Antitumor Vaccines

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Synthetic Antitumor Vaccines from Tetanus Toxoid Conjugates of MUC1 Glycopeptides with the Thomsen–Friedenreich Antigen and a Fluorine-Substituted Analogue**

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Dedicated to Professor Albert Eschenmoser on the occasion of his 85th birthday

An active immunization against human tumor cells would offer entirely new possibilities for cancer therapy. For this purpose, vaccines are required through which a strong immune response directed against tumor cells is induced. The tumor-associated mucin MUC1,[1–3] which is strongly over-expressed on epithelial tumor cells, constitutes a promising candidate for the development of such a vaccine. Compared to the MUC1 on normal epithelial cells, it carries truncated and prematurely sialylated saccharide antigens such as the T_N, the Thomsen–Friedenreich (T), and the sialyl-T_N antigens[1–4] in its extracellular tandem-repeat region. MUC1 isolated from biological material, that is, from tumor cell membranes, is not suitable for vaccinations, because of the biological microheterogeneity of its glycans which display antigens from healthy cells as well as from cancer cells. In contrast, synthetic glycopeptides that contain structurally defined tumor-associated saccharides bound to tandem-repeat peptide sequences of MUC1 should facilitate the induction of immune reactions selectively directed against epithelial tumor cells.

The immunogenicity of natural glycopeptides and peptides is too low to elicit a strong immune response. Even their conjugates with bovine serum albumin[6,7] induce only moderate immune reactions and antibodies of the IgM type. In contrast, strong, selective immune responses of the IgG type were observed in transgenic mice immunized with fully synthetic conjugates consisting of tumor-associated MUC1 glycopeptides and T-cell epitope peptides from ovalbumin.[8,9] However, so far only one third of the immunized mice gave this immune response. Exceptionally strong immune reactions were induced with a synthetic vaccine, in which the MUC1 glycopeptide containing a sialyl-T_N saccharide side chain is coupled to tetanus toxoid as the carrier protein.[10] Apparently, tetanus toxoid contains efficient murine T-cell epitopes. Tetanus-toxoid-based MUC1 glycopeptide vaccines[10] have the particular advantage that they are suitable for application to humans.[11] A selective immune response was also induced in all mice treated with a fully synthetic vaccine[12] consisting of a MUC1 glycopeptide carrying a T-antigen saccharide and a Toll-like receptor 2 ligand lipopeptide.[13]

These insights led us to synthesize the vaccine described herein consisting of tetanus toxoid and a MUC1 glycopeptide with a T-antigen side chain, in particular, since in the meantime an analogue of the T-antigen glycosyl amino acid building block is also available which carries fluorine substituents in 6- and 6′-positions of the saccharide.[14] Comparison of the properties of the vaccine having the natural structure with those of the mimetic T-antigen structure should reveal whether saccharide structures of the vaccines that are sensitive to enzymatic degradation can be replaced by analogues that are more stable against enzymatic degradation without reducing the immunological efficiency of the vaccine, and therefore whether this approach might be advantageous in long-term treatments.

The solid-phase synthesis of the MUC1 tandem-repeat glycopeptides which contain either the T-antigen or the difluoro-T-antigen saccharide side chain and are N-terminally extended by a triethylene glycol spacer amino acid was performed according to a known procedure[17] using a Tentagel resin[16] loaded with Fmoc-protected proline attached by means of a trityl linker [15] (Scheme 1). The Fmoc-protected amino acids were applied in 10 equivalents and coupled using HBTU/HOBt[18] in *N*-methylpyrrolidone (NMP). The more reactive HATU/HOAt reagent also in NMP was used for the coupling of the Fmoc-protected T-antigen threonine building block[17] **1** and the Fmoc-protected difluoro-T-antigen building block[14] **2** (2.5 equivalents).

