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Enhanced Binding of Antibodies to the DTR Motif of MUC1 Tandem Repeat Peptide Is Mediated by Site-specific Glycosylation[1]

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ABSTRACT

The epithelial mucin MUC1 is an important tumor marker of breast cancer and other carcinomas. Its immunodominant DTR motif, which is the principal target for immunotherapeutic approaches, has been assumed until recently not to be glycosylated in both normal and tumor MUC1 and to acquire its immunogenic conformation by virtue of a certain number of tandem repeats. We present evidence that the antigenicity of the single repeat toward a considerable number of antibodies to the DTR motif is greatly enhanced if it is glycosylated within this motif, and *only* in this position. Twenty-eight monoclonal anti-MUC1 antibodies with DTR specificity were tested for binding to synthetic 21-mer (AHG21) or 20-mer (HGV20) tandem repeat peptides *O*-glycosylated with galactose β1–3*N*-acetylgalactosamine α or *N*-acetylgalactosamine α at defined Thr or Ser positions. Binding was measured in ELISA experiments using the glycopeptides as plate-immobilized antigens or as inhibitors in solution. At least 12 antibodies revealed significantly enhanced binding to the peptides glycosylated at the DTR motif (Thr-10) as compared to positional isomers glycosylated at Thr-5, Ser-6, Ser-16, or Thr-17 and to the nonglycosylated peptides. Six antibodies (VU-3-C6, A76-A/C7, Ma552, VU-11-D1, VU-12-E1, and VU-11-E2) that were unreactive with the monomeric repeat peptide did bind to the DTR-glycosylated peptide. Several lines of evidence suggest that glycosylation with *N*-acetylgalactosamine is sufficient for the observed enhancement effect. Our results are of special interest in conjunction with the recent observation that the DTR motif of lactation-associated MUC1 is *O*-glycosylated *in vivo* (Müller *et al.*, J. Biol. Chem., *272:* 24780–24793, 1997). They may have consequences for the design of efficient tumor vaccines.

INTRODUCTION

The glycosylation status of mucins represents a complex and yet regulated epigenetic phenomenon that is susceptible to tumor-associated alterations. In particular, the widely distributed epithelial mucin, MUC1, has been described in numerous reports to be aberrantly processed in cancer cells. Both immunochemical and chemical evidence obtained for human mammary carcinoma cells agree in the observation that the tumor-associated deletion of Galβ1–3GalNAc[3]/β-6-glucosaminyl-transferase leads to the truncation of polylactosamine-type glycans and to the accumulation of core-type chains (1). Reduced glycosylation of MUC1 is assumed to permit the immune system access to the peptide core of the mucin. The preferred target for most peptide-specific antibodies generated to the tumor mucin is located within the tandem repeat of MUC1 and comprises the DTR motif (2). Besides a strong humoral response, cytotoxic T cells that recognize the nonglycosylated DTR motif in a non-MHC-restricted manner have been detected in breast cancer patients (3). Consequently, the development and evaluation of effective tumor vaccines based on the MUC1 tandem repeat peptide is currently under study (4). The immunodominance of the DTR motif has been attributed to its knob-like secondary structure (5) and to the assumption that threonine within the motif represents a poor substrate for peptide *N*-acetylgalactosaminyltransferases (GalNAc-T) (6). In accordance with this, *in vitro* glycosylation studies have revealed that GalNAc-T(s) from different sources and also the available recombinant enzymes are not able to glycosylate the DTR motif (7–9). These findings are contrasted, however, by the recent localization of *O*-glycosylation sites on lactation-associated MUC1 (10) and by the demonstration that all putative sites within the tandem repeat are glycosylation targets *in vivo*. With this important finding in mind, it appeared challenging to reexamine the antigenicity of the immunodominant region, and especially to compare its glycosylated and unglycosylated forms in this respect. This undertaking was possible because a new series of synthetic 21-mer MUC1-derived glycopeptides became available due to newly developed methods of synthesis. These glycopeptides carry Galβ1–3GalNAcα (TF antigen) or GalNAcα (Tn antigen) at different single or multiple positions. The results revealed that the majority of mAbs generated to MUC1 bind significantly stronger to the peptide if it is glycosylated with the core-type glycans at the Thr within the immunodominant DTR motif, and *only* at this position.

MATERIALS AND METHODS

Antibodies. The 29 antibodies used in this study were chosen from the panel of 56 MUC1-reactive mAbs submitted to the International Society for Oncodevelopmental Biology and Medicine TD-4 (MUC1) Workshop, San Diego, CA, held on November 17–22, 1996 (2). The purified antibodies were supplied as standardized solutions (1 mg/ml PBS) and are listed in Table 1 with reference to their immunoglobulin classes and subtypes and to their epitopes. The antibodies have been selected on the basis of their epitopes, which overlap at the DTR motif of the tandem repeat peptide. Antibodies with nonreactivity to the monomeric repeat peptide TAP25 (2) are marked in the table. Eleven mAbs were selected for quantitative binding studies (as indicated in Table 1) according to the following rules: (*a*) having a confirmed epitope within the immunodominant region of the MUC1 repeat; and (*b*) representing a balanced panel of mAbs with respect to the type of immunogen used (synthetic peptide versus native MUC1) and to the type of reactivity toward synthetic peptides of different length. Antibody 115D8, which does not bind to the DTR motif but to a MUC1-specific, NeuAc-containing epitope, was used as a negative control.

Synthesis of Glycopeptides. To synthesize glycopeptides, glycosylamino acid building blocks that already contain the oligosaccharide chain and threonine or serine are required. The syntheses of these building blocks have been described (26). The multiple column solid-phase synthesis was carried out in a semimanual 20-column multiple synthesizer (26), and Wang resin was selected as support material. The Wang resin (2.5 g) was placed in a glass reactor, swelled in dichloromethane (15 cm^3 for 10 min) and washed. A mixture of Fmoc-Ala-OH (3.40 mmol), 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (3.40 mmol) and methylimidazole (3.40 mmol) in dichloromethane (15 cm^3) was added. After 2 h, the resin was washed, and the unchanged amino groups were acetylated with acetic anhydride/dimethylformamid (1:1; 15 cm^3). The derivatized resin was packed for the glycopeptide synthesis in the 20 columns of the synthesizer. The reaction and washing solvent was DMF, and

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[3] The abbreviations used are: GalNAc, *N*-acetylgalactosamine; Gal, galactose; TF, Thomsen-Friedenreich; VNTR, variable number of tandem repeats; mAb, monoclonal antibody; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; NMR, nuclear magnetic resonance.

