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Enzymatic large-scale synthesis of MUC6-Tn glycoconjugates for antitumor vaccination

Teresa Freire[2], Richard Lo-Man[3], Friedrich Piller[4], Véronique Piller[4], Claude Leclerc[3], and Sylvie Bay[1,2]

[2]Unité de Chimie Organique URA CNRS 2128, Institut Pasteur, Paris, France; [3]Unité de Biologie des Régulations Immunitaires INSERM E352, Institut Pasteur, Paris, France; and [4]Centre de Biophysique Moléculaire, CNRS UPR 4301 affiliated to INSERM and the Université d'Orléans, Orléans, France

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In cancer, mucins are aberrantly *O*-glycosylated, and consequently, they express tumor-associated antigens such as the Tn determinant (α-GalNAc-*O*-Ser/Thr). As compared with normal tissues, they also exhibit a different pattern of expression. In particular, MUC6, which is normally expressed only in gastric tissues, has been detected in intestinal, pulmonary, colorectal, and breast carcinomas. Recently, we have shown that the MCF7 breast cancer cell line expresses MUC6-Tn glycoproteins *in vivo*. Cancer-associated mucins show antigenic differences from normal mucins, and as such, they may be used as potential targets for immunotherapy. To develop anticancer vaccines based on the Tn antigen, we prepared several MUC6-Tn glycoconjugates. To this end, we performed the GalNAc enzymatic transfer to two recombinant MUC6 proteins expressed in *Escherichia coli*, using UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts), which catalyze *in vivo* the Tn antigen synthesis. We used either a mixture of ppGalNAc-Ts from MCF7 breast cancer cell extracts or a recombinant ppGalNAc-T1. In both cases, we achieved the synthesis of MUC6-Tn glycoconjugates at a semi-preparative scale (mg amounts). These glycoproteins displayed a high level of Tn antigens, although the overall density depends on both enzyme source and protein acceptor. These MUC6-Tn glycoconjugates were recognized by two anti-Tn monoclonal antibodies that are specific to human cancer cells. Moreover, the MUC6-Tn glycoconjugate glycosylated using MCF7 extracts as the ppGalNAc-T source was able to induce immunoglobulin G (IgG) antibodies that recognized a human tumor cell line. In conclusion, the large-scaled production of MUC6 with tumor-relevant glycoforms holds considerable promise for developing effective anticancer vaccines, and further studies of their immunological properties are warranted.

Key words: antitumor immunotherapy/glycoconjugate/MUC6/ppGalNAc-Ts/Tn antigen

Introduction

Malignant cells selectively express on their surface molecules that have functional importance in cell adhesion, invasion, and metastasis. Some of these tumor-associated structures are the result of a blockage in the glycosylation pathway. In particular, the incomplete elongation of *O*-glycan saccharide chains leads to the expression of shorter carbohydrate structures such as Tn, sialyl-Tn, or TF antigens (Hollingsworth and Swanson, 2004). The Tn antigen, defined as a GalNAc unit α-linked to a serine or threonine residue (α-GalNAc-*O*-Ser/Thr), is one of the most specific human cancer-associated structures. Tn is detected in about 90% of human carcinomas (Springer, 1984), and its expression is correlated with carcinoma aggressiveness (Springer, 1997). Moreover, under appropriate conditions, Tn is capable of inducing a strong immune response in mice and nonhuman primates, the resulting antibodies being capable of recognizing human cancer cells (Lo-Man *et al.*, 2001; Lo-Man *et al.*, 2004).

This *O*-linked epitope is usually expressed on mucins as their carbohydrate core structure (Hollingsworth and Swanson, 2004). Mucins are high-molecular-weight *O*-glycosylated proteins (50–80% of their mass is due to *O*-linked carbohydrate chains) that participate in the protection, lubrication, and acid resistance of the epithelial surface (Gendler and Spicer, 1995). To date, different mucins have been identified and numbered in chronological order of their description (MUC1–MUC20) (Filshie *et al.*, 1998; Moniaux *et al.*, 2001; Williams *et al.*, 2001; Yin and Lloyd, 2001; Gum *et al.*, 2002; Pallesen *et al.*, 2002; Chen *et al.*, 2004; Higuchi *et al.*, 2004). Although they do not show homology of sequence, all mucins present a large region composed of variable number of tandem repeats (VNTRs). These regions, usually called tandem repeats, are characterized by a high content in Ser, Thr (which constitute the potential *O*-glycosylation sites), and proline residues.

Each organ or tissue exhibits a unique pattern of *MUC* gene expression (Gendler and Spicer, 1995). This mucin expression profile can be modified under pathological conditions, especially during malignant transformation. The up-regulation, down-regulation, and *de novo* expression of mucin proteins have been reported in cancer epithelial cells and are thought to influence cell adhesion (Hilkens *et al.*, 1992) and contribute to tumor invasiveness (Segal-Eiras and Croce, 1997). Moreover, these cancer-associated mucins show antigenic differences from normal mucins and are highly immunogenic, and as such, they may be used as potential targets for immunotherapy (Apostolopoulos *et al.*, 1996; Agrawal *et al.*, 1998). In particular, MUC1 is undergoing several clinical trials as anticancer vaccine (Finn *et al.*, 1995; Gilewski *et al.*, 2000).

[1]To whom correspondence should be addressed; e-mail: sbay@pasteur.fr

MUC6 was first isolated from a human stomach library (Toribara *et al*., 1993), and it is expressed at high levels in the normal stomach and gall bladder, with weaker expression in the terminal ileum, right colon, and endocervix (Toribara *et al*., 1993; De Bolos *et al*., 1995; Ho *et al*., 1995; Reis *et al*., 2000). MUC6 has a tandem repeat unit of 169 amino acids (507 bp each) (Toribara *et al*., 1993), and Southern blot analyses of the shortest MUC6 alleles indicate that they contain at least 15 repeat units (Vinall *et al*., 1998). Although the whole *MUC6* gene was localized and identified, a full-length cDNA has not been completely sequenced yet (Rousseau *et al*., 2004). In addition to its normal expression in gastric tissues, MUC6 has been detected in Barrett's adenocarcinoma and metaplasia, intestinal adenoma and carcinoma (Guillem *et al*., 2000), pulmonary carcinoma (Nishiumi *et al*., 2003; Hamamoto *et al*., 2005), colorectal polyps (Bartman *et al*., 1999), and breast carcinoma (De Bolos *et al*., 1995; Pereira *et al*., 2001), whereas it is not expressed in the respective normal tissues. In some cases, MUC6 expression has been reported to be correlated with the degree of histopathology related to malignant potential (Bartman *et al*., 1999; Nishiumi *et al*., 2003; Hamamoto *et al*., 2005). We have recently shown that MUC6 is aberrantly glycosylated in MCF7 breast cancer cells, because it contains the Tn antigen (Freire *et al*., 2005). Several studies have shown that the carbohydrate structures on mucins (including the core Tn antigen) may be essential for the definition of the tumor-associated structures (Grinstead *et al*., 2003; von Mensdorff-Pouilly *et al*., 2005). Therefore, MUC6-Tn glycoconjugates represent attractive targets to be used in cancer immunotherapy. A specific anti-Tn antibody response should target cancer cells through the Tn antigen, which is expressed on their surface. Furthermore, the activation of mucin-specific cytotoxic T lymphocytes should be favored through the uptake of soluble MUC6-Tn immune complexes by Fc receptors on dendritic cells (Amigorena and Bonnerot, 1999).