After completion of the synthesis, the glycopeptide antigens **3** (23%) and **4** (29%), still protected in the carbohydrate portion, were detached from the resin using trifluoroacetic acid/triisopropylsilane/water (10:1:1). Con-

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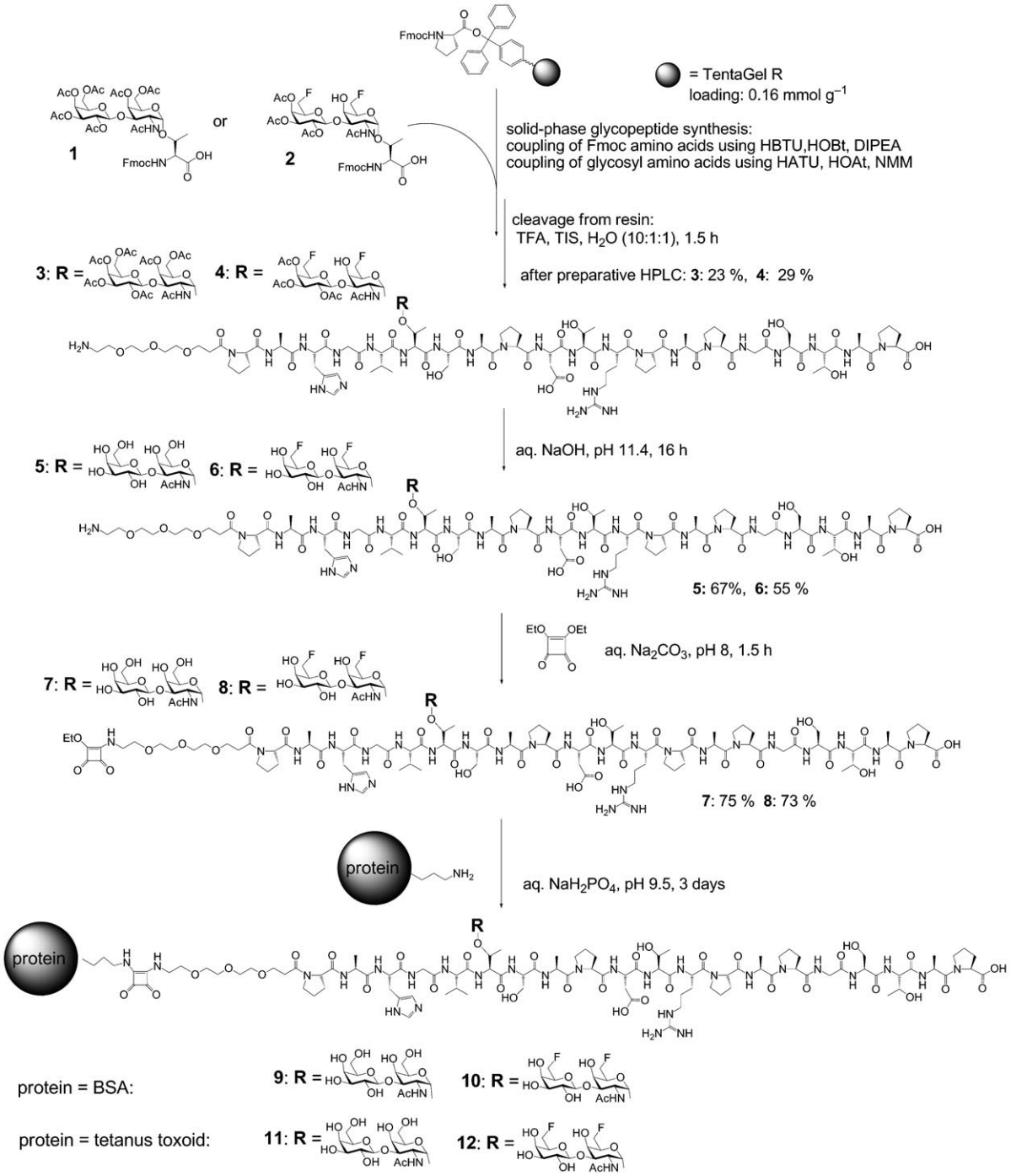

