acid residues attached by MALDI-TOF may be underestimated because of the labile nature of this sugar linkage. The sialylation is considered complete as evaluated by immunoreactivity pattern with anti-STn (positive) and anti-Tn (negative) MAbs. The core 1 T structure was produced using a recombinant β3Gal transferase (Ju *et al*., 2002). Glycopeptides formed are depicted on top of each MALDI-TOF profile. The mass scale of spectra shown are 5000–10,000 counts.

MUC1 glycopeptide ($MUC1_{60}STn_{15}$) yielded high antibody titers in both mice. The highest antibody titers were found with the MUC1 glycoform used as immunogen, but considerable reactivity with the other MUC1 glycoforms were found as well. Low reactivity with unglycosylated MUC1 was found particularly in the Tn immunized mice. Very low levels of anti-Tn and STn hapten antibodies were detected using the mucin ovine submaxillary mucin (OSM with mainly STn glycoform) and asialo-OSM (A-OSM with Tn glycoform) as antigens. No reactivity with non-MUC1 peptides or glycopeptides with Tn glycosylation were found.

Characterization of a MAb 5E5 that mimics the immune response elicited in wild-type and MUC1*.Tg mice immunized with Tn MUC1*

To further characterize and define the specificity of the immune response to the glycopeptides, we isolated a MAb (designated 5E5) from a mouse immunized with the complete Tn glycosylated MUC1 glycopeptide, which essentially mirrored the specificity of the polyclonal response found (Figure 3a). The antibody 5E5 reacted with all Tn and STn glycoforms of the MUC1 TR and showed no reactivity with unglycosylated MUC1 peptides and only very weak reactivity with the Tn hapten presented on non-MUC1