To further develop anticancer vaccines based on the Tn antigen, we established an *in vitro* enzymatic method for the preparation of MUC6-Tn glycoconjugates. To this end, we performed the GalNAc enzymatic transfer onto the Ser and Thr residues of the mucin, using UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferases (EC 2.4.1.41, ppGalNAc-Ts), which catalyze *in vivo* the first step of the mucin-type *O*-glycosylation pathway, that is, the Tn antigen synthesis (Ten Hagen *et al*., 2003). In this study, we used either a recombinant ppGalNAc-T1 or a microsome extract from MCF7 breast cancer cells containing ppGalNAc-Ts to better mimic the glycosylation of cancer cells. We show that this method is very efficient and allowed the preparation of semi-preparative quantities of different MUC6-Tn glycoproteins with high carbohydrate densities. The resulting MUC6-Tn glycoconjugates were shown to be antigenic as judged by the recognition by two anti-Tn monoclonal antibodies (mAbs) specific to human cancer cells. Moreover, the MUC6-Tn glycoconjugate glycosylated using MCF7 extracts as the ppGalNAc-T source was able to induce immunoglobulin G (IgG) antibodies that recognized a human tumor cell line.

Results

MUC6 recombinant protein production in Escherichia coli *and enzymatic synthesis of Tn-expressing MUC6 mucins*

To obtain semi-preparative amounts of Tn-expressing MUC6-Tn glycoproteins, we designed two recombinant MUC6 proteins cloned from the MCF7 breast cancer cell line and containing either a whole (MUC6-1) or a half (MUC6-2) tandem repeat (Figure 1A). These two different constructs were selected to study the glycosylation of two related proteins of different size, with different number of potential *O*-glycosylation sites (85 for MUC6-1 and 48 for MUC6-2). Slight amino acid changes were detected between the two cloned MUC6 cDNAs and the reported MUC6 tandem repeats cloned from gastric tissues (Figure 1A) (Toribara *et al*., 1993). This could be attributed to the high polymorphism found in mucin tandem repeats. MUC6-1 and MUC6-2 polypeptides were expressed in *Escherichia coli* and purified using Ni-NTA-agarose (Figure 1B and C). For MUC6-1, one additional step of purification using a C18 column was necessary (Figure 1B). As a result, purified MUC6-1 and MUC6-2 proteins were obtained with a purity level >95%, as estimated by high-performance liquid chromatography (HPLC), at a yield of 2 and 3.4 mg of protein per liter of culture, respectively.

These purified mucin proteins (MUC6-1 or MUC6-2) were subjected to *in vitro* transglycosylation reactions from uridine 5′-diphospho-*N*-acetylgalactosamine (UDP-GalNAc), using either a recombinant bovine ppGalNAcT1 (bppGalNAc-T1) or an MCF7 cell extract (Figure 2A). The reactions were performed at an analytical scale under different conditions (incubation time, UDP-GalNAc equivalents, and enzyme quantity). The course of the transfer was monitored by HPLC and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), and the reaction parameters giving the highest Tn density were selected (Freire *et al*., 2006). These conditions (Table I) were used to perform the semi-preparative-scale synthesis of MUC6-Tn (~0.3–3 mg). A maximal GalNAc transfer was achieved, because purified MUC6-Tn glycoconjugates were not further glycosylated after being subjected again to the same glycosylation reaction conditions (data not shown). The resulting glycoproteins were analyzed by HPLC (Figure 2B and C), purified, and then characterized by SELDI-TOF MS (Figure 2D and E). In all assays, the starting protein was totally converted into glycoconjugates. The SELDI-TOF MS profiles showed different GalNAc glycosylation levels of the protein (major peak ± 3 GalNAc) (Figure 2D and E). A similar polydispersity was observed on the crude mixtures and the purified glycoconjugates, independently of the protein acceptor and the enzyme source used (data not shown).

Physico-chemical characterization of the synthesized glycoconjugates

Four different MUC6-Tn glycoconjugates were synthesized by this enzymatic transglycosylation and purified by Ni-NTA-agarose and HPLC. Then, they were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis (Figure 3), confirming the presence of