Scheme 1. Solid-phase synthesis of the T-antigen-MUC1 glycopeptides **3** and **4** and their conjugation to proteins to yield vaccines **9**–**12**: Fmoc = fluorenyl-9-methoxycarbonyl, HBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxybenzotriazole, DIPEA = diisopropylethylamine (Hünig's base), NMM = *N*-methylmorpholine, NMP = *N*-methylpyrrolidone, TFA = trifluoroacetic acid, TIS = triisopropylsilane, BSA = bovine serum albumin.

comitantly all acid-sensitive protecting groups of the amino acid side chains were removed. Subsequently, the glycopeptides were treated with aqueous NaOH at pH < 11.4 resulting in the removal of the *O*-acetyl groups. The obtained free glycopeptides **5** and **6** were isolated after preparative HPLC in yields of 67% and 55%, respectively. Reaction of the terminal amino groups on the triethylene glycol spacer with the diethyl ester of squaric acid[20] in aqueous solution at pH 8 afforded the squaric acid monoamides of the glycopeptide antigens **7** (75%) and **8** (73%).[21] These compounds were stirred with bovine serum albumin (BSA) in aqueous phosphate buffer at pH 9.5 to give the BSA conjugates of T-antigen-MUC1 glycopeptide (**9**) and difluoro-T-antigen-MUC1 glycopeptide (**10**). In analogous reactions, the squaric

acid monoamides of the glycopeptides **7** and **8** were linked to tetanus toxoid in aqueous solution. The T-antigen-MUC1-TTox vaccine **11** and the F_2T-antigen-MUC1-TTox vaccine **12** were obtained after ultrafiltration (membrane 30 kDa).

Whereas the loading of the BSA conjugates could be determined by MALDI-TOF mass spectrometry to amount to on average seven molecules of glycopeptide antigen per molecule of protein (see Figure 1). The loading of the MUC1-TTox vaccine **11** and **12** had to be estimated by ELISA binding data.[10] Values of >20 molecules of glycopeptide antigen **7** or **8** per molecule of protein were obtained.

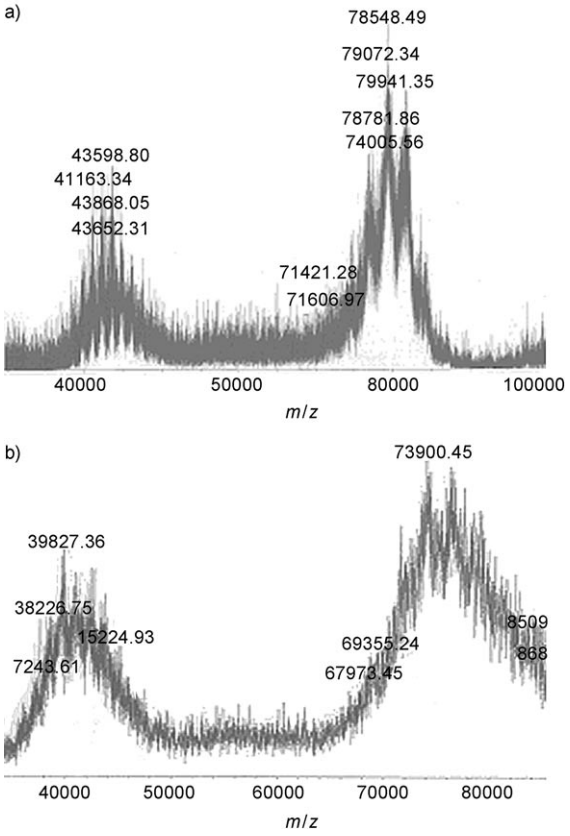

Figure 1. MALDI-TOF spectra of the T-antigen-MUC1-BSA conjugate **9** (a) and of the difluoro-T-antigen-MUC1-BSA conjugate **10** (b).