Table 1 *Antibodies included in this study*

mAb	Isotype	Epitope	Experiment[a]	Ref.
Ma552	IgG1	GVTSAPDTRPAP[b]	q, i	11
BC3	IgM	APDTR		12
HMPV	IgM	APDTR		13
VU-3-C6	IgG1	GVTSAPDTRPAP[b]	q, i	2
VU-12-E1	IgG1	PDTRPAP	q, i	2
MF06	IgG1	DTRPAP	q, i	2
VU-11-D1	IgG1	TSAPDTRP[b]	q, i	2
VA1	IgG1	TRPAP		14
BCP8	IgG2b	PDTRPA	i	15
DF3	IgG1	APDTRPAP[b]		16
BC2	IgG1	APDTR	q	12
B27.29	IgG1	PDTRPAP	i	17
VU-3-D1	IgG1	SAPDTRPAP		2
VU-4-H5	IgG1	APDTRPAP		2
BC4E549	IgG1-k	TSAPDTRPAP	i	18
VU-11-E2	IgG1	TSAPDTRP[b]	i	2
E29	IgG2a	APDTRP	q, i	19
GP1.4	IgG1	PDTRPAPGS		2
214D4	IgG1	PDTR	i	2
SM3	IgG1	APDTRP	q, i	20
BC4W154	IgM	DTRPAP		18
HMFG-1	IgG1	APDTR	q	21
VA2	IgG1	APDTRPA	q, i	14
B12	IgG1	PDTRPAP		22
C595	IgG3	TRPAP		23
BCP10	IgM	DTRPAP[b]		15
Mc5	IgG1-k	DTRPAP		24
A76-A/C7	IgG1-k	APDTRPAP[b]	q, i	2
115D8	IgG2b-k		q	25,2

[a] A screening of all 28 antibodies reactive to the DTR motif was performed in duplicate assays by the AP method on TAP25 and some of the glycopeptides (A1–A5 and A9). A selected panel of the antibodies was used for the quantitative binding studies performed in quadruplicate by the POD method (q). Another panel of antibodies was selected for the binding inhibition studies (i).

[b] Nonreactivity to monomeric repeat peptide TAP25.

the Fmoc deprotections were performed by treatment with piperidine (20%) in DMF (20 min). The amino acids were coupled as Fmoc amino acid pentafluorophenyl ester with dihydro-1,2,3-benzotriazine (3 mol equivalent). The Gal(1→3)GalNAc-containing building block was coupled with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate and *N*-ethyldiisopropylamine (1.5 mol equivalent). After 20 h of reaction time, the synthesis cycle was repeated to complete the assembly of each glycopeptide. After removal of the last Fmoc groups, the resins were washed, dried, treated with 95% aqueous trifluoroacetic acid (2 cm^3 for 2 h), and filtered off. Then, the compounds were treated with catalytic amounts of 1% CH_3ONa in methanol at pH 8.5 to remove the acetylic groups of the saccharide part, and purified by preparative reversed phase-high-performance liquid chromatography. The pure *O*-glycopeptides were obtained in yields of 16–57% after lyophilization.

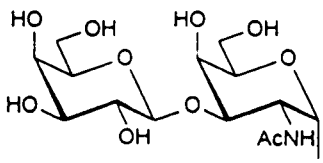
The glycopeptides listed in Table 2 were analyzed by ^1H NMR spectroscopy (400 MHz; Table 3) as described (26) and confirmed with respect to their calculated molecular masses by matrix-assisted laser desorption ionization mass spectrometry (10). The peptides A1–A9 correspond to a 21-mer of the MUC1 tandem repeat domain starting with the AHG motif (AHG21) and carrying one to five *O*-linked disaccharides Galβ1–3GalNAc in varying positions. Glycopeptides A11, A12, and A13 carrying GalNAc in defined positions of a 20-mer (HGV20) or 21-mer (AHG21) were synthetized similarly as described above (Table 2). Control peptides TAP25 (corresponding to one repeat and five overlapping amino acids and starting with the TAP motif) or PAH60, PAH100, and PAH120 (corresponding to three, five, and six repeats, respectively, and starting with the PAH motif) were kindly provided by Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London, United Kingdom) or Dr. Jo Hilgers (Department of Obstetrics and Gynecology, Free University, Amsterdam, The Netherlands).

Binding and Inhibition Analyses in Enzyme-linked Immunoassays. Two types of enzyme immunoassays (ELISA) were used. In the antigen-coated mode, 96-well microtest plates of tissue culture type (Nunc, Wiesbaden, Germany) were coated with 50 μl per well of a solution of 10 μg/ml of the respective glycopeptide or of the unglycosylated reference peptide TAP25 in 0.1 M carbonate buffer, pH 9.6, at 37°C, which resulted in drying of the antigen

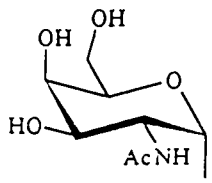
Table 2 *Synthetic glycopeptides used in this study*[a]

A1	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A2	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A3	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A4	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A5	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A6	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A7	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A8	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A9	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A11	His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A12	His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
A13	Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala
Position	1 5 6 10 16 17

[a] The peptides A1–A9 correspond to a 21-mer of the MUC1 tandem repeat domain starting with the AHG motif (AHG21) and carrying one to five *O*-linked disaccharides Galβ1-3GalNAc in varying positions (*; see below *left*). Glycopeptides A11, A12, and A13 carry GalNAc in defined positions (*; see below *right*).



Galβ1-3GalNAc



GalNAc

Table 3 *Data of synthesized glycopeptides: MS data by matrix-assisted laser desorption ionization time-of-flight mass spectrometry*

From the ^{1}H NMR spectra (400 MHz) only the 1-H values (δ ppm, coupling Hz in parentheses) of the GalNAc units are shown, demonstrating the number of carbohydrate side chains of the peptides. The complete NMR data are available on request.