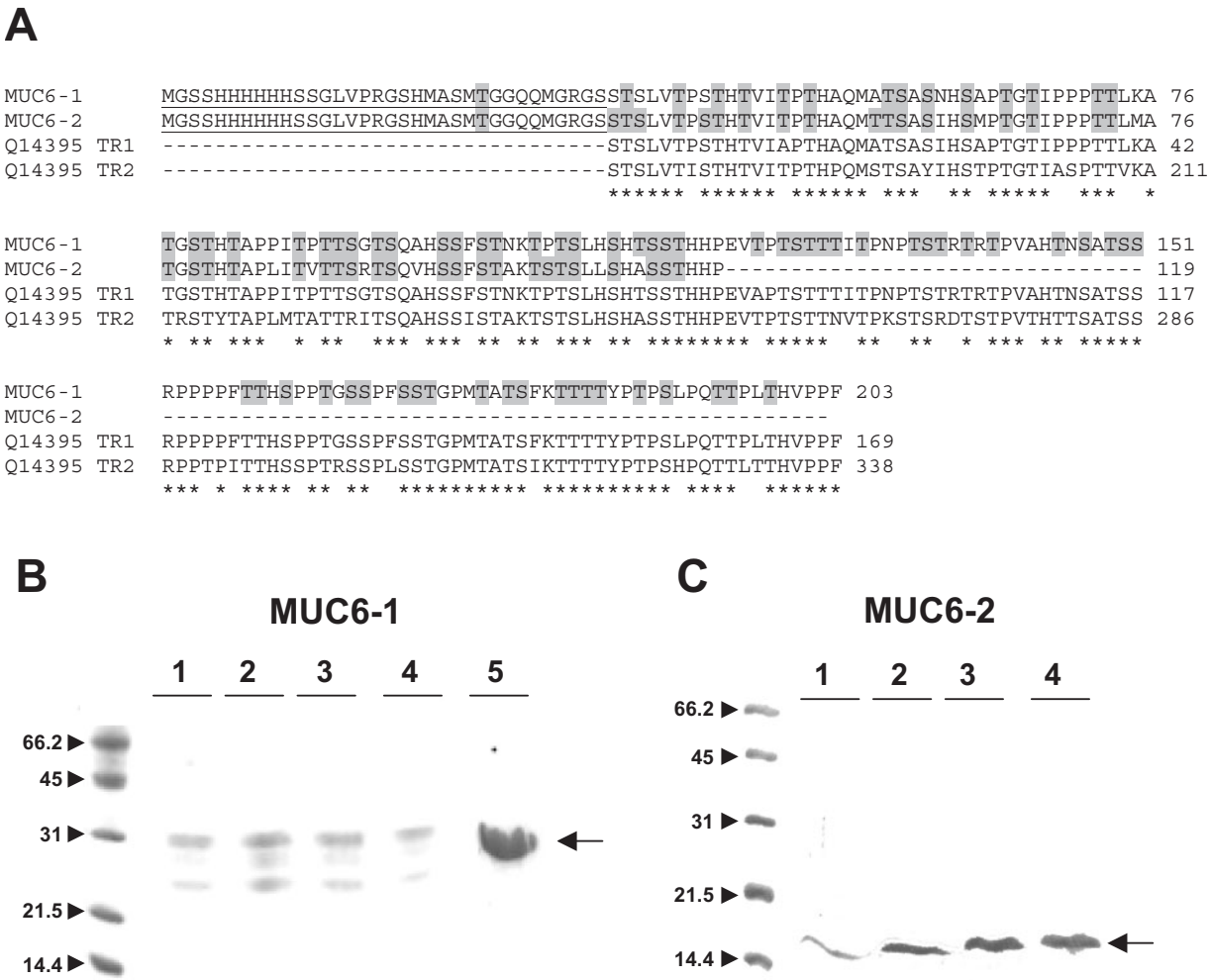

Fig. 1. (**A**) Alignment of the cloned MUC6 proteins with two known MUC6 tandem repeats. Alignment was performed with ClustalW using the obtained predicted sequences of cloned MUC6 proteins (MUC6-1 and MUC6-2) from MCF7 breast cancer cells and with already-reported MUC6 tandem repeats (TR1 and TR2) amplified from a normal gastric library (accession number Q14395). The potential sites of *O*-glycosylation were determined using the NetOGlyc3.1 server at http://www.cbs.dtu.dk/services/NetOGlyc/ and are shaded in gray. The sequences corresponding to the fusion His-tag are underlined. Identical amino acids are marked with an asterisk. (**B**) Purification of MUC6-1 recombinant protein as analyzed by SDS–PAGE. MUC6-1 recombinant protein was purified using Ni-NTA-agarose and HPLC. Fractions were resolved by SDS–PAGE (13%) and stained with Coomassie Blue. Lanes 1–4, Ni-NTA-agarose elution fractions; Lane 5, purified MUC6-1 after HPLC. Molecular markers are expressed in kDa. (**C**) Purification of MUC6-2 recombinant protein as analyzed by SDS–PAGE. MUC6-2 recombinant protein was purified using Ni-NTA-agarose. Fractions were resolved by SDS–PAGE (13%) and stained with Coomassie Blue. Lanes 1–4, Ni-NTA-agarose elution fractions. Molecular markers are expressed in kDa.

purified glycoproteins at the expected molecular weights. The MUC6-Tn glycoproteins presented different Tn contents depending on the mucin backbone and on the different ppGalNAc-T source used (Table I). When the MCF7 breast cancer cell extract was used, an average of 54–58% of potential *O*-glycosylation sites was glycosylated, representing 30–32% of the total molecular mass, independently of the mucin used as acceptor. A different Tn density was obtained when the mucin proteins were glycosylated by the purified bppGalNAc-T1. MUC6-2 was less glycosylated (20 GalNAc, representing 42% of total *O*-glycosylation sites). By contrast, MUC6-1 was much more glycosylated by bppGalNAc-T1 because 64% of the potential *O*-glycosylated sites were glycosylated (54 GalNAc).

MUC6-Tn glycoconjugates are recognized by anti-Tn mAbs

The MUC6-Tn glycoproteins were identified by western blotting using anti-Tn (83D4) and anti-His mAbs (Figure 4). As expected, the anti-His mAb recognized all MUC6 proteins (including the nonglycosylated MUC6) (Figure 4A). On the contrary, the anti-Tn mAb 83D4 only recognized the MUC6-Tn glycoconjugates (Figure 4B).

The antigenicity of these MUC6-Tn glycoconjugates was analyzed by enzyme-linked immunosorbent assay (ELISA) using two anti-Tn mAbs raised against human cancer cells (MLS128 and 83D4) and a polyclonal anti-MUC6 serum. Figure 4C and D shows that both the anti-Tn mAbs, although at different rates, recognized the MUC6-Tn glycoconjugates, but not the corresponding nonglycosylated MUC6 proteins. The anti-Tn mAb 83D4 similarly

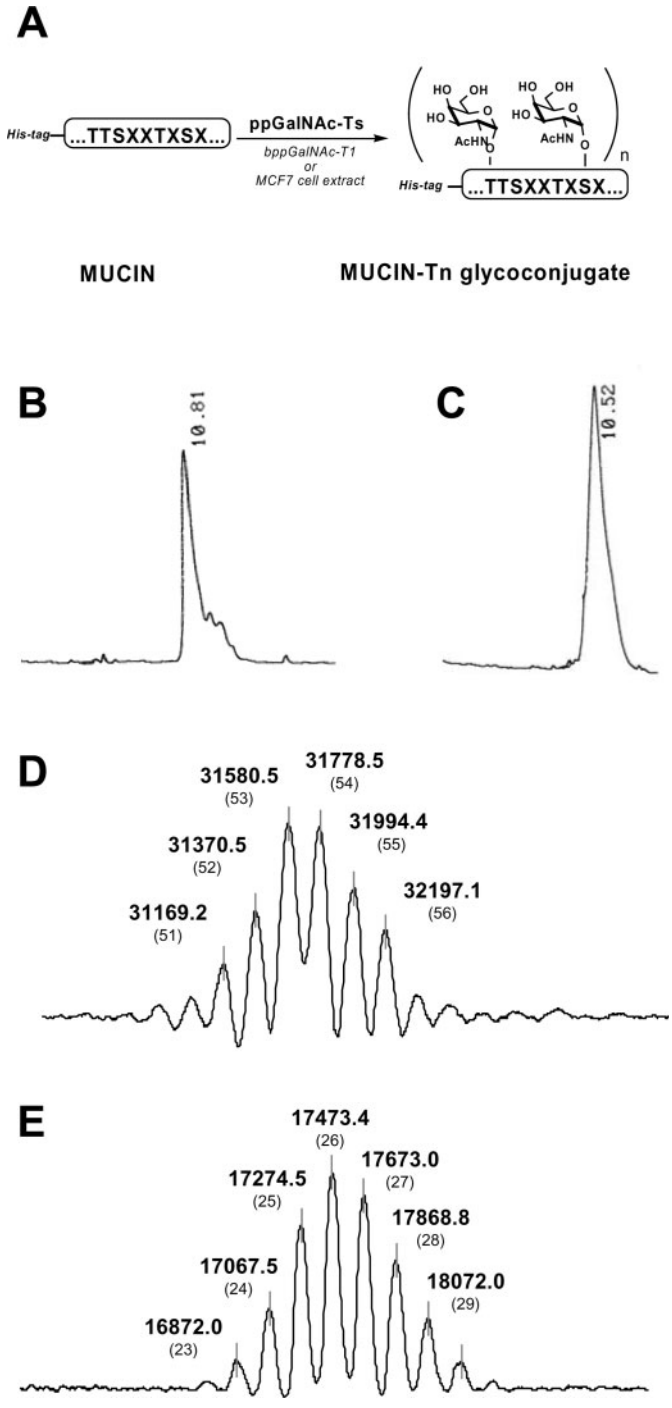

Fig. 2. GalNAc transfer into MUC6 proteins and GalNAc number determination. The transglycosylation reaction (**A**) was performed either with MUC6-1 purified mucin (3 mg, 0.14 µmol) and UDP-GalNAc (7.8 mg, 11.5 µmol) using bppGalNAc-T1 (300 µg) or with purified MUC6-2 mucin (1 mg, 0.082 µmol) and UDP-GalNAc (3.2 mg, 4.92 µmol, two times) using MCF7 cell extracts (12 mg, two times). The MUC6-1 : Tn(T1) glycoprotein was directly analyzed by HPLC (**B**). The MUC6-2 : Tn(MCF7) glycoprotein was purified using Ni-NTA-agarose and analyzed by HPLC (**C**). After purification by HPLC, MUC6-1 : Tn(T1) (**D**) and MUC6-2 : Tn(MCF7) (**E**) were analyzed by SELDI-TOF MS. Each peak is labeled with the mass/charge (*m*/*z*) value in daltons. The corresponding number of incorporated GalNAc units is indicated in brackets.

recognized MUC6-1 and MUC6-2 glycoconjugates (Figure 4C), whereas MLS128 showed less reactivity with the MUC6-2 : Tn(T1) glycoconjugate, which was the one with the lowest Tn density (Figure 4D). All glycosylated and nonglycosylated MUC6 proteins were differently recognized by the anti-MUC6 serum (Figure 4E). The nonglycosylated MUC6 proteins were slightly more reactive than the MUC6-Tn glycoconjugates, probably because of the lack of accessibility to the protein backbone in highly glycosylated proteins.