In order to evaluate the immunological properties three balb/cJ mice were immunized with the T-antigen-MUC1-TTox vaccine **11** (mice 1–3), and three were immunized with the difluoro-T-antigen-MUC1-TTox vaccine **12** (mice 4–6) under addition of complete Freund's adjuvant (CFA). After 21 days booster immunizations with vaccines **11** and **12** and incomplete Freund's adjuvant (IFA) were performed. On the fifth day after the third immunization, blood was drawn from the tail vein of the mice, and the sera were tested for induced antibodies. A very strong immune response effectual enough to override the natural tolerance was observed for all six vaccinated mice. The titers of the ELISA tests were determined to be approximately 80000 for mice immunized with the natural T-antigen-MUC1-TTox vaccine **11** (mice 1–3). For these tests, the microtiter plates were coated with the T-antigen-MUC1-BSA conjugate **9** (Figure 2a). The immune responses were more than 25 times stronger than in the case of the strongest responding mice[8,9] immunized with the synthetic MUC1-OVA-T-cell-epitope vaccine, and more than 100 times stronger than that of the Pam_3Cys-lipopeptide-MUC1 vaccine.[12] The mice immunized with the difluoro analogue **12** (mice 4–6) showed titers of approximately 40000 (Figure 2b). In these ELISA tests, the microtiter plates were coated with the F_2T-antigen-MUC1-BSA-conjugate **10**. The antibodies induced by vaccine **11** strongly recognized, in particular, the glycopeptide antigen of BSA conjugate **9**, which corresponds to the glycopeptide structure present in vaccine **11** (Figure 2a and line 1, Figure 2c). The glycopeptide-BSA conjugate containing the T-antigen structure within the 2,6-sialyl-T antigen on the identical MUC1 sequence[22] was bound with similar affinity (line 5, Figure 2c). The recognition of the BSA conjugates representing related tumor-associated antigens with sialyl-T_N antigen[9] (line 2), with one sialyl-T_N antigen, and one T_N antigen[9] (the latter bound to threonine 18, line 3) and the conjugate with non-glycosylated MUC1 peptide (line 4) were found slightly weaker. The glycopeptide sequence from MUC1[23] truncated to 12 amino acids is only weakly recognized (line 6). The antibodies induced by vaccine **12** (Figure 2d) show very similar behavior. It is noteworthy that their binding to the natural T-antigen-MUC1-BSA conjugate **9** (line 1, Figure 2d) is as strong as that to the fluoro-substituted conjugate **10** (Figure 2b).

The determination of the isotypes of the induced antibodies using isotype-selective secondary antibodies shows that the antibodies induced by vaccine **11** and **12** predominantly are of the IgG_1 type. Virtually no antibodies of the IgM type were induced. Thus, the immune responses are selective and indicate the establishment of an immunological memory. They are MHCII restricted. In the case of the natural T-antigen-MUC1 vaccine **11**, besides IgG_1 also $IgG_{2a,b}$ antibodies are induced to some extent; these were evident to a distinct degree in the sera induced by F_2T-antigen-MUC1 vaccine **12** (Figure 3a,b).

It is of particular importance for this overall concept that the antibodies induced by the synthetic vaccines **11** and **12** strongly bind to cells of the epithelial tumor cell line MCF-7.[24] To visualize this binding, the tumor cells were incubated with the induced antisera. After washing, secondary anti-mouse-IgG antibodies from the goat carrying a fluorescent label (AlexaFluor 488) were added to the cells. These cells were then counted in the FACS analyzer (fluorescent-activated cell sorter) by light scattering (Figure 4, left area). The cells recognized by the antibodies from the mouse antisera show fluorescence and were counted separately (right area). As is shown in Figure 4a, cells treated with buffer solution as a control do not bind fluorescent-labeled anti-mouse antibodies and, therefore, do not show fluorescence and appear in the left area. The MCF-7 cells incubated with the 1000-fold diluted serum of mouse 2 immunized with vaccine **11** are all labeled and appear in the right area (Figure 4b). The MCF-7 cells were also strongly recognized by 1000-fold diluted serum of mouse 5, which was immunized