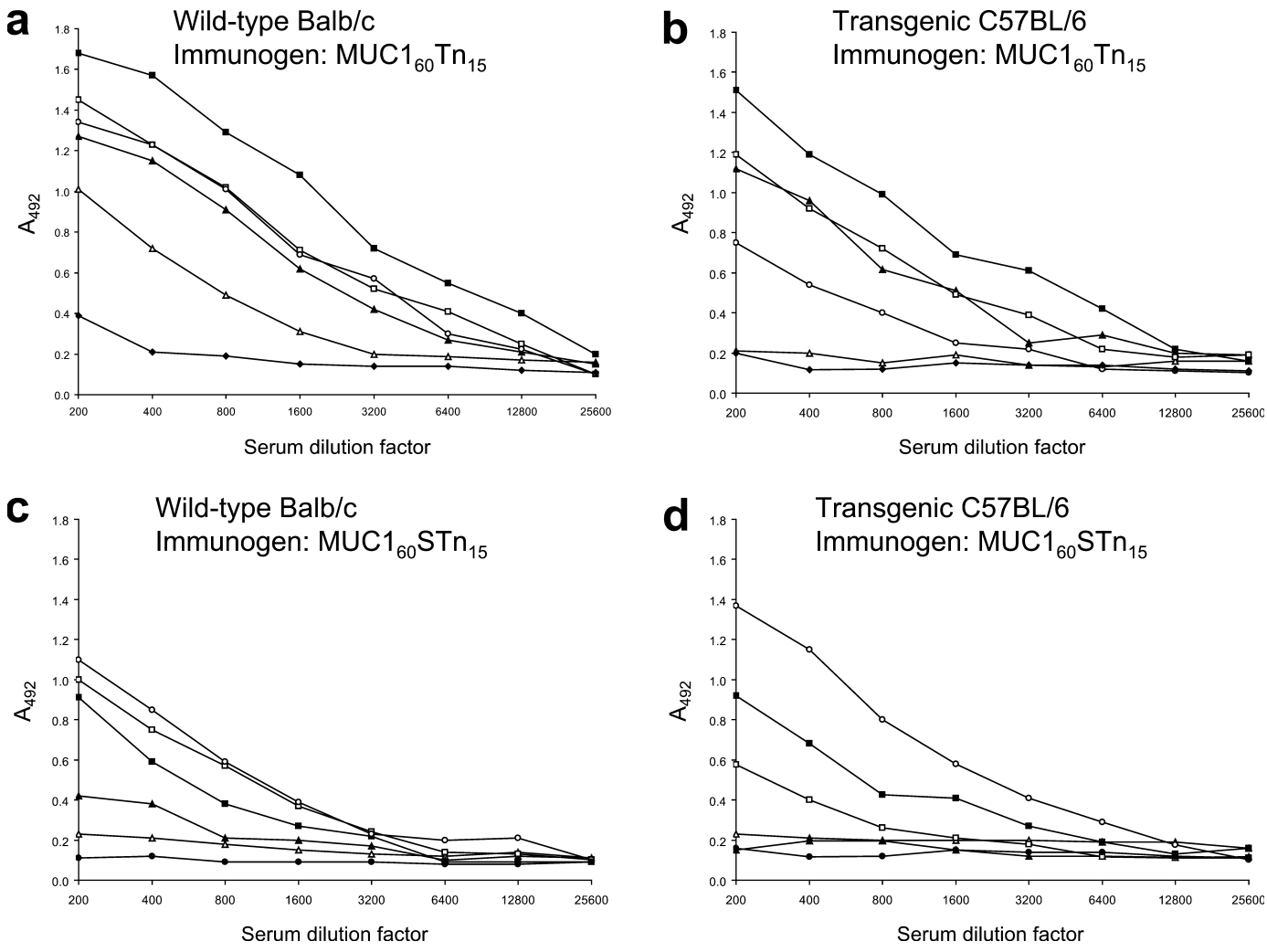

Fig. 2. MUC1 glycopeptides with complete *O*-glycan attachment are most immunogenic, and Tn and STn glycopeptides elicit strong antibody responses in MUC1.Tg mice. (**a**) ELISA of serum from one representative (of four) wild-type Balb/c mouse immunized with complete Tn glycosylated MUC1 ($MUC1_{60}Tn_{15}$). Designations are as follows: ■, $MUC1_{60}Tn_{15}$; ❑, $MUC1_{60}Tn_{9}$; ○, $MUC1_{60}STn_{15}$; ●, OSM (STn); ▲, $MUC1_{60}Tn_{6}$; Δ, MUC160; ◆, A-OSM (Tn). Additional peptides tested which gave no reactivity include unglycosylated MUC2, Tn MUC2, and Tn MUC4. (**b**) ELISA of serum from one representative (of four) wild-type mouse immunized with the complete STn glycosylated MUC1 glycopeptide ($MUC1_{60}STn_{15}$). The highest antibody titers were found with the MUC1 glycoform used as immunogen, but considerable reactivity with the other MUC1 glycoforms were found as well. Low reactivity with unglycosylated MUC1 was found, in particular, in the Tn immunized mice. Very low levels of anti-Tn and STn hapten antibodies were detected using the mucin OSM (with mainly STn glycoform) and A-OSM (with Tn glycoform) as antigens. No reactivity with non-MUC1 peptides or glycopeptides with Tn-glycosylation were found. (**c**) ELISA of serum from one (of four) MUC1.Tg mice immunized with complete Tn glycosylated MUC1 ($MUC1_{60}Tn_{15}$). (**d**) ELISA of serum from one (of four) MUC1.Tg mice immunized with the complete STn glycosylated MUC1 glycopeptide ($MUC1_{60}STn_{15}$). The highest antibody titers were found with the MUC1 glycoform used as immunogen, but considerable reactivity with the other MUC1 glycoforms was found as well. No reactivity was detected with unglycosylated MUC1 as well as the mucins OSM (STn) and A-OSM (Tn) and non-MUC1 Tn glycopeptides.

peptide backbone. To evaluate the range of *O*-glycan structures involved in the specificity, we took advantage of the Chinese hamster ovary (CHO) ldlD cell system. CHO ldlD cells lack the UDP-Gal/GalNAc epimerase and are deficient in GalNAc O-glycosylation and galactosylation in the absence of exogeneous addition of GalNAc and Gal, respectively (Kingsley and Krieger, 1984). CHO ldlD cells stably transfected with the full-coding human MUC1 gene (CHO ldlD/MUC1) were grown in the presence of GalNAc, in the presence of Gal and GalNAc, or in the absence of both, yielding cells expressing Tn, ST, or unglycosylated MUC1 glycoforms, respectively. As shown in Figure 3b, the CHO ldlD MUC1 cells express MUC1 as detected by the general anti-MUC1 antibody HMFG2 regardless of the addition of sugars to the growth medium. Cells grown in GalNAc alone express as expected only Tn antigen and not T or ST, whereas cells grown in Gal and GalNAc as expected only express ST. Interestingly, cells grown in GalNAc alone do not express the STn structure, which indicate that the CHO ldlD cells do not express significant amounts of ST6GalNAc-I (Marcos *et al.*, 2004). The staining of the anticarbohydrate antibodies was highly dependent on the expression of MUC1, because nontransfected CHO ldlD cells only showed very weak reactivity (not shown). Further