MUC6-2 : Tn(MCF7) glycoconjugate induces antibodies that recognize tumor cells

The immunogenicity of one of the MUC6-Tn glycoconjugates was studied. BALB/c mice were immunized with the MUC6-2 : Tn(MCF7) glycoconjugate or with the nonglycosylated MUC6-2 protein in alum plus CpG, and sera were tested for their capacity of recognizing the human tumor cell line Jurkat (Figure 5). Control mice received only CpG in alum. It is worth noting that all MUC6-Tn glycoproteins showed very low levels of endotoxins (<2 EU/mg of protein). Immunization with the MUC6-2 : Tn(MCF7) glycoconjugate, but not with the nonglycosylated MUC6-2 protein, induced IgG antibodies that recognized the Jurkat human tumor cell line (Figure 5A). To confirm that these antibodies recognized the Tn antigen on these cells, we carried out inhibition assays using either asialo-ovine submaxillary mucin (aOSM, carrying the Tn antigen) or deglycosylated-OSM. As shown in Figure 5B, only aOSM inhibited the recognition of the Jurkat cells by sera obtained after immunization with MUC6-2 : Tn(MCF7) and by the anti-Tn mAb 83D4 (used as control). In contrast, the binding of an anti-CD4 antibody to Jurkat cells was not affected in either of the two cases (Figure 5B). These experiments clearly show that MUC6-2 : Tn(MCF7) can induce anti-Tn antibodies that recognize Tn+ tumor cells.

Discussion

The aim of cancer immunotherapy is to elicit protective immunity against cancer cells, without causing collateral autoimmune damage. One approach is based on the induction of tumor-specific immune responses by cancer-associated antigens. To this end, mucins can be used as immunogens in vaccines designed to elicit therapeutic anti-tumor immunity.

Cancer-associated changes occur not only in mucin protein expression between normal and abnormal tissues, but also in the pattern of *O*-glycosylation that distinguishes cancer mucins from normal mucins. Indeed, mucins are normally highly glycosylated, and thus, the antigenic peptide core is physically inaccessible to the immune system, particularly to antibodies. However, in tumor cells, they present cancer-associated truncation of *O*-linked carbohydrate chains, creating the tumor-specific TF, Tn, and sialyl-Tn antigens (Hollingsworth and Swanson, 2004). This suggests that such glycosylated mucins can be used as targets for the treatment of specific cancers.

Table I. Conditions used for transglycosylation assays and characteristics of the resulting glycoconjugates

	UDP-GalNAc equivalents[a]	Enzyme or extract amount/μg of mucin	Obtained quantity (mg)	Product yield (%)[b]	Experimental molecular mass (Da)[c]	Average GalNAc number[c]	Tn (% weight)	Glycosylated sites (%)[d]
MUC6-1	–	–	–	–	20,833.7	–	–	–
MUC6-1 : Tn(T1)	1 eq.: 24 h/37°C	0.1 μg	2.5	59	31,778.5	54	34	64
MUC6-1 : Tn(MCF7)	(0.5 eq.: 24 h/37°C) × 2[e]	(6 μg) × 2[e]	0.35	25	30,718.5	49	32	58
MUC6-2	–	–	–	–	12,157.5	–	–	–
MUC6-2 : Tn(T1)	1 eq.: 24 h/37°C	0.1 μg	2.5	69	16,220.5	20	25	42
MUC6-2 : Tn(MCF7)	(0.5 eq.: 24 h/37°C) × 2[e]	(6 μg) × 2[e]	0.5	34	17,473.4	26	30	54

[a]The equivalent amount is expressed as compared with the total potential *O*-glycosylation sites (i.e., total Ser and Thr residues).
[b]Isolated product yields refers to the obtained yield after the glycosylation reaction and purification of the resulting glycoprotein. MUC6-Tn glycoconjugates were tested for the endotoxin levels and found to be lower than 2 EU/mg of glycoprotein in all cases.
[c]The average molecular mass and GalNAc number of the glycoconjugate were calculated from the medium peak.
[d]The % of obtained glycosylated sites was calculated taking into account the obtained GalNAc number for each glycoconjugate as compared with the total number of Ser and Thr residues in the proteins (85 for MUC6-1 and 48 for MUC6-2) (100%).
[e]The same quantity of UDP-GalNAc equivalents and enzyme was added at the beginning of the reaction and then at 24 h.

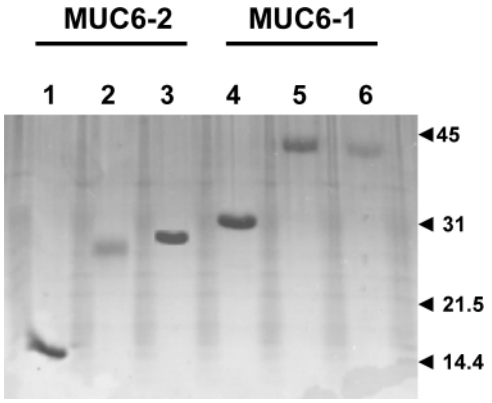

Fig. 3. SDS–PAGE of purified MUC6-1 and MUC6-2 and their glycoconjugates. Purified glycoconjugates (0.5 μg) were separated in a 13% SDS–PAGE and stained with Coomassie Blue. Lane 1, MUC6-2; Lane 2, MUC6-2 : Tn(T1); Lane 3, MUC6-2 : Tn(MCF7); Lane 4, MUC6-1; Lane 5, MUC6-1 : Tn(T1); Lane 6, MUC6-1 : Tn(MCF7). Molecular markers are expressed in kDa.

Various vaccines based on the Tn structure have been developed and tested in preclinical or clinical models. Desialylated ovine submaxillary mucin (expressing high Tn levels) (Singhal *et al.*, 1991) and Tn–protein conjugates (Longenecker *et al.*, 1987; Toyokuni *et al.*, 1994; Kuduk *et al.*, 1998) induced high Tn-specific antibody titers in mice, resulting in protection against tumor challenge. In humans, desialylated red blood cells (rich in Tn and T antigens) allowed a protection against the recurrence of advanced breast cancer (Springer *et al.*, 1993). More recently, a clinical trial with a Tn–protein conjugate resulted in an antitumor effect as determined by a decline in the prostate-specific antigen (PSA) slope (Slovin *et al.*, 2003). We also reported the preparation of a fully synthetic vaccine based on the Tn antigen, the multiple antigenic glycopeptide (MAG) (Bay *et al.*, 1997). MAG : Tn vaccines are capable of inducing, in mice and nonhuman primates, strong tumor-specific anti-Tn antibodies that can mediate antibody-dependent cell cytotoxicity against human tumor cells (Lo-Man *et al.*, 2004). However, the large-scale preparation of such conjugates is limited by the complexity of the whole synthesis process.

To further extend the scope of our approach to clinical trials, we propose to enzymatically attach the Tn antigen to a mucin core protein. To this end, we chose the MUC6 mucin that is aberrantly expressed in different cancers and may constitute a target antigen itself. Indeed, MUC6 has been detected in intestinal, pulmonary, colonic, and mammary adenocarcinomas, whereas it is not expressed in the respective normal tissues (De Bolos *et al.*, 1995; Bartman *et al.*, 1999; Guillem *et al.*, 2000; Pereira *et al.*, 2001; Nishiumi *et al.*, 2003; Hamamoto *et al.*, 2005). Furthermore, our preliminary data suggest that MUC6 carries the Tn antigen in MCF7 breast cancer cells (Freire *et al.*, 2005).

In the present work, we describe the enzymatic synthesis of Tn-expressing MUC6-Tn glycoconjugates. To produce high amounts of MUC6-Tn glycoconjugates, we performed the GalNAc transfer to a recombinant MUC6 protein expressed in *E. coli*, using ppGalNAc-Ts. This large family of enzymes catalyzes *in vivo* the linking of a GalNAc residue to Ser or Thr (i.e., the synthesis of the Tn antigen). To date, 15 ppGalNAc-Ts have been identified in mammals and the functional profile of each member of the family have been established, showing that these enzymes have not only different substrate specificities but also specific tissue-expression patterns (Ten Hagen *et al.*, 2003; Cheng *et al.*, 2004).