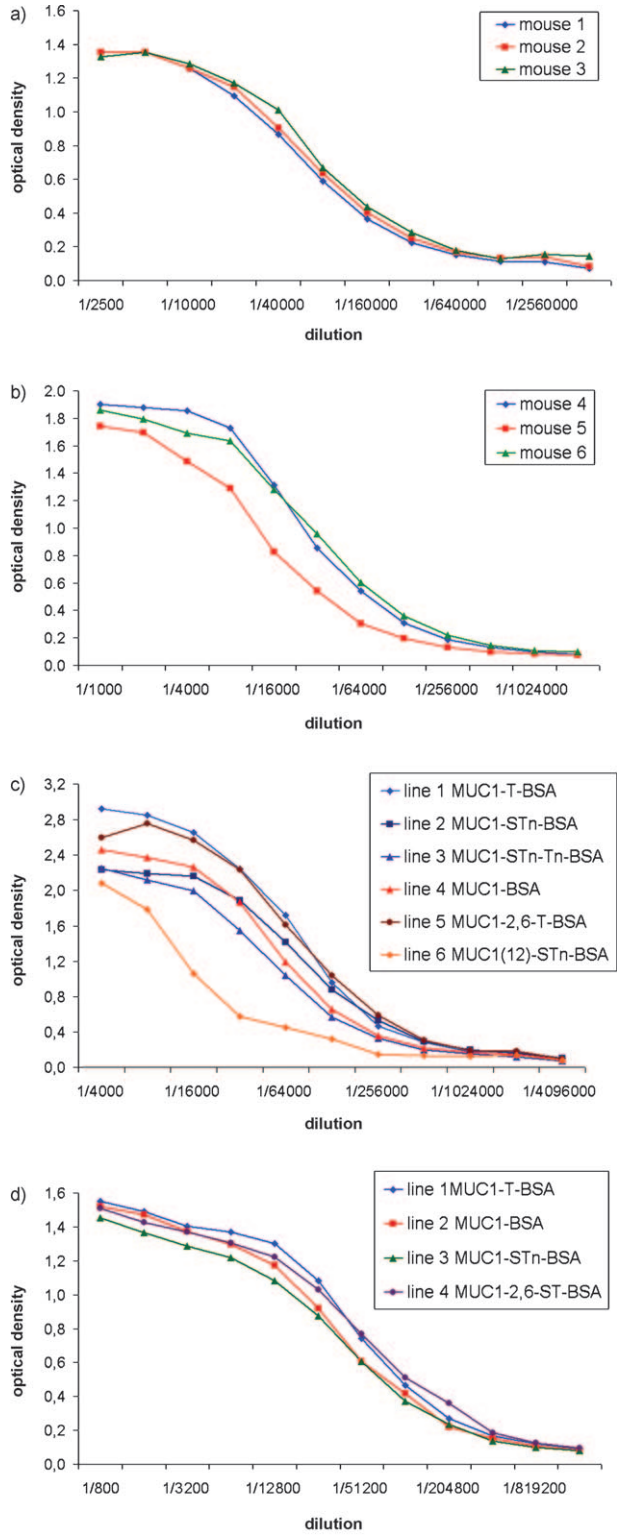

Figure 2. ELISA of the antisera induced by the T-antigen-MUC1 vaccine: a) Antisera of mice immunized with vaccine **11**, binding to BSA conjugate **9**; b) antisera of mice immunized with vaccine **12**, binding to BSA conjugate **10**; c) binding of antibodies induced by **11** (serum mouse 2) to related tumor-associated saccharide antigen-BSA conjugates; d) binding of antibodies induced by **12** (serum mouse 6) to related tumor-associated saccharide antigen-BSA conjugates.