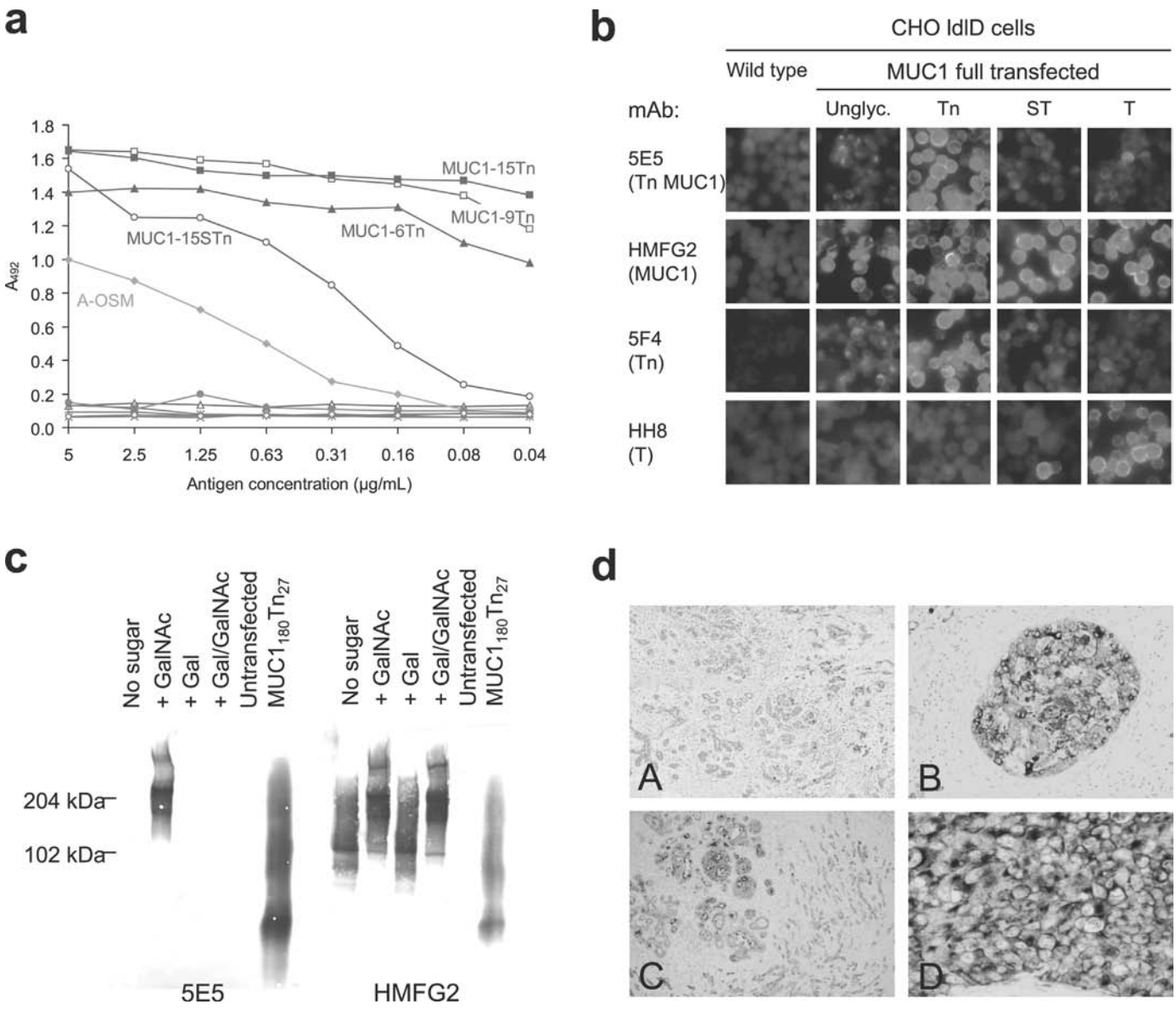

Fig. 3. Characterization of a MAb 5E5 that mimics the immune response elicited in wild-type and MUC1.Tg mice immunized with Tn MUC1. (**a**) ELISA with MAb 5E5 shows strong reactivity with all glycoforms of the MUC1 TR sequence, but no reactivity with the unglycosylated MUC1 peptide. Weak reactivity was also observed with A-OSM, but no reactivity was detected with other Tn glycopeptides. Designations as in Figure 2. Negative control peptides include unglycosylated MUC2, Tn MUC2, Tn MUC4Tn$_1$, and Tn MUC4Tn$_3$. (**b**) Immunofluorescence staining with MAb 5E5 (**top** row) showing reactivity with CHO ldlD cells expressing the Tn MUC1 glycoform, but no reactivity with cells expressing unglycosylated MUC1, ST MUC1, or T MUC1 (after pretreatment with neuraminidase) glycoforms as well as wild-type CHO ldlD cells. Control antibodies to MUC1 (HMFG2), Tn (5F4), and T (HH8) were included to confirm the expression of MUC1 and the respective glycoforms Tn, T, and ST as indicated (**c**) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) western blot analysis of culture medium of CHO ldlD cells secreting different MUC1 glycoforms. MAb 5E5 exhibits strict specificity for the secreted Tn MUC1 glycoform, whereas HMFG2 reacts with all glycoforms as well as unglycosylated MUC1. (**d**) Immunohistochemical staining of breast tissues with MAb 5E5. Primary breast infiltrating ductal carcinoma grade II stained with 5E5. Note that surrounding normal tissue is negative (**A**). Ductal carcinoma *in situ* (DCIS) stained with 5E5 (**B**). Grade II ductal carcinoma showing areas of DCIS. Both infiltrating and DCIS are staining with 5E5 (**C**). Primary breast infiltrating ductal carcinoma grade III stained with 5E5 (**D**).

confirmation of the MUC1 glycoforms produced by CHO ldlD cells have been achieved by mass spectrometric analysis of a secreted *MUC1*-IgG chimeric construct grown with or without sugars (Backstrom *et al.*, 2003) (results to be published elsewhere). 5E5 specifically reacted with the Tn glycoform of recombinant MUC1 expressed in the CHO ldlD cells and did not react with unglycosylated or further glycosylated T and ST MUC1 glycoforms (Figure 3b and c). 5E5 defined a cancer-associated glycoform of MUC1 strongly expressed in most breast cancers (Table I, Figure 3d). 5E5 stained all ductal carcinomas (n = 18) and two lobular carcinomas. The percentage of positive cells varied among <25 to >75. Six cases of benign lesions were examined, of these only two (one fibrosis and one fibroadenoma) showed positive staining with 5E5, and in these cases <25% of the cells stained. This staining pattern closely followed that of