Glycosyltransferases have been extensively used as tools to perform transglycosylation reactions, because they are an attractive alternative to the total chemical synthesis of large glycosyl amino acids (Marcaurelle and Bertozzi, 2002). The synthesis of glycopeptides and glycoconjugates with *O*-linked glycans has already been reported, especially for the sialyl-Tn (George *et al.*, 2001) and sialyl-TF antigens (Ajisaka and Miyasato, 2000; George *et al.*, 2001). ppGalNAc-Ts have also been used successfully for the *in vitro* synthesis of glycopeptides. Most of the studies aimed at investigating the specificities of these different enzymes (either from recombinant or from cell extracts) for various peptide substrates from MUC1 (Takeuchi *et al.*,

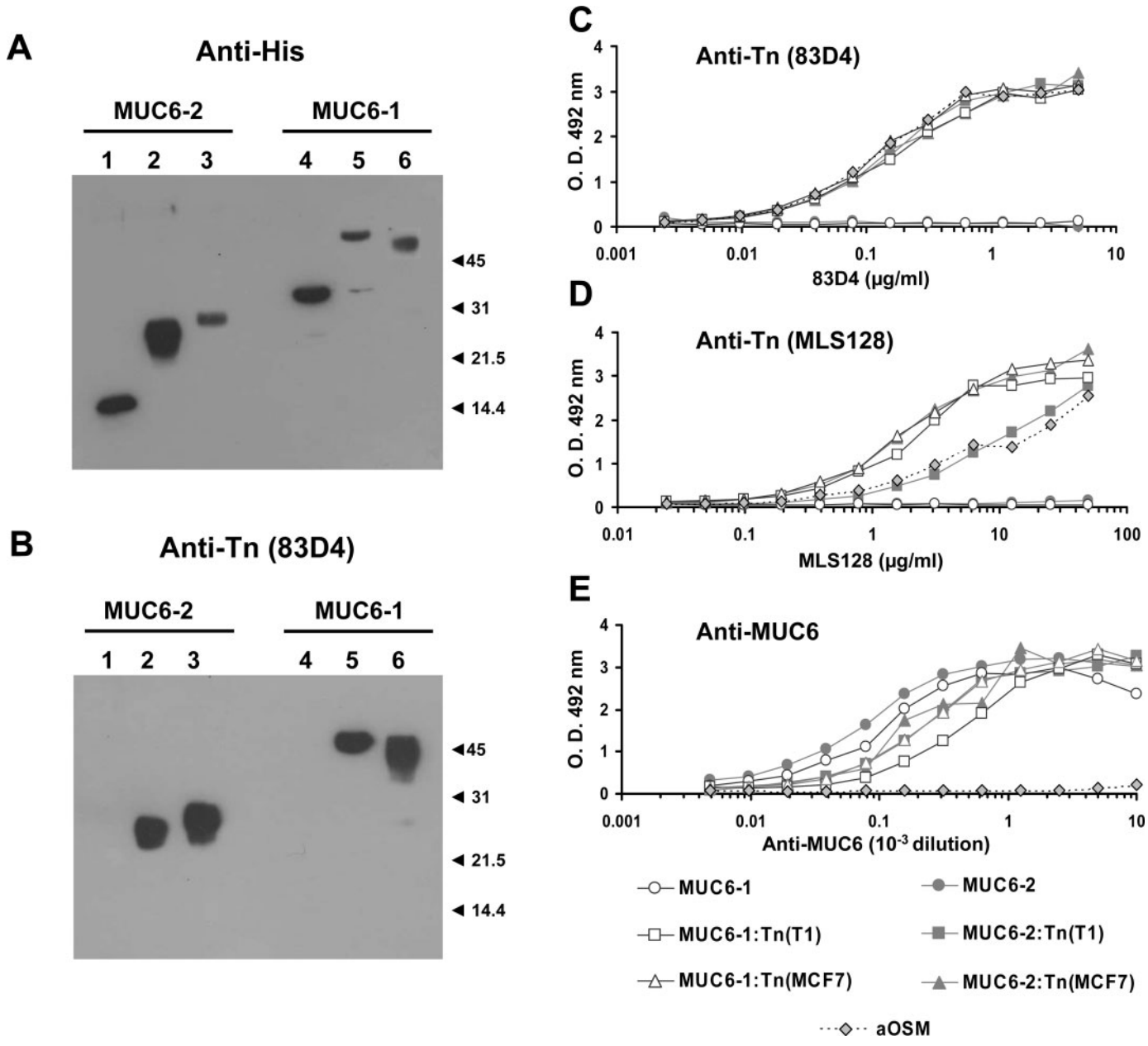

Fig. 4. Recognition of MUC6-Tn glycoconjugates by anti-Tn mAbs by western blotting (**A** and **B**) and ELISA (**C**–**E**). Glycoconjugates were separated in a 13% SDS–PAGE and transferred onto nitrocellulose sheets. An anti-His mAb (**A**) and the anti-Tn mAb 83D4 (**B**) were added followed by an anti-mouse peroxidase conjugate, and the reaction was developed with enhanced chemiluminiscence. Lane 1, MUC6-2; Lane 2, MUC6-2 : Tn(T1); Lane 3, MUC6-2 : Tn(MCF7); Lane 4, MUC6-1; Lane 5, MUC6-1 : Tn(T1); Lane 6, MUC6-1 : Tn(MCF7). Molecular markers are expressed in kDa. The recognition of the MUC6-Tn glycoconjugates by anti-Tn mAbs 83D4 (**C**), MLS128 (**D**), and a polyclonal anti-MUC6 serum (**E**) was also tested by ELISA. aOSM, a Tn-rich mucin, was used as a control.

2002) or MUC2 (Irimura *et al*., 1999; Kato *et al*., 2001), and they were performed at the analytical scale (0.1–10 μg range). Interestingly, however, recombinant ppGalNAc-T2 and ppGalNAc-T4 allowed the preparation of MUC1-Tn glycopeptides that were used for immunization purposes (Kagan *et al*., 2005). In addition, a comprehensive study about the synthesis of MUC1-Tn peptides using ppGalNAc-T2, ppGalNAc-T4, and ppGalNAc-T11 was published during the course of examination of this article (Sorensen *et al*., 2005).

In this study, we used either ppGalNAc-Ts from cancer cell extracts or purified recombinant bovine ppGalNAc-T1 to achieve the maximal GalNAc transfer to the Ser and Thr residues of a MUC6 recombinant protein. These two ppGalNAc-T sources were chosen for two reasons. On the one hand, breast cancer cell extracts were used to better mimic the *in vivo O*-glycosylation sites of MUC6 in cancer cells. On the other hand, the recombinant bppGalNAc-T1 has a very broad specificity, and the *in vitro* glycosylation assays using a purified recombinant protein are expected to