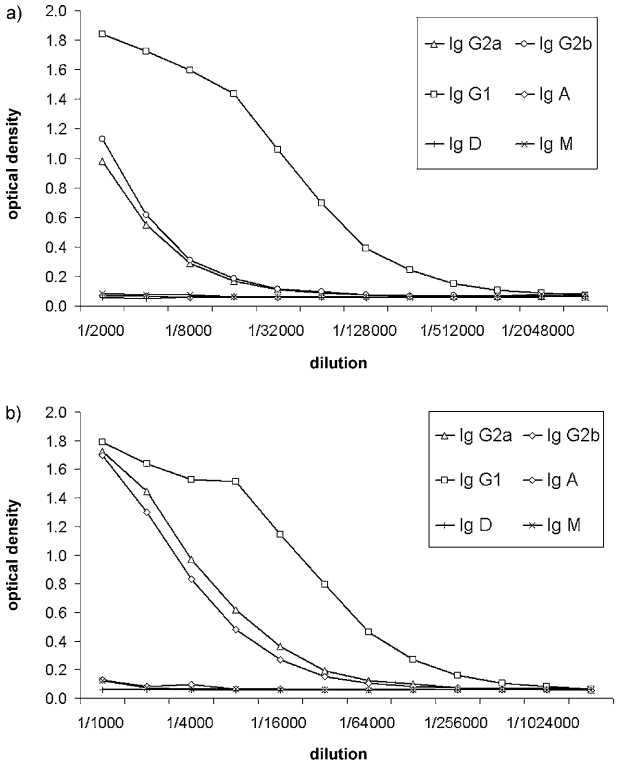

Figure 3. Determination of the isotypes of the antibodies induced by vaccine **11** and **12**: a) antiserum of mouse 1, treated with **11**; b) antiserum of mouse 5, treated with **12**.

with the fluorine-substituted T-antigen-MUC1 vaccine **12** (Figure 4c). The binding of the antibodies induced by the synthetic vaccines to the MCF-7 tumor cells was neutralized by addition of MUC1 glycopeptides. When the T-antigen-MUC1 glycopeptide **5** (3 $\mu g\,mL^{-1}$) was added to the MCF-7 cells incubated with the antiserum from mouse 2 (vaccinated with **11**), the binding of the antibodies to MCF-7 cells was almost completely neutralized (Figure 4e). Similarly, glycopeptide **6** containing the difluoro-T-antigen (3 $\mu g\,mL^{-1}$) neutralized the binding of the antiserum of mouse 5 (vaccinated with **12**) to the tumor cells (Figure 4f).

The serum of mouse 5 was neutralized also by the natural glycopeptide antigen **5**, and the serum of mouse 2 was neutralized by the fluorinated glycopeptide antigen **6**. In contrast, the recognition of the tumor cells cannot be neutralized by a glycopeptide from a different mucin (MUC4),[17] which has a different peptide sequence (Figure 4d). These effects give evidence that the tumor-cell recognition through the antibodies induced by the synthetic T-antigen-MUC1-TTox vaccines is selectively directed to tumor-associated glycopeptide antigens such as **5** and **6**.

These results constitute essential progress in the development of a synthetic antitumor vaccine for active immunizations. Extraordinarily strong immune reactions were elicited by the T-antigen-MUC1-tetanus-toxoid vaccines **11** and **12** which override the natural tolerance of the immune system against structures intrinsically occurring on mammalian cells. In contrast to the immune reaction induced with MUC1-T-cell-epitope peptide vaccines in transgenic mice,[8,9] not only