Glycopeptide	Formula	$M_{calc}/M + H_{found}$	1-H of GalNAc units of the side chains
A1	$C_{97}H_{155}N_{27}O_{39}$	2323.5/2324.5	4.91 (3.6)
A2	$C_{97}H_{155}N_{27}O_{39}$	2323.5/2324.3	4.85 (3.7)
A3	$C_{97}H_{155}N_{27}O_{39}$	2323.5/2323.5	4.90 (3.6)
A4	$C_{97}H_{155}N_{27}O_{39}$	2323.5/2323.9	4.81 (3.6)
A5	$C_{97}H_{155}N_{27}O_{39}$	2323.5/2323.5	4.85 (3.6)
A6	$C_{111}H_{178}N_{28}O_{49}$	2688.9/2691.0	4.86 (3.6); 4.84 (3.7)
A7	$C_{125}H_{201}N_{29}O_{59}$	3054.3/3056.0	4.82 (3.6); 4.79 (3.2); 4.78 (3.5)
A8	$C_{139}H_{224}N_{30}O_{69}$	3419.5/3420.5	4.89 (4.0); 4.87 (4.0); 4.83 (3.6); 4.81 (4.0)
A9	$C_{153}H_{247}N_{31}O_{71}$	3784.4/3785.0	4.92 (3.6); 4.91 (3.2); 4.87 (3.5); 4.86 (3.6); 4.82 (3.5)
A11	$C_{98}H_{155}N_{27}O_{39}$	2334.1/2335.5	4.86 (3.8); 4.85 (3.8)
A12	$C_{120}H_{192}N_{30}O_{53}$	2901.3/2902.3	4.94 (3.9); 4.93 (3.9); 4.89 (3.6); 4.87 (3.6); 4.84 (3.7)
A13	$C_{91}H_{145}N_{27}O_{34}$	2143.3/2144.2	4.78 (3.4)

onto the plastic. For quantitative binding studies with a selected panel of antibodies, the purified mAbs, diluted with PBS/Tween containing 1% BSA to a concentration of 10 μg/ml, were added after three washes with PBS/0.05% Tween 20. The plates were incubated in a moist chamber at 37°C for 2 h, washed three times as above, and then incubated with peroxidase-labeled rabbit antimouse immunoglobulin serum (DAKO, Hamburg, Germany) diluted 1:2000. After three final washes as above, color development was accomplished with *o*-phenylenediamine for 15 min, and stopped with 2.5 N sulfuric acid. Absorbance was read with a Spectra photometer (SLT Labinstruments, Salzburg, Austria) at 492 nm. Blank values were substracted, and means were calculated from four or more individual measurements. In some cases, carbohydrate-selective periodate oxidation of the antigen (10 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.5, for 1 h at 25°C) and reduction of active aldehydes (50 mM $NaBH_4$ in PBS for 30 min) was performed as described by Woodward *et al.* (27). The efficiency of this treatment was checked in binding assays with TF- or Tn-specific mAbs. Statistical analysis was done using one-way ANOVA or *t* tests performed with the GraphPad Prism program (GraphPad Software, San Diego, CA).

For the screening of 29 mAbs on a selected panel of glycopeptides (A1–A5 and A9) and on nonglycosylated peptides TAP25 or PAH100, the antigens were coated on polystyrene microtitration plates by drying in a desiccator for 2 h. After blocking the active surface with a solution of 5% BSA in PBS for 1 h at 37°C, the primary antibodies were incubated in duplicate for 18 h at 4°C. Bound antibody was measured by a double sandwich technique using rabbit antimouse immunoglobulin (Z259, DAKO) and mouse antialkaline phosphatase-alkaline phosphatase complex as described (10). Color was developed with *p*-nitrophenylphosphate (1 mg/ml) in 50 mM diethanolamine buffer, pH 9.3, and the absorbance was read with a Multiscan photometer (Labsystems, Bornheim Hersel, Germany) at 405 nm.

For inhibition assays, the nonglycosylated peptides TAP25 and PAH100 (1 μg/ml) were immobilized by drying in a desiccator as above. To the blocked wells, the primary antibodies (10 μg/ml) were added, together with serial 2-fold dilutions of the peptide (or glycopeptide) inhibitors (starting from 50 μg/ml 0.5% BSA in PBS) and incubated for 18 h at 4°C. Quantification of bound antibody was performed in the antialkaline phosphatase-alkaline phosphatase technique as described above. With respect to the concentration of binding-active repeat monomers, 50 μg/ml of the glycopeptide inhibitors correspond to 22 (TAP25), 22 (A2 and A3) or 26 (PAH100) μM. The assays were performed in duplicate, and mean values of the measured absorbances at 405 nm and the calculated ID_{50} concentrations are presented in Table 4.

RESULTS

Of the 28 DTR-specific mAbs included in this study, 7 were nonreactive to the monomeric tandem repeat peptide of MUC1 (TAP25) as previously shown (Ref. 2; Table 1). Six of these were grouped to a cluster of antibodies with high binding activities on the oligomeric tandem repeat peptide (PAH100; Ref. 2). The remaining antibodies exhibited varying binding affinities to the monomeric repeat peptide (Table 1).

Quantitative Binding Assays of Antibodies on Glycopeptides. Glycopeptides corresponding to the MUC1 repeat carrying either Galβ1–3GalNAcα (TF) or GalNAcα (Tn) at one or more of the five possible glycosylation sites were coated to ELISA plates and reacted with anti-MUC1 antibodies specific for the immunodominant DTR motif. Although all of the mAbs recognized the 120-mer (consisting of 6 VNTR units), four of the mAbs tested in quantitative binding assays did not bind to single VNTR units (20-mers). The surprising result of the present experiments was that glycosylation with TF or Tn at the Thr within the epitope led to a substantial enhancement of binding by these antibodies and to a significantly stronger binding by some of the remaining antibodies. In detail, the following patterns of reactivity were observed (Figs. 1–3).

Type A: MF06. This antibody reacted with single unsubstituted VNTR peptide in ELISA (Table 4). Of the 11 glycopeptides tested (Fig. 1*A*), 8 were almost identical to the unsubstituted VNTR peptide in their

Table 4 *Summary of antibodies with preferential binding to the glycosylated DTR motif as revealed by inhibition analyses*

	Binding to coated single or multiple VNTR peptides[a]			% Binding activity remaining on inhibition with 0.01 mg/ml (ID_{50} values, μM)		
Antibody	20-mer	60-mer	120-mer	TAP25[b] or PAH100[c]	A2	A3
Ma552	38	74	142	72 (13)[c]	65 (6)	102 (>22)
VU-3-C6	93	145	375	62 (7)[c]	19 (0.3)	107 (>22)
A76-A/C7	0	65	218	37 (2)[c]	5 (0.3)	99 (11)
VU-11-D1	26	110	336	32 (0.4)[c]	29 (0.9)	73 (7)
VU-11-E2	23	116	374	35 (3)[c]	30 (2)	65 (11)
E29	389	1080	1034	2 (<0.75)[b]	1 (<0.75)	6 (<0.75)
SM-3	297	534	678	42 (3)[b]	8 (0.4)	46 (4)
VU-12-E1	300	902	1251	40 (2)[b]	16 (0.9)	85 (>22)
MF06	458	715	842	56 (4)[b]	14 (0.7)	69 (9)
214D4	624	891	1036	14 (0.9)[b]	4 (0.2)	16 (0.9)
BCP8	464	886	937	14 (0.75)[b]	6 (<0.75)	27 (2)
B27.29	315	672	923	85 (>50)[b]	46 (8)	70 (>50)

[a] Absorbance × 1000.
[b] Antibodies reactive to monorepeat peptides were tested on TAP25.
[c] Antibodies with exclusive reactivity to oligomeric repeat peptides were tested on PAH100.