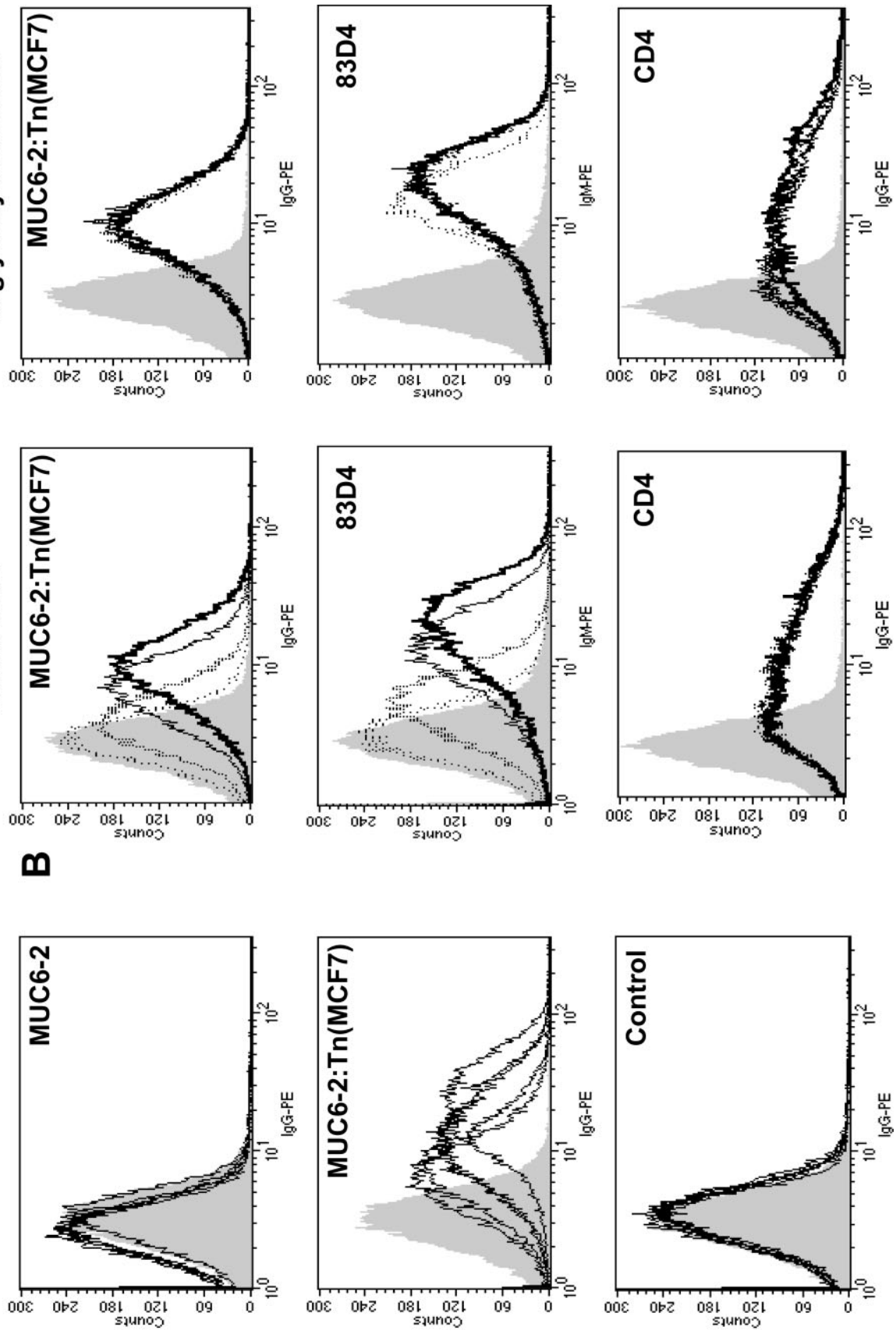

Fig. 5. Recognition of human tumor cell line Jurkat by sera from MUC6-2 : Tn(MCF7)-immunized mice. (**A**) Flow cytometry analyses were carried out on human Tn+ Jurkat tumor cells incubated with individual sera (diluted 1:500) collected from BALB/c mice (five per group) immunized with MUC6-2, MUC6-2 : Tn(MCF7), or alum plus CpG alone (control group). (**B**) For inhibition assays, cells were incubated with a pool of sera from mice immunized with MUC6-2 : Tn(MCF7), together with various concentrations of aOSM (Tn positive mucin) or deglycosylated-OSM (Tn negative mucin). The anti-Tn IgM mAb 83D4 and an anti-CD4 IgG mAb were used as controls. Antibody binding was detected using PE-labeled antibodies specific to mouse immunoglobulin. Concentrations used for aOSM or deglycosylated-OSM were 0 μg/mL (….), 0.01 μg/mL (----), 1 μg/mL (——), and 100 μg/mL (——). The results obtained with mouse sera are the result of two independent experiments.

give more reproducible results and allow easier purification. Indeed, the product yield obtained using both ppGalNAc-T sources was different, being higher when using the recombinant bppGalNAc-T1 (59–69% for bppGalNAc-T1 and 25–34% for MCF7 extracts). This difference is due to an additional step needed to purify the MUC6-Tn glycoconjugates from the reaction mixture containing MCF7 cell extract.

Although a significant progress has recently been made in the synthesis of glycoconjugates, the access to this type of macromolecules remains very difficult, particularly when large quantities are required. To our knowledge, this is the first time that a Tn-glycosylated recombinant protein is obtained in semi-preparative amounts, by the use of ppGalNAc-Ts. By selecting the best conditions for maximal GalNAc transfer, we obtained MUC6-Tn glycoconjugates carrying high densities of Tn antigen. These different Tn densities—54 GalNAc for MUC6-1 : Tn(T1), 49 GalNAc for MUC6-1 : Tn(MCF7), 20 GalNAc for MUC6-2 : Tn(T1), and 26 GalNAc for MUC6-2 : Tn(MCF7)—are the result of independent and reproducible experiments. Depending on the enzyme source, we produced glycoconjugates with different Tn levels. This could be explained by the specificity of ppGalNAc-Ts (not all Thr and Ser residues are recognized by one ppGalNAc-T) (Ten Hagen *et al*., 2003).

We also studied the glycosylation of two MUC6 recombinant proteins of different sizes (203 amino acids for MUC6-1 and 119 amino acids for MUC6-2) to evaluate whether the GalNAc transfer is influenced by the length of the protein. When using the recombinant bppGalNAc-T1, we obtained an average of 54 and 20 incorporated GalNAc residues of 85 and 48 potential *O*-glycosylation sites (total number of Thr and Ser residues) for MUC6-1 and MUC6-2, respectively, representing 64 and 42% of *O*-glycosylation sites, approximately. Structural studies would be necessary to determine whether these distinct glycosylation rates are due to a lack of accessibility of bppGalNAc-T1 to the acceptor sites in the mucin protein.

Of primary importance in the design of vaccines against cancer is that the antigen in the vaccine mimics the antigen in the tumor. To synthesize structures close to the native Tn clusters present in cancer cells, we also performed the GalNAc transfer using MCF7 breast cancer cell extracts, which may express various ppGalNAc-T isoforms, as already shown on other human cancer cell lines (Mandel *et al*., 1999; Marcos *et al*., 2003; Freire *et al*., 2005). In this case, similar GalNAc density was obtained, being 49 for MUC6-1 and 26 for MUC6-2, representing 58 and 54%, respectively, of potential *O*-glycosylation sites. Thus, the degree of glycosylation obtained with the purified recombinant bppGalNAc-T1 and MCF7 cell extracts is different, and surprisingly, the Tn density is not necessarily higher in the latter case. This could be explained by the presence of different ppGalNAc-Ts in the cell extract, which act in a coordinate and sequential manner and may contribute positively or negatively to the overall glycosylation of the protein.

Most of anti-Tn antibodies raised against cancer cells or tissues recognize groups of adjacent Tn epitopes usually called Tn clusters. Indeed, 83D4 and MLS128 anti-Tn mAbs require the presence of at least two consecutive Tn residues for substrate recognition (Nakada *et al*., 1993; Osinaga *et al*., 2000). Taking into account that two-thirds of the Thr and Ser residues in MUC6-1 and MUC6-2 are arranged in clusters, it is highly probable that most of the Tn antigens will be presented, at least, as clusters of two Tn. The potential relevance of the MUC6-Tn glycoconjugates for tumor immunotherapy is evidenced by the analysis of their antigenicity. Indeed, the Tn antigen on MUC6 was recognized by Tn-specific mAbs such as MLS128 and 83D4 and this confirms the presence of Tn clusters. The analyses of the *O*-glycosylation in MUC6 proteins to determine the glycosylation sites in the different MUC6-Tn glycoconjugates are in progress.