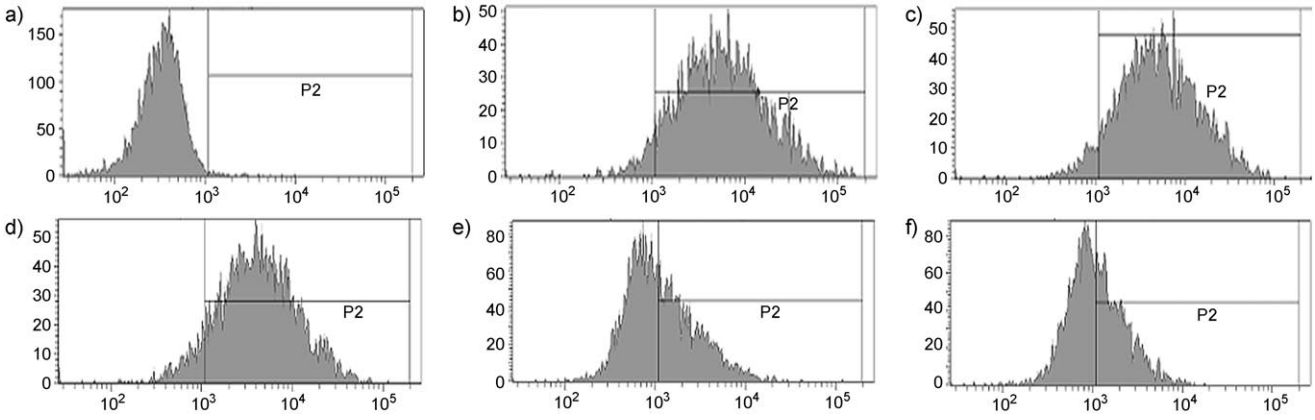

Figure 4. FACS analysis of the binding of the antisera to MCF-7 cells: a) MCF-7 tumor cells treated with buffer solution; b) MCF-7 cells treated with serum of mouse 2 which had been vaccinated with **11**; c) MCF-7 cells treated with serum of mouse 5 which had been vaccinated with **12**; d) the binding displayed in (c) is not neutralized by the addition of a glycopeptide from of different mucin (MUC4);[17] e) Neutralization of the binding of the antibodies from mouse 2 by T-antigen-MUC1 glycopeptide **5**. f) neutralization of the binding of the antibodies from mouse 5 by F_2T-antigen-MUC1 glycopeptide **6**. Horizontal axes: fluorescence intensity; vertical axes: counts (of cells).

every third mouse reacted, but all wild-type mice vaccinated with **11** and **12** responded very strongly. The immune responses are selective and elicit antibodies of the IgG type. The induced antibodies bind strongly to epithelial tumor cells of the MCF-7 cell line. The primary OH groups of the T-antigen carbohydrate part of the glycopeptide antigen can be replaced by fluorine without reducing the immunogenicity and structure-selectivity of the vaccine. Of particular value is that the vaccine consisting of a synthetic MUC1 glycopeptide and tetanus toxoid can also be applied to humans, as already has been shown by a series of practically applied TTox-based vaccines.[11]