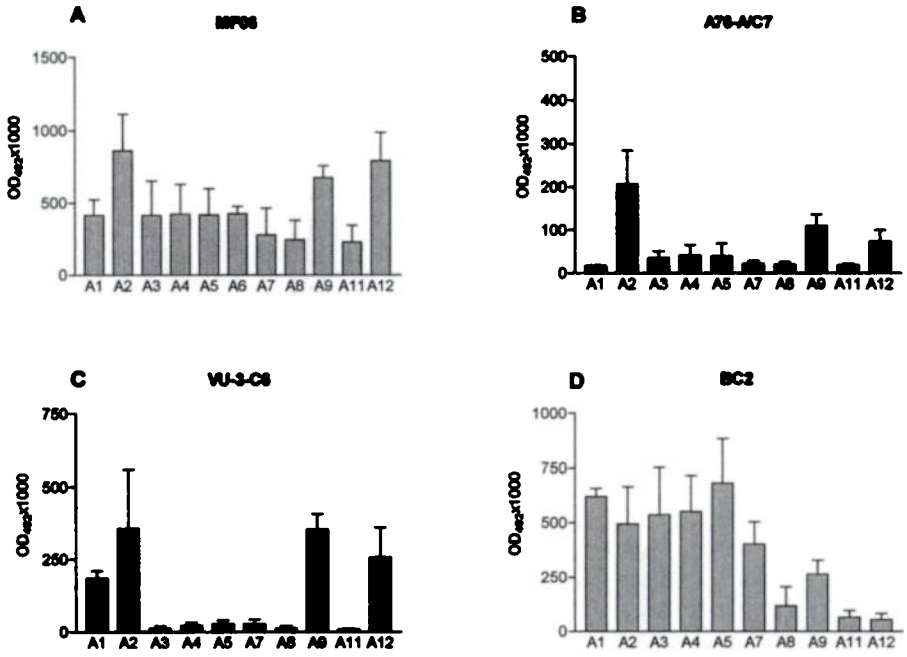


Fig. 1. Binding patterns of mAbs known to react with the DTR motif of MUC1 with a series of synthetic glycopeptides. The peptide backbone is a 21-mer (A1–A9 and A13) or 20-mer (A11 and A12) corresponding to an overlapping VNTR unit of MUC1 (see Table 2). Peptides A1–A9 carry TF-disaccharide at one or more of the five potential glycosylation sites, Thr or Ser; A11, A12, and A13 carry Tn at different positions (see Table 2). *Columns*, means; *bars*, SD. Statistical calculations were performed with one-way ANOVA, yielding the following results: in *A* and *B*, A2, A9, and A12 values are significantly different from the rest; in *C*, the same holds true, with the exception that A1 is not sufficiently different from A2; and in *D*, the differences among A1, A2, and A3 and between A11 and A12 are not significant.

binding. Three glycopeptides revealed significantly higher absorbance values; the only structural difference in common, as compared to the weaker binding glycopeptides, was the fact that they were substituted at the DTR motif with TF (A2 and A9) or with Tn (A12). A similar pattern was observed with mAbs VU-12-E1 (Fig. 2*A*), E29 (Fig. 2*C*), and SM-3 (Fig. 2*D*). The enhancement effect exerted by the TF-disaccharide on the peptide antigenicity of A2 was also observed with A13 (Fig. 2), which represents the structural counterpart of A2 by carrying a Tn-monosaccharide at the DTR motif (Table 2).

Type B: A76-A/C7. This antibody did not react with the single unsubstituted VNTR peptide, although an epitope containing the DTR motif could be assigned to it (Table 1). As expected, glycosylation did not improve antibody binding, with the exception of A2, A9, and A12 (Fig. 1*B*). The most obvious explanation of this surprising observation is that glycosylation with either TF or Tn at the Thr-10 position within the epitope renders the VNTR peptide antigenic toward this antibody. This pattern of reactivity [enhancement relative to the unsubstituted peptide exerted by TF-disaccharide (A2 or A9) or Tn-monosaccharide (A12 or A13)] was not restricted to a single antibody but instead shared with several others, among them Ma552 (Fig. 2*B*), VU-11-D1 (not shown), and, with the possible exception of A1 reactivity, VU-3-C6 (Fig. 1*C*).

Type C: BC2. This antibody reacted similarly to MF06 with the single unsubstituted VNTR peptide (Table 1). However, the glycopeptides A2, A9, A12, and A13 did not show enhanced binding. On the contrary, multiple glycan side chains appeared to inhibit antibody binding (Fig. 1*D*). A similar but not identical binding pattern was observed with mAbs HMFG-1 (Figs. 2*E* and 3*B*) and VA2 (data not shown).

mAb 115D8 was unreactive with all peptides whether glycosylated with TF/Tn (Fig. 2*F*) or not (2). According to the screening of a panel of 29 mAbs, several others (Mc5, GP1.4, and VU-3-D1) could potentially be included in the list of mAbs with enhanced binding to the DTR motif (Table 4). However, these antibodies were not tested either in quantitative binding analyses or by inhibition assays.

Effect of Periodate Oxidation of Glycopeptides on Antibody Binding. To examine further the possible role of glycosylation in modifying and/or stabilizing the DTR epitope, carbohydrate-specific periodate oxidation according to Woodward (27) was applied to the glycopeptides coated to microtest plates before probing with antibodies. This was performed with three mAbs (VU-3-C6, HMFG-1, and A76-A/C7). As shown in Fig. 3 for mAb VU-3-C6, binding of the Tn-carrying peptide A13 was strongly reduced after periodate treatment, whereas binding to the TF-carrying peptide A2 was unchanged. Because periodate oxidation requires vicinal OH groups, in case of TF the penultimate GalNAc cannot be attacked. The obvious interpretation, therefore, is that GalNAc alone is sufficient for the enhanced antigenicity of the DTR motif toward the antibodies in question. This confirms our observation that TF and Tn were found to be equally effective if present at the Thr in the DTR motif [A2 *versus* A13 (see Fig. 2, *A–D*) and A9 *versus* A12 (see Fig. 1, *A–C*)].