As an example, we have also shown that one of the MUC6-Tn glycoconjugates is immunogenic. Indeed, MUC6-2 : Tn(MCF7) induced IgG antibodies in mice, which were capable of recognizing human tumor cells through a Tn-dependent mechanism. To our knowledge, this is the first work reporting the induction of human tumor cell-specific antibodies after immunization with a mucin-derived protein carrying the Tn antigen, without a protein carrier. Indeed, the mucin-derived glycopeptides used so far as immunogens have been conjugated to keyhole limpet hemocyanin (Kagan *et al*., 2005; Sorensen *et al*., 2005). Such conjugates have limitations regarding their application to anticancer immunotherapy in humans. The immune response to the carrier molecule results in a low level of the desired antibodies as compared with the total amount of antibodies produced. This may lead to carrier-induced suppression of the immune response directed against the haptenic molecule (Schutze *et al*., 1985). Furthermore, contrary to KLH conjugates, the structure and the composition of the MUC6-Tn vaccine can be determined by mass spectrometry. This feature is essential to meet the requirements of regulatory bodies for approval in humans.

In conclusion, the transglycosylation method of a recombinant mucin protein presented here is very convenient and effective, because 100% of the starting protein is converted into glycosylated species. Furthermore, a high glycosylation ratio is achieved. The ability to produce recombinant MUC6 with tumor-relevant glycoforms in large amounts is unique and will be extremely valuable for preclinical, immunological, and tumor-protection studies. The study of antitumor potency of MUC6-Tn glycoconjugates is currently underway.

Materials and methods

MUC6 cloning and expression in E. coli

A cDNA clone containing one tandem repeat of human MUC6 was isolated from total cDNA of MCF7 breast cancer cells by reverse transcription–polymerase chain reaction (RT-PCR) and cloned into pGem-T (Promega, Charbonnières-les-Bains, France). The PCR products were designed to encode one tandem repeat of human MUC6 (MUC6-1, 169 amino acids) or a half tandem repeat (MUC6-2, 87 amino acids), which were amplified with *Pfu* DNA polymerase and the primers MUC6-F, 5′-cgggatccTCCACCTCCTTGGTGACT-3′ and MUC6–1R, 5′-ggaagcttTTAGAAAGGTGGAACGTG-3′

(for MUC6-1) or MUC6–2R 5′-ggaagcttATTAGGATGGTGTGTGGA-3′ (for MUC6-2) (lowercase characters indicate restriction sites for BamHI and HindIII in the forward and reverse primers, respectively). Following digestion with BamHI and HindIII, each product was cloned into the pET28a(+) vector (Novagen, Fontenay-sous-Bois, France), so as to encode for a protein carrying a six-histidine tail at the N-terminus. *Escherichia coli* DH5α transformants were selected on Luria Bertani (LB) agar plates containing 50 μg/mL of kanamycin, and the positive clones were confirmed by PCR and sequencing. Plasmids were purified from selected clones and used to transform *E. coli* BLi5 chemically competent cells. The recombinants were expressed in *E. coli* Bli5 by induction with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and purified over Ni^{2+}-nitriloacetic acid columns under denaturing conditions according to the manufacturer's (Qiagen, Hilden, Germany) instructions. MUC6-1 protein was further purified by HPLC using a Perkin-Elmer pump system with a UV detector at 230 nm. The column was a Symmetry 300 C18 (5 μm, 300 Å, 3.9 × 250 mm) (Waters, St. Quentin-en-Yvelines, France). Elution was carried out with a linear gradient of 10–60% acetonitrile in 0.1% trifluoracetic acid in water at a flow rate of 1 mL/min (over 30 min). The MUC6 proteins were characterized by amino acid analysis (AAA) and SELDI-TOF MS. These analyses, together with an N-terminal sequencing, showed that both proteins lack the N-terminal methionine residue.

Breast cancer cell line extract

Breast cancer cell line MCF7 was grown to 90% confluence in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Cergy Pontoise, France) with 10% fetal bovine serum, 1 mM pyruvate, 2 mM glutamine, and 5% CO_2 at 37°C. After trypsinization, cells were washed three times with phosphate-buffered saline (PBS), resuspended in 250 mM sucrose, and homogenized. Cells were then centrifuged at 3000 × *g* for 10 min at 4°C. The resulting supernatant was again centrifuged at 100,000 × *g* for 1 h at 4°C. The pellet was resuspended in 0.1 M imidazole, pH 7.2, and 0.1% Triton X-100. The cell extract was aliquoted and stored at –80°C. Protein concentration was determined by the bicinchoninic acid (BCA) method (Sigma Chemical Co., St. Louis, MO).

Recombinant bovine ppGalNAc-T1

A soluble form of the bovine ppGalNAc-T1 (bppGalNAc-T1) was expressed in the yeast *Pichia pastoris* KM71H strain (Invitrogen, Cergy Pontoise, France) and was purified from the culture supernatant. The cDNA coding region for the soluble form of the bppGalNAc-T1 (from amino acids 52 to 559) was introduced in 3′ of the α-factor sequence signal coding region of a pPICZαA expression vector (Invitrogen) modified to introduce an N-terminal 6His-tag and a C-terminal FLAG-tag. The KM71H strain was made competent using the *Pichia* EasyComp kit (Invitrogen) and transformed according to the manufacturer's instructions. After 120 h of induction in 0.5% methanol, the secreted bppGalNAc-T1 was purified on Ni-NTA-agarose (Qiagen), as previously described (Duclos *et al.*, 2004). Fractions containing enzymatic activity were pooled and dialyzed against ultra-pure water; the protein was freeze-dried and stored at –20°C until use. The specific activity of the recombinant bppGalNAc-T1 was tested, as previously described (Duclos *et al.*, 2004), and estimated at 3 U/mg protein (1 unit transfers 1 μmol of GalNAc per min at 37°C to the acceptor peptide $[STP]_5$).

In vitro *GalNAc transfer to MUC6 proteins*

Using MCF7 extracts. Optimal conditions for *in vitro* glycosylation of both MUC6 proteins were selected after testing assays at an analytical scale under different conditions and characterizing the resulting glycoproteins by SELDI-TOF MS (Ciphergen Biosystems, Fremont, CA), as described separately (Freire *et al.*, 2006). Briefly, a microsome extract of MCF7 breast cancer cells was incubated at 37°C with UDP-GalNAc and purified MUC6-1 or MUC6-2 in 50 mM imidazole pH 7.2 containing 15 mM $MnCl_2$ and 0.1% Triton X-100. Aliquots were taken at different times and were frozen at –20°C. IMAC30 chip array surfaces were activated with 100 mM $NiCl_2$ at room temperature for 15 min and then washed with water and PBS. Spots were incubated with the crude glycosylation mix aliquots for 40 min at room temperature using the bio-processor adaptor and then washed with 0.1% Triton X-100 in PBS (2 × 5 min), PBS (3 × 2 min), and 5 mM 4-(2-hydroxyethyl)-piperazine-1-ethane sulfonic acid (HEPES) (2 × 5 min). Chips were then read in the instrument (Ciphergen ProteinChip Reader, PBS II), and each array spot was laser sampled. Spectra were treated using the Ciphergen ProteinChip software 3.2.1.