Table I. Immunochemical staining of human breast tissue with anti-MUC1 MAbs

Pathology	Grade	Node	5E5		HMFG2	
			Proportion of tumor cells stained	Intensity	Proportion of tumor cells stained	Intensity
Normal			–	–	+	+
Normal			–	–	–	–
Normal			–	–	–	–
Normal			–	–	+	++
Normal			–	–	+	++
Normal			–	–	–	–
Normal			–	–	–	–
Duct hyperplasia			–	–	+	++
Duct hyperplasia			–	–	–	–
Fibrocystic, duct hyperplasia			–	–	<25%	++
Fibrocystic, duct hyperplasia			–	–	<25%	+
Fibrosis			<25%	+++	<25%	++
Fibroadenoma			<25%	+++	<25%	+++
Lobular carcinoma		–	50–75%	++	50–75%	+++
Lobular carcinoma		+	25–50%	+++	ND	ND
Ductal carcinoma	II	–	50–75%	+++	50–75%	+++
Ductal carcinoma	II	–	25–50%	+++	25–50%	+++
Ductal carcinoma	II	–	50–75%	+++	50–75%	+++
Ductal carcinoma	II	+	50–75%	+++	<25%	+
Ductal carcinoma	III	–	25–50%	+++	25–50%	+++
Ductal carcinoma	III	–	<25%	+++	<25%	+++
Ductal carcinoma	III	–	>75%	+++	>75%	+++
Ductal carcinoma	III	+	<25%	+++	25–50%	+++
Ductal carcinoma	III	+	50–75%	+++	50–75%	+++
Ductal carcinoma	III	+	50–75%	+++	ND	ND
Ductal carcinoma	III	+	>75%	+++	ND	ND
Ductal carcinoma	I	–	50–75%	+++	ND	ND
Ductal carcinoma	I	–	25–50%	+++	ND	ND
Ductal carcinoma	I	+	25–50%	+++	ND	ND
Ductal carcinoma	I	+	25–50%	+++	ND	ND
Ductal carcinoma	I	+	<25%	+++	ND	ND
Ductal carcinoma	I	+	50–75%	+++	ND	ND
Ductal carcinoma	I	+	–	–	ND	ND

ND, not determined.
Intensity: –, no staining; +, weakly positive; ++, moderately positive; +++, strongly positive.

MAb HMFG2 (Burchell *et al.*, 1987) in cancer, but 5E5 was more restricted in normal breast and benign lesions. This further indicates that Tn and STn MUC1 TR glycopeptides represent prime vaccine candidates.

Production of MUC1 glycopeptide-specific responses restricted to cancer-associated MUC1 glycoforms by MUC1.*Tg mice immunized with Tn and STn MUC1 glycopeptides*

MUC1 TR peptide vaccines have generally been ineffective in inducing humoral responses to the cancer-associated MUC1, when the mucin is expressed as a self-antigen, presumably because of tolerance (Goydos *et al.*, 1996; Karanikas *et al.*, 1997; Rowse *et al.*, 1998; Adluri *et al.*, 1999; Acres *et al.*, 2000; Soares *et al.*, 2001). However, as shown in Figure 2, both the Tn and STn 60-mer MUC1 glycopeptides induced strong antibody responses to the glycopeptides in *MUC1*.Tg mice. The specificities of the antibody responses were essentially identical to those found in wild-type mice. The Ig subclass distribution was primarily of IgG1, but responses to STn 60-mer MUC1 included IgG2A and IgG2B subclasses indicating significant class switching