Inhibition of Antibody Binding by Glycopeptides. To confirm the above observations by an independent method that is not affected by different plate binding efficiencies of the antigens, a selected panel of antibodies was tested for inhibition by the solubilized glycopeptides A2 and A3 and by the peptides TAP25 and PAH100 (Figs. 4 and 5). Six of the seven tested antibodies that were reactive to the monomeric repeat peptide exhibited the same binding pattern as that revealed by the direct binding assays (Fig. 4 and Table 4). However, although the immobilized peptides generally bound more antibody molecules than the glycopeptides (data not shown), the inhibitory capacity of A2 in solution exceeded that of TAP25/PAH100 by a factor of 1.5–23 (mean, 6.7) on a molar basis (Table 4). The enhancement of binding activity of the glycosylated monorepeat (A2) relative to the nonglycosylated mono- and oligorepeat (TAP25 and PAH100) was measured in the presence of equimolar concentrations of binding-active repeats. The actual enhancement induced by glycosylation of the DTR motif may even be larger, considering the higher avidity of the oligomeric repeat peptide (demonstrated by binding inhibition of SM-3 antibody; Fig. 6*B*). The discrepancy between coated and soluble peptide (or glycopeptide) antigen could be explained by the expected higher binding efficiencies of the nonglycosylated peptides to the plastic surface. Only VA2 did not show a preferential binding to any of the tested glycopeptides (Fig. 4*D*), whereas BC4E549 bound more avidly to the nonglycosylated pentameric VNTR peptide (not shown).

Among the series of antibodies with nonreactivity to the monomeric repeat peptide TAP25, six of the seven tested mAbs were very strongly inhibited by A2, showed intermediate inhibition by PAH100, and were only weakly affected by A3 (Fig. 5 and Table 4). In summary, 12 antibodies tested in these inhibition assays exhibited an unexpected

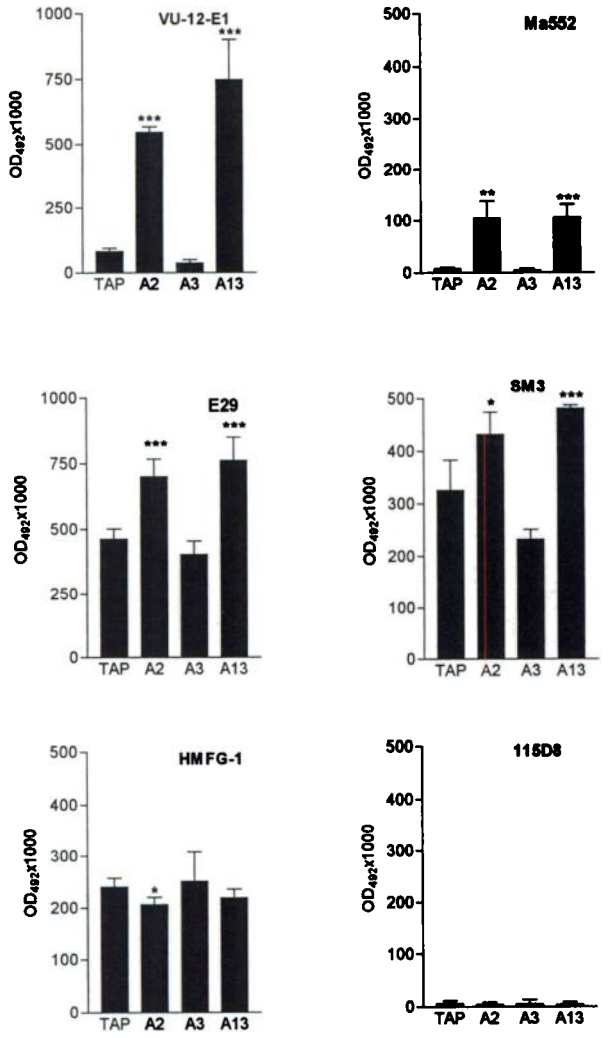


Fig. 2. Binding patterns of a panel of anti-MUC1 mAbs with VNTR peptide unglycosylated or glycosylated with a single TF or Tn side chain. The occupied positions are as follows: TAP, none; A2, Thr-10; A3, Thr-17; A13, Thr-10 (see Table 2). Statistical calculations were performed with *t* test (TAP *versus* A2 and A3 *versus* A13). *Columns*, mean; *bars*, SD. Values that differ significantly from each other are marked as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. mAb 115D8 recognizes a nonVNTR epitope of MUC1.

binding pattern (Table 4). Three of these antibodies, SM-3, MF06, and VU-3-C6, were also tested with the GalNAcα-substituted VNTR peptides A11, A12, and A13, which resulted in the same pattern of binding inhibition as that observed with the Galβ1–3GalNAcα-substituted peptides (Fig. 6, *A* and *C*). In conclusion, the binding (Fig. 2) and inhibition (Fig. 6*C*) experiments with the glycopeptide A13 corroborate that the enhancement of peptide antigenicity is already exerted on the monosaccharide level without measurable differences in the efficacy compared to the TF-disaccharide carrying inhibitor peptide A2.

DISCUSSION

We here report on an observation that was unexpected and in striking contrast to a hitherto unquestioned assumption: our data suggest that glycosylation of the immunodominant DTR motif leads to enhanced antigenicity of the MUC1 repeat peptide.

On the basis of the presented data from a panel of 28 mAbs, it can be assumed that the observations are not a singular effect shown by a particular antibody but seem to reflect a phenomenon of general relevance. Core-type glycans like *O*-GalNAc at the DTR motif may stabilize a particular conformation of the peptide sequence recognized by a panel of mAbs that have been generated to the MUC1 tandem repeat peptide. The glycosylation-induced effect cannot be interpreted in terms of a peptide-carbohydrate mimicry because different glycan-substituents, GalNAc and Galβ1–3GalNAc, had similar effects on antibody binding to the peptide motif. It can be hypothesized that the immunogenic structure of the *ex vivo* immunogens used for the generation of several of the DTR-specific antibodies were similar to or identical with the glycosylated DTR motif. This assumption is in contrast to the generally accepted concept that the DTR motif of MUC1 tandem repeats represents an effective target for B and T cells only if it is not glycosylated. However, indirect evidence, namely the observed blocking of antibody binding by the TF-specific lectin PNA in gastric mucosae (28), had already pointed toward the possibility that Thr in the DTR motif might be carrying TF. The enhanced reactivity toward Thr-10-substituted glycopeptides was not shared by all of the 28 mAbs with DTR specificity; however, it was common among those antibodies with exclusive binding to the *oligomeric* repeat. Antibodies reactive to the *monomeric* repeat exhibited a more complex binding pattern; some antibodies were additionally or exclusively affected by glycosylation at vicinal positions of the DTR motif. Sterical masking or indirect conformational influences have to be kept in mind when interpreting the binding and inhibition data. Within the series of glycopeptides, the preferential binding to the glycosylated DTR motif (A2) could have resulted from negative effects being exerted by vicinal glycosylation in the series of reference glycopeptides (A1, A3, A4, and A5). However, as revealed particularly in the inhibition experiments, a real enhancement of binding activity can be concluded for several of the antibodies when comparing the A2 inhibition curves to those of the nonglycosylated mono- or oligomeric repeat peptides as reference. Particular structural elements of the carbohydrate substituents, which remain to be identified, should be involved in the stabilization of the binding epitope.