As a result, the following conditions were chosen and used for semi-preparative-scale glycosylation transfer assays. Purified MUC6-1 or MUC6-2 (40–80 μM) was incubated with MCF7 extract (6 μg protein/μg mucin) and UDP-GalNAc (2 equivalents per Thr/Ser equivalent in mucin glycoproteins) in 50 mM imidazole, pH 7.2, containing 50 mM $MnCl_2$ and 0.1% Triton X-100 at 37°C. After 24 h incubation, the same amounts of MCF7 extract and UDP-GalNAc were added and incubated for another 24 h. The resulting MUC6-1 : Tn or MUC6-2 : Tn glycoproteins were purified using Ni-NTA-agarose (Qiagen) and then subjected to reverse-phase HPLC using a Perkin-Elmer pump system with an UV detector at 230 nm. The column was a Symmetry 300 C18 (5 μm, 300 Å, 3.9 × 250 mm) (Waters). Elution was carried out with a linear gradient of 10–60% acetonitrile in 0.1% trifluoracetic acid in water at a flow rate of 1 mL/min (over 30 min). The peak was collected and then lyophilized. The MUC6-1 : Tn and MUC6-2 : Tn glycoproteins were characterized by AAA and mass spectrometry.

Using bppGalNAc-T1. Optimal semi-preparative conditions of GalNAc transfer using bppGalNAc-T1 were set up using the Ciphergen technology, as described for the MCF7 extracts. Purified MUC6-1 or MUC6-2 proteins (40–80 μM) were incubated with UDP-GalNAc (two equivalents per Thr/Ser equivalent in mucin glycoproteins) and bppGalNAc-T1 (0.1 μg/μg mucin) in 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 6.5, containing 15 mM $MnCl_2$ for 24 h at 37°C. The resulting MUC6-1 : Tn or MUC6-2 : Tn were directly subjected to reverse-phase HPLC and purified as explained above. The peak was

collected, lyophilized, and characterized by AAA and mass spectrometry.

Antibodies

The mAb 83D4 (IgM) (kindly given by Dr E. Osinaga, Facultad de Medicina, Montevideo, Uruguay), which recognizes specifically the Tn antigen (Osinaga *et al*., 2000), was produced from a mouse immunized with cell suspensions obtained from formalin-fixed paraffin-embedded sections of an invasive human breast cancer (Pancino *et al*., 1990). It was then precipitated from ascitic fluids by dialysis against demineralized water at 4°C, dissolved in a small volume of 0.5 M NaCl in PBS, and purified by gel-filtration chromatography on Sephacryl S-200.

The anti-Tn mAb MLS128 (IgG_1), kindly given by H. Nakada, was obtained from a mouse immunized with human colonic cancer cells (LS180) (Numata *et al*., 1990) and purified by affinity chromatography on protein A-Sepharose. Both anti-Tn mAbs recognize Tn residues organized in clusters (Nakada *et al*., 1993; Osinaga *et al*., 2000).

A MUC6-2 anti-serum was obtained by injecting BALB/c mice with 10 μg of purified MUC6-2 (see Immunization of mice) in alum (1 mg) and CpG (10 μg). Mice were injected i.p. at days 0, 21, and 42 and bled at days 20, 28, and 49. MUC6-2 anti-serum reactivity against MUC6-1 and MUC6-2 was confirmed by ELISA assays, and the serum was stored at –20°C until use.

Recognition of MUC6-1 : Tn and MUC6-2 : Tn by anti-Tn mAbs and anti-MUC6 serum

Microtiter plates (Nunc, Roskilde, Denmark) were coated with the *in vitro*-synthesized glycoproteins (0.1 μg/mL) and dried overnight. Plates were washed three times with 0.1% Tween-20 in PBS (PBS/T), and nonspecific binding sites were blocked with 1% gelatin in PBS (PBS/G) for 2 h at 37°C. After washing, anti-Tn mAbs (83D4 or MLS128) or a polyclonal anti-MUC6 serum was added and incubated for 2 h at 37°C. After three washes with PBS/T, plates were incubated with goat anti-mouse IgM or anti-IgG peroxidase conjugates (Sigma) diluted in PBS/TG for 1 h at 37°C. The plates were revealed using *o*-phenylenediamine/H_2O_2 and read photometrically at 492 nm in an ELISA autoreader (Dynatech, Marnes la Coquette, France).

Western blot analysis of MUC6-Tn glycoconjugates

MUC6-Tn glycoproteins were analyzed by western blotting using an anti-His mAb (Qiagen) and the anti-Tn mAb 83D4. (Glyco)conjugates were separated in a 13% SDS–PAGE and transferred to nitrocellulose sheets (Amersham, Saclay, France) at 30 V overnight in 20 mM Tris–HCl, pH 8.3, 192 mM glycine and 10% ethanol, as previously described (Towbin *et al*., 1979). Residual protein-binding sites were blocked by incubation with 3% bovine serum albumin (BSA) in PBS at 37°C for 2 h. The nitrocellulose was then incubated with either the anti-His mAb or the anti-Tn mAb 83D4 for 2 h at 37°C. After three washes with PBS containing 0.1% Tween-20 and 1% BSA, the membrane was incubated for 1 h at room temperature with goat anti-mouse immunoglobulins conjugated to peroxidase (Sigma) diluted in PBS containing 0.1% Tween-20 and 1.5% BSA, and reactions were developed with enhanced chemiluminiscence (ECL) (Amersham, Saclay, France). The same procedure was performed omitting the antibodies as a negative control.

Endotoxin level determination

The endotoxin level was determined in all glycosylated and nonglycosylated MUC6 proteins according to the instructions of the manufacturer, using the Limulus Amebocyte Lysate QCL-1000 kit (Cambrex, Emerainville, France).

Immunization of mice

Six- to eight-week-old female BALB/c mice were purchased from Janvier (Le Genest Saint-Isle, France). Mice were injected i.p. three times with MUC6-2 or MUC6-2 : Tn(MCF7) (10 μg) mixed with alum (1 mg) (Serva, Heidelberg, Germany) plus CpG (10 μg) (Proligo, Paris, France) at 3-week intervals (five mice per group). Control mice received alum plus CpG alone. Sera were collected after each immunization and tested for the presence of anti-MUC6 and anti-Tn antibodies by ELISA and fluorescence-activated cell sorting.

Flow cytometry

Mouse sera were tested at 1:500 dilution by flow cytometry on the human tumor cell line Jurkat. Cells were first incubated for 15 min with sera at 4°C in PBS containing 5% fetal bovine serum and 0.1% sodium azide. Then, they were incubated for 15 min with an anti-mouse IgG goat antibody conjugated to phycoerythrin (Caltag, Burlingame, CA). Paraformaldehyde-fixed cells were analyzed on an FACScan flow cytometer (Becton Dickinson, San Jose, CA), and analyses were performed with CellQuest software (Becton Dickinson). For inhibition assays, cells were incubated with sera first mixed with serial dilutions of aOSM or deglycosylated-OSM (kindly given by Dr E. Osinaga) for 15 min at 4°C. Then, the binding of antibodies to cells was revealed using an anti-mouse IgG goat antibody conjugated to PE. The anti-Tn mAb 83D4 was used as a positive control. An anti-CD4 mAb (Caltag) was also used to verify that the binding of this mAb to the cells was not affected by the OSM proteins.

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Conflict of interest statement

None declared.

Abbreviations

AAA, amino acid analysis; aOSM, asialo-ovine submaxillary mucin; BSA, bovine serum albumin; ELISA, enzyme-linked

immunosorbent assay; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ppGalNAc-T, UDP-*N*-acetylgalactosamine: polypeptide *N*-acetylgalactosaminyltransferase; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; Ser, serine; Thr, threonine; UDP-GalNAc, uridine 5′-diphospho-*N*-acetylgalactosamine.

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