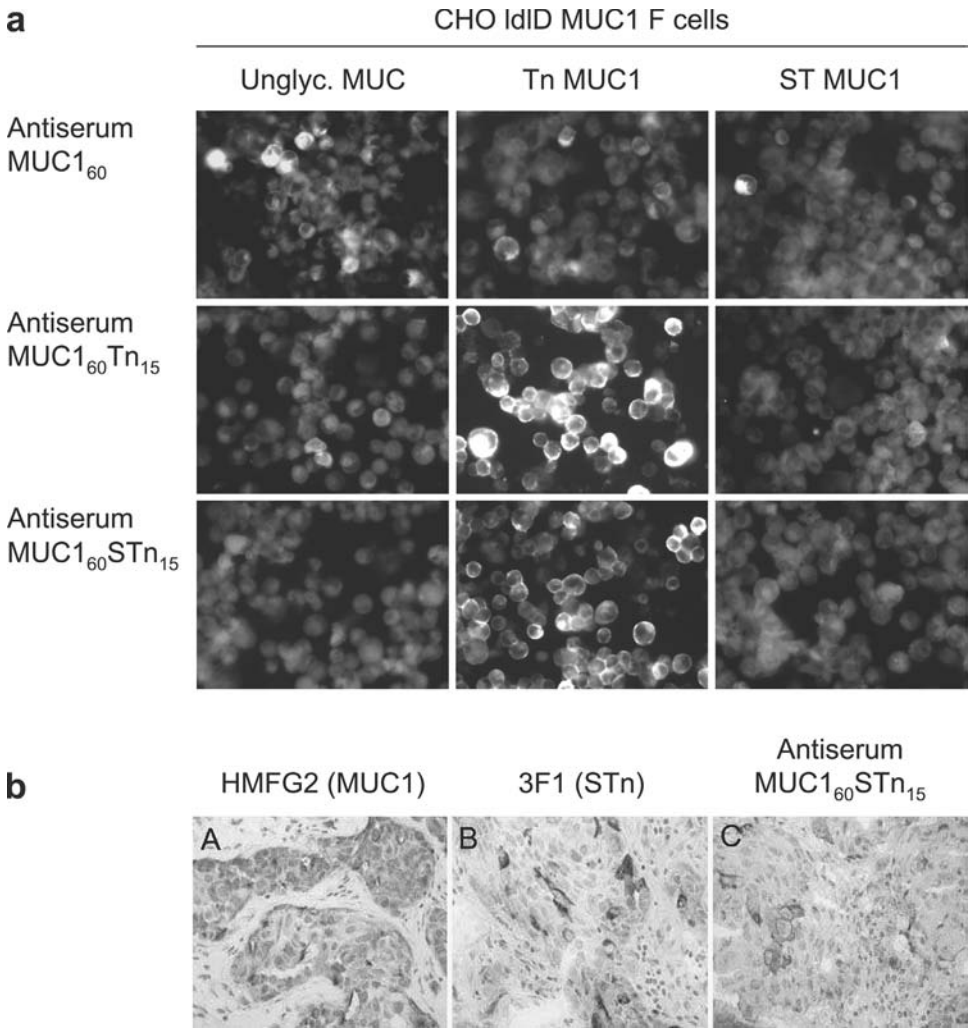

Fig. 4. MUC1.Tg mice immunized with MUC1 Tn and STn glycopeptides produce MUC1 glycopeptide-specific responses restricted to cancer-associated MUC1 glycoforms. (**a**) Sera from MUC1.Tg mice immunized with MUC1 Tn or STn glycopeptides reacted with Tn MUC1 but not unglycosylated or T/ST glycoforms of MUC1 expressed in CHO ldlD cells. Sera from Tg mice immunized with unglycosylated MUC1 reacts preferentially but weakly with CHO ldlD cells expressing unglycosylated MUC1. (**b**) Sera from Tg mice immunized with MUC1 glycopeptides recognize MUC1 expressed by cancer cells. Immunohistochemical staining of a primary breast carcinoma expressing both STn (**B**) and MUC1 (**A**) (determined by MAbs HB-STn and HMFG2) with serum from one Tg mouse immunized with $MUC1_{60}STn_{15}$(**C**).

(not shown). The elicited antibodies reacted with recombinant Tn MUC1 expressed in CHO ldlD cells (Figure 4a) similar to wild-type sera (not shown) and the MAb 5E5 (Figure 3b). Furthermore, sera from mice immunized with Tn MUC1 glycopeptides reacted strongly with the human breast cancer cell line T47D, which mainly carry Tn but also some T and ST *O*-glycans. Sera raised against $MUC1_{60}Tn_{15}$ showed strong staining of T47D cells. Sera from mice immunized with MUC1 60-mer carrying two or three Tn per TR sequence showed intermediate levels of reactivity with the tumor cell line. Sera from *MUC1*.Tg mice immunized with $MUC1_{60}Tn_{15}$ showed intermediate staining of T47D. Another breast cancer cell line, MCF7, has been shown to express MUC1 with *O*-glycans partially based on core two structures and thus has a glycosylation pattern that more closely resembles the pattern found in normal epithelial cells. Sera from mice immunized with $MUC1_{60}Tn_{15}$ showed lower reactivity with MCF7 than with T47D cells. MCF7 was not stained by sera raised against $MUC1_{60}STn_{15}$. All sera demonstrated very low reactivity with the nontumorigenic epithelial cell line MTSV1–7, which expresses high levels of C2GnT1 and produces MUC1 carrying core 2-based *O*-glycans (Dalziel *et al.*, 2001). Finally, antisera from the *MUC1*.Tg mice immunized with $MUC1_{60}STn_{15}$ reacted with primary breast carcinomas expressing MUC1 and STn (Figure 4b).

Discussion

The initial step in mucin-type O-glycosylation involves attachment of GalNAc residues to Thr and Ser amino acids. This process is carried out by a large family of UDP-GalNAc : polypeptide *N*-acetylgalactosaminyltransferases (GalNAc transferases), and because of subtle differences in their acceptor peptide substrate specificities *O*-glycan