The 28 DTR-specific mAbs included in this study had been gen-

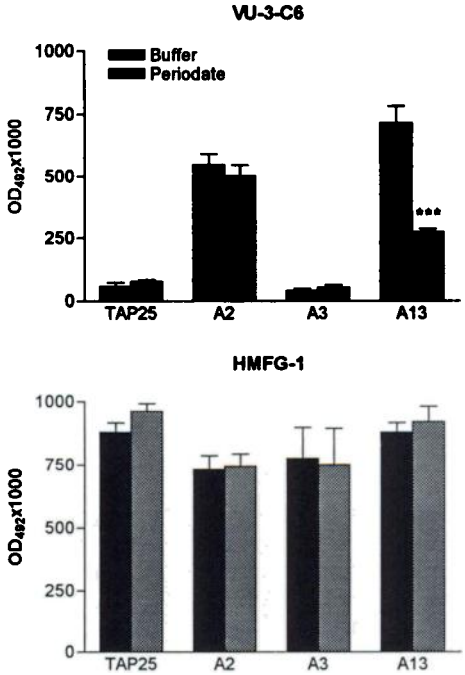


Fig. 3. Binding of antibodies VU-3-C6 and HMFG-1 to selected MUC1-derived glycopeptides before and after periodate oxidation. *Columns*, means; *bars*, SE. Periodate treatment strongly reduced the binding of antibody VU-3-C6 in the case of peptide A13 ($P < 0.001$) but did not significantly change antibody binding in the case of peptide A2. Binding of antibody HMFG-1 to the VNTR peptide is not influenced by glycosylation with TF or Tn and, accordingly, is also not influenced by carbohydrate-selective periodate oxidation.

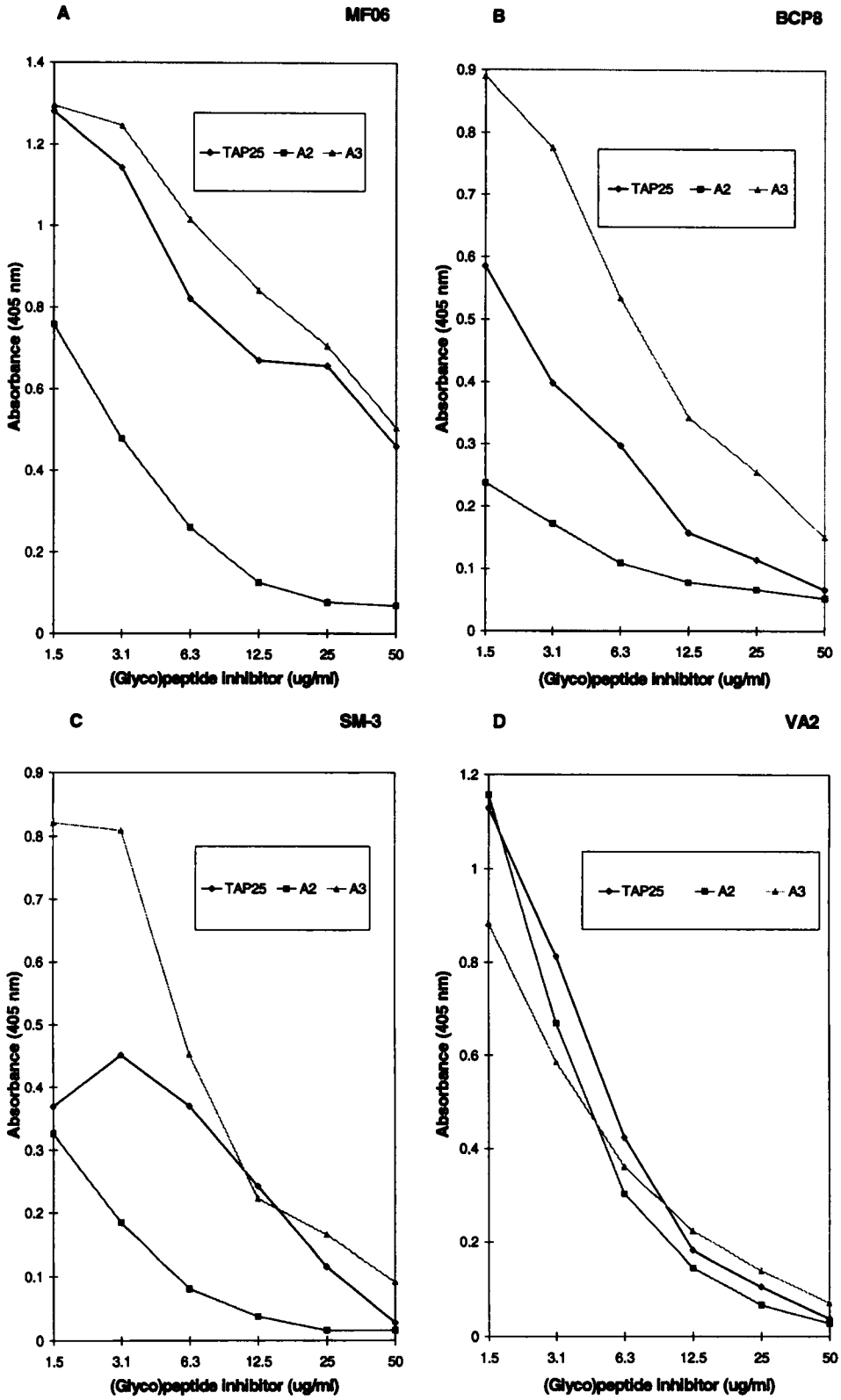


Fig. 4. Binding inhibition of antibodies reactive with the monomeric MUC1 repeat peptide. Binding of antibodies in the presence of inhibitory glycopeptides and peptides was measured on TAP25. The glycopeptide inhibitors A2 and A3 were 21-mers corresponding to the VNTR peptide of MUC1 and carrying TF in position Thr-10 and Thr-17, respectively (see Table 2). Fifty μg/ml of each of the glycopeptides and peptides A2 and A3 and of TAP25 corresponded to a concentration of 22 μM. *A*, mAb MF06; *B*, mAb BCP8; *C*, SM-3; *D*, VA2.