attachment sites can be controlled using different combinations of isoenzymes (Figure 1) (Hassan *et al*., 2000a). Thus, the 13 functional GalNAc transferase isoforms characterized to date exhibit distinct substrate specificities with some overlap, although consensus amino acid sequences defining the substrate specificities of individual GalNAc transferases are at present undefined. A further level of complexity is that GalNAc glycosylation of mucin sequences with a high density of *O*-glycan attachment sites requires an orchestrated action of multiple GalNAc transferase isoforms. Specifically, three of the five potential glycosylation sites in the MUC1 TR can be glycosylated by one of several GalNAc transferases, but only one isoform, GalNAc-T4, is capable of glycosylating the other two sites (Bennett *et al*., 1998). Interestingly, the GalNAc-T4 enzyme requires prior partial GalNAc glycosylation by other isoforms because its unique specificity is directed by a lectin-mediated interaction with GalNAc residues (Bennett *et al*., 1998; Hassan *et al*., 2000b). In this study, we used combinations of three human recombinant GalNAc transferases to synthesize 60-mer MUC1 TR glycopeptides carrying two, three, or five *O*-glycans per repeat (Figure 1). The 60-mer peptide design was chosen as it represents the minimum length of MUC1 TR peptides shown to adopt conformation and still allow detailed structural analysis by MALDI-TOF (Fontenot *et al*., 1995). Availability of some other recombinant glycosyltransferases acting in the mucin-type O-glycosylation pathway opens possibilities for the synthesis of more complex structures. The chemoenzymatic synthesis used in this study relies on the chemical synthesis of long MUC1 TR peptides (Kotera *et al*., 1994; Fontenot *et al*., 1995). To increase the versatility of this approach, longer recombinant peptides based on the TR of MUC1 and mucins have been expressed in bacteria and used successfully as substrates for *in vitro* O-glycosylation (not shown).

We have previously used a short glycopeptide vaccine based on the *MUC2* TR sequence (23 amino acids) carrying a high density of Tn epitopes and found that this glycopeptide linked to KLH elicited strong IgG1 responses reactive only with the glycopeptide or the corresponding glycosylated MUC2 mucin, but not with the hapten sugar or the unglycosylated peptide (Reis *et al*., 1998b). Such antibodies presumably recognize conformational peptide epitopes induced by the glycosylation with or without actual contact to the carbohydrate unit. In this report, we describe the induction of similar antibodies to MUC1 glycopeptides, not only in wild-type mice but also in mice expressing the human *MUC1* transgene from its own promoter. Initially, we examined humoral immune responses to the 60-mer MUC1 TR peptide with zero, two, three, and five Tn epitopes per repeat. In both wild-type Balb/c mice and *MUC1*.Tg mice, the glycopeptides carrying three and five Tn epitopes per repeat elicited the strongest antibody response reacting with MUC1 expressed in breast cancer cell lines. The unglycosylated peptide induced antibodies to the peptide used as immunogen, but these showed no or only weak reactivity with Tn glycosylated peptides and cell lines. The fully Tn and STn glycosylated peptides produced antibody responses that were highly reactive with both the STn and the Tn glycoforms but only weakly reactive with the unglycosylated MUC1 peptide in both wild-type and MUC1.Tg mice (Figure 2). In agreement with our previous studies, only low levels of antibodies reactive with the carbohydrate haptens Tn and STn were found (Reis *et al*., 1998b). Although the most effective design was the MUC1 glycopeptides with complete *O*-glycan occupancy, the elicited antibodies showed a range of reactivity with all Tn and STn glycoforms of MUC1. Importantly, no reactivity with the T glycoform was found indicating that further extension of *O*-glycans toward the normal glycosylation pattern (ST and core 2 structures) masks the epitope.

Although MUC1 is a self-protein, it is clear that cancer cells selectively express glycoforms that are antigenically distinct from the mucin produced by the normal epithelial cells from which they derive. The specific changes in patterns of glycosylation have been well characterized in breast cancer, where the cancer-associated glycoforms can carry short unbranched *O*-glycans which are simpler than the branching core 2-based structures carried on the mucin produced by the normal breast. These changes relate to changes in the levels and expression of glycosyltransferases in breast cancers. An increase in the level of expression of the sialyltransferase, ST3Gal-I, which competes for the same substrate as the core 2-branching β6GlcNAc transferase, C2GnT1, has been observed in breast carcinomas (Burchell *et al*., 1999; Dalziel *et al*., 1999, 2001), and the expression of the sialyltransferase, ST6GalNAc-I, which is not found in normal breast, is seen in ~30% of breast carcinomas (Sewell *et al*., manuscript in preparation). Moreover, studies of breast cancer cell lines have shown that the density of *O*-glycans in the MUC1 TR sequence is high as compared with MUC1 isolated from milk (Muller *et al*., 1997, 1999; Muller and Hanisch, 2002; Hanisch *et al*., 2003).

Many murine MAbs have been generated to the TR sequence of MUC1, and several of these exhibit cancer-associated reactivity patterns reflecting the changes in the profile of mucin-type O-glycosylation. One classic example is the SM3 antibody whose reactivity is abolished by core 2 O-glycosylation of the TRs (Burchell *et al*., 1987; Dalziel *et al*., 2001) and enhanced by glycosylating all sites in the TR with the simpler *O*-glycans (Karsten *et al*., 1998; Reis *et al*., 1998a). One MAb, VU-2-G7, generated to a MUC1 TR peptide with a single GalNAc residue (Tn glycoform) attached to each repeat at the immunodominant region in wild-type mice (-PDTR-) was also found to exhibit a cancer-associated reactivity pattern (Ryuko *et al*., 2000). These findings may be in agreement with MUC1 in cancer cells having short *O*-glycans and higher density of O-glycosylation.

The finding that *MUC1*.Tg mice elicit a strong humoral immune response with specificity for cancer-associated glycoforms of MUC1 suggests that the vaccine design used can overcome tolerance previously shown to be a problem in humans. The prevailing specificity of the immune response is directed to the Tn and STn glycoforms of MUC1 with strongest reactivity with the complete *O*-glycan-covered MUC1 glycopeptide. The prevailing specificity is mimicked in the MAb 5E5, which also exhibits remarkable cancer specificity and may have diagnostic and therapeutic value. In conclusion, this report presents a novel promising vaccine design based on the highly abundant cell surface mucin MUC1 that warrant preclinical studies in appropriate animal models for tumor protection and rejection.

Materials and methods

Chemoenzymatic synthesis of multimeric Tn and STn MUC1 glycopeptides

MUC1 60-mer $(VTSAPDTRPAPGSTAPPAHG)_{n=3}$ peptide was synthesized, as originally reported by Fontenot *et al.* (1993). Control peptides used were derived from TR of MUC2 (PTTTPISTTTMVTPTPTPTC) and MUC4 (CPLPVTDTSSASTGHATPLPV). Peptides were glycosylated *in vitro* using purified recombinant human glycosyltransferases polypeptide GalNAc-T2, GalNAc-T4, and GalNAc-T11 (Bennett *et al.*, 1998; Schwientek *et al.*, 2002). The GalNAc-substituted peptides were subsequently sialylated using purified recombinant mouse ST6GalNAc-I (Kurosawa *et al.*, 2000). GalNAc glycosylation of the peptides was performed in a reaction mixture (1 mg peptide/mL) containing 25 mM cacodylate buffer (pH 7.4), 10 mM $MnCl_2$, 0.25% Triton X-100, and 2 mM UDP-GalNAc. Glycosylation of 1 mg 60-mer peptide with two GalNAc per TR ($MUC1_{60}Tn_6$) was obtained using GalNAc-T11. Incorporation of three GalNAc per TR ($MUC1_{60}Tn_9$) was obtained using GalNAc-T2. Substitution of all five putative O-glycosylation sites in the MUC1 TR ($MUC1_{60}Tn_{15}$) was performed using $MUC1_{60}Tn_9$ as substrate in a reaction with GalNAc-T4. Sialylation was performed in a reaction mixture (1 mg peptide/mL) containing 20 mM Bis–Tris buffer (pH 6.5), 20 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, and 2 mM cytidine-5′-monophospho-N-acetylneuraminic acid sodium salt (Sigma, Brondby, Denmark). Glycosylation was monitored using nano-scale reversed-phase columns (Poros R3, PerSeptive Biosystems, Framingham, MA) and MALDI-TOF mass spectrometry. The glycopeptides were purified by high-performance liquid chromatography (HPLC) on a Zorbax 300SB-C3 column (9.4 mm × 25 cm) (Agilent Technologies, Palo Alto, CA) in an 1100 Hewlett Packard system (Avondale, PA) using 0.1% trifluoroacetic acid (TFA) and a gradient of 0–80% acetonitrile. Quantification and estimation of yields of glycosylation reactions were performed by comparison of HPLC peaks by UV 210 absorbance using 10 μg weighed peptide as standard. GalNAc glycosylation of peptides generally yielded 80–90% recovery, whereas the sialylation step was more variable with yields from 60 to 80%. Purified glycopeptides were characterized by MALDI-TOF mass spectrometry on a Voyager DE or Voyager DE Pro MALDI-TOF mass spectrometer (PerSeptive Biosystems) equipped with delayed extraction. The MALDI matrix was 2,5-dihydroxybenzoic acid 10 g/L (Aldrich, Milwaukee, WI) dissolved in 2:1 mixture of 0.1% TFA in 30% aqueous acetonitrile. Samples dissolved in 0.1% TFA to a concentration of ~1 pmol/μL were prepared for analysis by placing 1 μL of sample solution on a probe tip followed by 1 μL of matrix. All mass spectra were obtained in the linear mode. Data processing was carried out using GRAMS/386 software (Galactic Industries, Salem, NH).

Immunization protocol

Glycopeptides were coupled to KLH (Pierce, Rockford, IL) using glutaraldehyde. Efficiency of conjugation was assessed by analyzing the reaction by size exclusion chromatography on a PD-10 column using anti-MUC1 ELISA of fractions. Essentially all reactivity was found with the excluded fraction and insignificant reactivity in the included fractions expected to contain peptides. Further evaluation included comparative titration analysis of the KLH conjugate with the corresponding glycopeptide in ELISA. Both analyses indicated that the conjugation was near complete, which should result in a KLH to glycopeptide ratio of 1:300. *MUC1*.Tg mice homozygous for the transgene expression were originally developed on an H2-k background (Peat *et al.*, 1992). Subsequently, these mice have been backcrossed onto a Balb/c strain for 15 generations to give a pure Balb/c (H2-d) background (Graham and Taylor-Papadimitriou, unpublished data). Female Balb/c wild-type and *MUC1*.Tg mice were injected subcutaneously with 10 or 15 μg of (glyco)peptide in a total volume of 200 μL (1:1 mix with Freunds adjuvant, Sigma). Mice received four immunizations 14 days apart, and blood samples were obtained by tail or eye bleeding 1 week following the third and fourth immunization.