erated against a variety of natural or synthetic immunogens. Among 12 antibodies showing the above reported enhancement of binding on glycosylated DTR, 5 were produced by immunization with the breast cancer cell line ZR75–1 (VU-3-C6, VU-12-E1, VU-11-D1, VU-11-E2, and Ma552), 1 was generated from immunization with neuraminidase-treated T47D cells (A76-A/C7), 3 were antibodies to mucin preparations from different sources (MF06, B27.29, and SM3), 1 was an antibody to human milk fat globules (E29), 1 was an antibody to a synthetic VNTR-peptide (BCP8), and 1 was an antibody to a VNTR fusion protein (214D4). Although for the latter two a glycosylation of the DTR motif can be excluded, for the cellular immunogens and also for the mucin preparations, the possible involvement of glycosylated

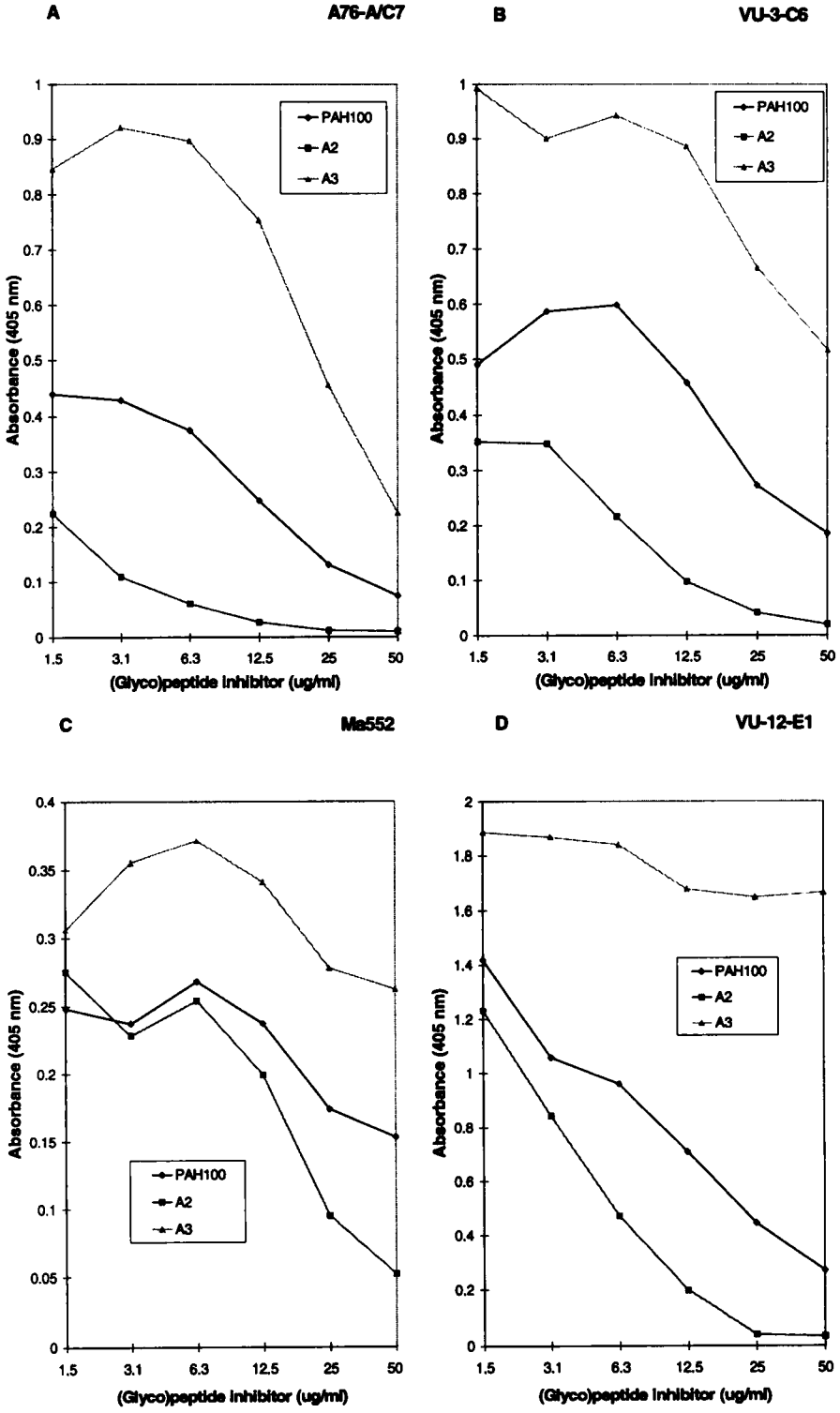


Fig. 5. Binding inhibition of antibodies nonreactive with the monomeric MUC1 repeat peptide. Binding of antibodies in the presence of inhibitory glycopeptides and peptides was measured on PAH100. The same glycopeptide inhibitors, A2 and A3, were used as in Fig. 4 (see Table 2). Fifty μg/ml of PAH100 corresponded to a concentration of 26 μM of binding active repeats. *A*, A76-A/C7; *B*, VU-3-C6; *C*, Ma552; *D*, VU-12-E1.

DTR in eliciting the respective B-cell response appears likely. This holds also true for mAb SM3, which had been generated to deglycosylated milk mucin (20). We have recently shown that the DTR motif within MUC1 tandem repeats is a target for *O*-glycosylation in mammary epithelial cells during lactation (10). Chemical deglycosylation of mucins under various conditions has been reported by several groups to be incomplete, with the core-GalNAc partially remaining bound to the protein (10, 29). Clearly, our hypothesis that the glycosylated DTR motif may represent the more effective immunogen compared to the unglycosylated motif needs further experimental evidence. Structural studies on the conformation of the glycopeptides are in progress.[4] Because BCP8, which had been generated to a nonglycosylated VNTR-peptide, binds even more strongly to the glycosylated DTR within a monorepeat than to the nonglycosylated pentameric repeat, we assume that in this and most likely also in other cases a glycosylation-

[4] H. Paulsen, unpublished data.

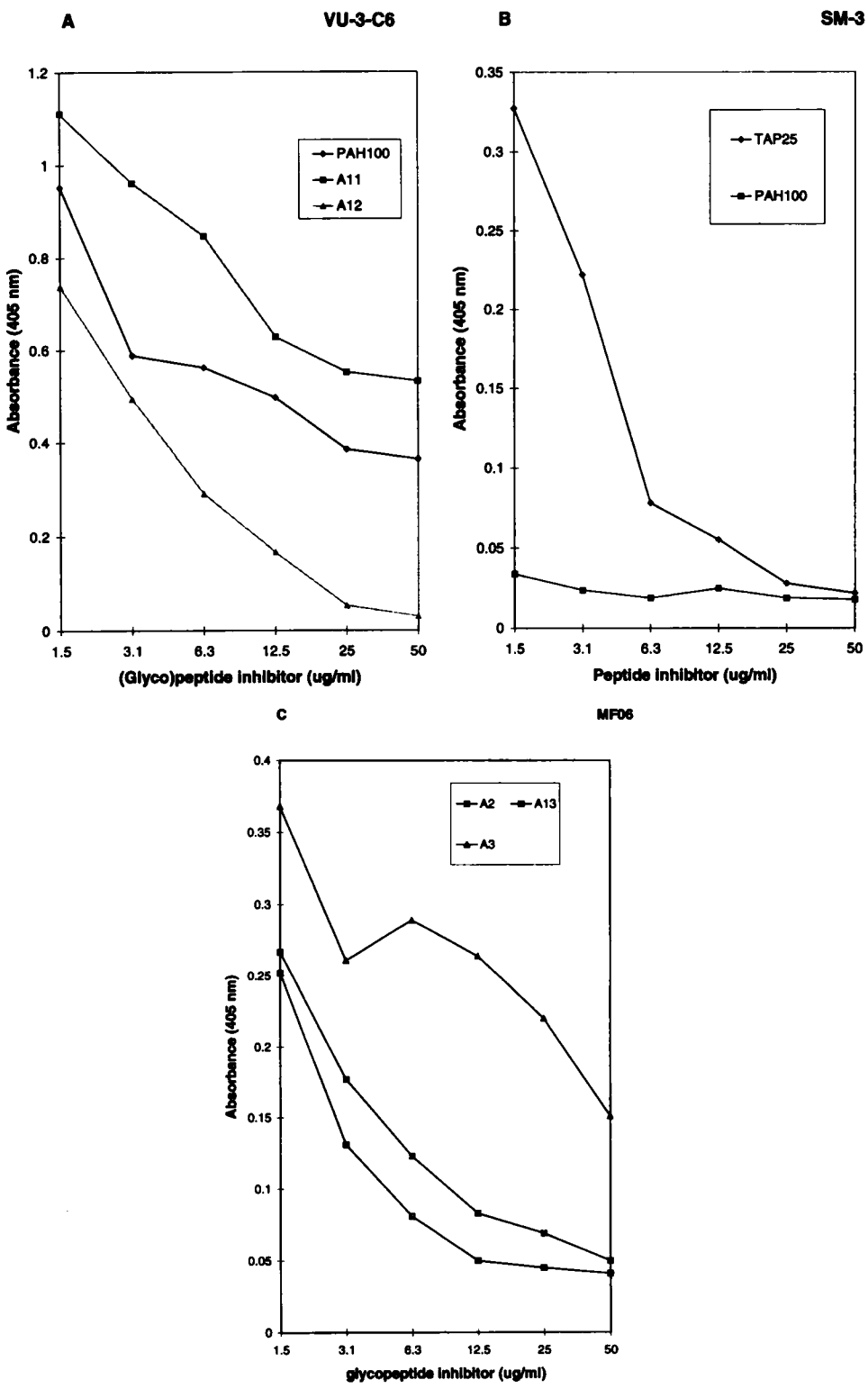


Fig. 6. Binding inhibition of antibodies by Tn-substituted peptides and peptides of different repeat numbers. Binding of antibodies in the presence of inhibitory glycopeptides was measured on TAP25 (SM-3) and PAH100 (VU-3-C6). *A*, binding of antibody VU-3-C6 was analyzed in the presence of PAH100, A11, and A12 glycopeptides. Fifty μg/ml of inhibitor corresponded to a concentration of 26 μM (PAH100), 22 μM (A11), or 17 μM (A12) of binding active repeats. *B*, binding of antibody SM-3 was analyzed in the presence of the monomeric repeat peptide TAP25 or the pentameric PAH100. Fifty μg/ml correspond to a concentration of 22 (TAP25) or 26 (PAH100) μM of binding active repeats. *C*, the relative inhibitory capacities of glycopeptides A2, A3, and A13 were tested in the blocking of antibody MF06 binding to TAP25. Fifty μg/ml of glycopeptide corresponded to a concentration of 22 (A2 and A3) or 23 (A13) μM.

induced change in the peptide conformation is responsible for the observed enhancement effect. This assumption is also supported by the results from periodate oxidation of glycopeptides A2 and A13 demonstrating that destruction of the Gal in Galβ1–3GalNAcα (A2) does not reduce the glycosylation-induced binding by VU-3-C6, whereas destruction of the GalNAcα residues on A13 strongly reduces antibody binding. A glycosylation-induced enhancement of antibody binding to the MUC1 repeat peptide has previously been reported for mAb C595 (30); however, the observed effect resulted from GalNAc substitution of Thr within the GSTA motif. In another paper (31), the MUC1 repeat peptide carrying GalNAc at all putative sites was shown to retain its binding capacity for antibidies SM-3 and HMPV.

The reported finding could be of utmost importance in the context of the design of tumor vaccines. Impressive progress has been made over the past decade in the identification of potential target antigens and the development of efficient strategies for the induction of cellular immune responses in cancer patients. Several different approaches to immunizing against breast cancer using MUC1-VNTR peptides conjugated to various

carriers have been tried (4). It could be shown that a MUC1 fusion protein coupled under oxidizing conditions to mannan is able to elicit a strong cellular (T1-type) immune response with the result of a significant tumor protection. Clinical trials are in progress to evaluate the immunogenicity of MUC1 and its efficacy in active specific immunization. Evidence has also been obtained for a potential contribution of glycosylated epitopes on MUC1 in specific T-cell reactions. Alloreactive cytotoxic T-cell clones from patients with colorectal carcinoma were shown to kill preferentially MUC1-expressing target cells by recognizing an underglycosylated VNTR peptide in conjunction with an internal carbohydrate epitope, the TF-disaccharide (32). Whether the glycosylation-induced effects on the peptide antigenicity observed in the present study are related to this carbohydrate-mediated enhancement of cytotoxicity remains to be elucidated. We hypothesize that MUC1 tandem repeat peptides with core-type glycosylation at DTR may also represent a more effective target for the stimulation of cellular immune responses necessary for attacking tumor cells. Ongoing NMR studies may be able to decide to what extent glycosylation at the DTR motif induces an epitope conformation similar to or superior to that provided by multiple tandem repeats (33). Another question to be answered in further studies is whether both factors (length of the peptide and DTR glycosylation) might act synergistically with respect to their influence upon the immunogenicity of MUC1 peptides.

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