Generation of mouse MAb anti-Tn/STn-MUC1

A MAb was produced, as described previously (Reis *et al.*, 1998b), from a wild-type Balb/c mouse immunized with the fully GalNAc-glycosylated 60-mer MUC1 glycopeptide coupled to KLH. Screening was based on glycopeptide ELISA followed by immunocytology with breast cancer cell lines (MCF7, T47D, MTSV1–7) and immunohistology with breast cancer tissues. Selection was based on reactivity pattern similar to total sera of the same mouse.

ELISA

ELISA were performed using 96-well MaxiSorp plates (Nunc, Denmark). Plates were coated overnight at 4°C with 1 μg/mL of glycopeptides in bicarbonate–carbonate buffer (pH 9.6), blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), followed by incubation with sera (diluted in PBS) or MAbs for 2 h at room temperature. Bound antibodies were detected with peroxidase-conjugated rabbit anti-mouse immunoglobulins (DakoCytomation, Glostrup, Denmark) or isotype-specific antibodies peroxidase-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology Associates, Birmingham, AL). Plates were developed with *O*-phenylenediamine tablets (DakoCytomation) and read at 492 nm. Control antibodies included anti-MUC1 antibodies HMFG2 and SM3 (Burchell *et al.*, 1987) and anticarbohydrate antibodies 5F4 (Tn) and 3F1 (STn) (Mandel *et al.*, 1991). Control sera included mice immunized with MUC4 mucin peptide linked to KLH.

Cell lines

The human mammary cell lines MCF7 (Soule *et al.*, 1973), MTSV1–7 (Bartek *et al.*, 1991), and T47D (Keydar *et al.*, 1979) and the murine pancreatic carcinoma cell line Panc02 were cultured, as previously described. CHO ldlD cells were stably transfected with full-coding *MUC1* containing 32 TRs and grown with or without the addition of Gal/GalNAc as indicated. Confluent cultures of CHO ldlD cells in six-well MaxiSorp plates (Nunc, Denmark) were grown in

HAM'S F12 with 10% fetal calf serum (FCS) without GalNAc and Gal, in presence of 1 mM GalNAc or in the presence of 1 mM GalNAc and 0.1 mM Gal (Sigma Aldrich, Brondby, Denmark). The medium was harvested after 48 h of growth and used for immunoassays. Cells were trypsinized, washed, and air-dried on coverslides for immunocytology.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis western blot

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) western blot analysis was performed, according to manufacturer's instructions (4–12% gradient gel, Biowhittaker Molecular Applications, Walersville, ME). Membranes were blocked in 15% skimmed milk powder (Merck Eurolab, Dorset, UK), incubated with MAbs 5E5 and HMFG2 overnight at 4°C, followed by incubation with biotinylated goat anti-mouse IgG1 (0.5 μg/mL) (Southern Biotechnology Associates) for 1 h at room temperature. Membranes were incubated with avidin horseradish peroxidase conjugate (0.36 μg/mL) (DakoCytomation) for 30 min at room temperature, followed by 50 mM Tris–HCl buffer (pH 7.6) containing 0.04% 4-chloro-1-naphthol (Sigma) and 0.025% H_2O_2.

Immunocytochemistry

Cell lines were fixed for 10 min in ice-cold acetone or in methanol : acetone. Fixed cells were incubated overnight at 5°C with mouse sera (1:200/1:400/1:800) or MAbs, followed by incubation for 45 min at room temperature with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins (DakoCytomation). Slides were mounted in glycerol containing *p*-phenylenediamine and examined in a Zeiss fluorescence microscope (FluoresScience, Hallbergmoos, Germany).

Immunohistochemistry

Formalin fixed, paraffin wax embedded tissues of breast carcinoma were obtained from the tissue bank at Guy's Hospital, London. All cases were conventionally classified by histological type. The avidin–biotin peroxidase complex method was used for immunostaining. Paraffin sections were dewaxed, rehydrated, and treated with 0.5% H_2O_2 in methanol for 30 min. Sections were rinsed in TBS and incubated for 20 min with rabbit nonimmune serum. Sections were rinsed and incubated overnight at 5°C with primary antibody. Sections were rinsed and incubated with biotin-labeled rabbit anti-mouse serum (DakoCytomation) diluted 1:200 in TBS for 30 min, rinsed with TBS, and incubated for 1 h with avidin–biotin peroxidase complex (DakoCytomation). Sections were rinsed with TBS and developed with 0.05% 3,3′-diaminobenzidine tetrahydrochloride freshly prepared in 0.05 M TBS containing 0.1% H_2O_2. Sections were stained with hematoxylin, dehydrated, and mounted.

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Abbreviations

CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assays; KLH, keyhole limpet hemocyanin; MAbs, monoclonal antibodies; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; *MUC1*.Tg, *MUC1* transgenic; OSM, ovine submaxillary mucin; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TR, tandem repeat.

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