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[1] a) S. J. Gendler, C. A. Lancaster, J. Taylor-Papadimitriou, T. Dulugm, N. Peat, J. Burchell, L. Pemberton, E. Lalani, D. Wilson, *J. Biol. Chem.* **1990**, *265*, 15286; b) J. Taylor-Papadimitriou, J. Burchell, D. W. Miles, M. Daziel, *Biochim. Biophys. Acta Mol. Basis Dis.* **1999**, *1455*, 301.
[2] a) F.-G. Hanisch, S. Müller, *Glycobiology* **2000**, *10*, 439; b) T. Ninkovic, L. Kinarsky, K. Engelmann, V. Pisarev, S. Sherman, O. J. Finn, F. G. Hanisch, *Mol. Immunol.* **2009**, *47*, 131.
[3] A. L. Sørensen, C. A. Reis, M. A. Tarp, U. Mandel, K. Ramachandran, V. Sankaranarayanan, T. Schientek, R. Graham, J. Taylor-Papadimitriou, M. A. Hollingworth, J. Burchell, H. Clausen, *Glycobiology* **2006**, *16*, 96.
[4] T. Ju, R. P. Aryal, C. J. Stowell, R. D. Cummings, *J. Cell Biol.* **2008**, *182*, 531.
[5] S. Muller in *Synthetic Peptides as Antigens*, *Vol. 28* (Eds.: S. Pillai, P. C. van der Vliet) Elsevier, Amsterdam, **1999**, pp. 79–131.
[6] T. Peters, Jr., *Adv. Protein Chem.* **1985**, *37*, 161.
[7] H. Kunz, S. Birnbach, *Angew. Chem.* **1986**, *98*, 354; *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 360.
[8] S. Dziadek, A. Hobel, E. Schmitt, H. Kunz, *Angew. Chem.* **2005**, *117*, 7803; *Angew. Chem. Int. Ed.* **2005**, *44*, 7630.
[9] U. Westerlind, A. Hobel, N. Gaidzik, E. Schmitt, H. Kunz, *Angew. Chem.* **2008**, *120*, 7662; *Angew. Chem. Int. Ed.* **2008**, *47*, 7551.
[10] A. Kaiser, N. Gaidzik, U. Westerlind, D. Kowalczyk, A. Hobel, E. Schmitt, H. Kunz, *Angew. Chem.* **2009**, *121*, 7688; *Angew. Chem. Int. Ed.* **2009**, *48*, 7551.
[11] See for example: T. Nolan, S. Lambert, D. Roberton, H. Marshall, P. Richmond, C. Streeton, J. Poolman, D. Boutriau, *Vaccine* **2007**, *25*, 8487, and references therein.
[12] A. Kaiser, N. Gaidzik, T. Becker, C. Menge, K. Groh, H. Cai, Y.-M. Li, B. Gerlitzki, E. Schmitt, H. Kunz, *Angew. Chem.* **2010**, *122*, 3772; *Angew. Chem. Int. Ed.* **2010**, *49*, 3688.
[13] R. Spohn, U. Buwitt-Beckmann, R. Brock, G. Jung, A. J. Ulmer, K.-H. Wiesmüller, *Vaccine* **2004**, *22*, 2494.
[14] a) C. Mersch, S. Wagner, A. Hoffmann-Röder, *Synlett* **2009**, 2167; b) S. Wagner, C. Mersch, A. Hoffmann-Röder, *Chem. Eur. J.* **2010**, DOI: 10.1002/chem.200903294.
[15] J. M. J. Fréchet, K. E. Haque, *Tetrahedron Lett.* **1975**, *16*, 3055.
[16] Rapp Polymere, Tübingen, Germany.
[17] C. Brocke, H. Kunz, *Synthesis* **2004**, 525.
[18] L. A. Carpino, *J. Am. Chem. Soc.* **1993**, *115*, 4397.
[19] V. Dourtoglou, B. Gross, V. Lambropoulou, C. Ziodrou, *Synthesis* **1984**, 572.
[20] L. F. Tietze, M. Arlt, M. Beller, K.-A. Glüsenkamp, E. Jäde, M. F. Rajewski, *Chem. Ber.* **1991**, *124*, 1215.
[21] **7**:15.6 mg (64%), colorless lyophilisate, $[\alpha]_D^{23}=-82.3$ ($c=1.0$, H_2O); $t_R=17.8$ min (Phenomenex Jupiter, gradient: CH_3CN/H_2O + 0.1% TFA (10:90)→(25:75), 30 min, $\lambda=218$ nm). MALDI-TOF-MS (dihydroxybenzoic acid): *m/z*: 2580.88 ($[M+H]^+$, calcd: 2579.19). ESI-MS: *m/z*: 1290.17 ($[M+2H]^{2+}$, calcd: 1290.10), 860.77 ($[M+3H]^{3+}$, calcd: 860.40). ^{1}H NMR (400 MHz, D_2O, COSY, HSQC; selected signals): $\delta=8.50$ (d, 1H, $J_{H\varepsilon,H\delta}=1.3$ Hz, H_ε), 7.20 (d, 1H, H_δ, $J_{H\varepsilon}=1.1$ Hz, H_δ), 4.83 (d, 1H, $J_{H1,H2}=3.8$ Hz, 1-H), 4.63–4.03 (m, 24H, D^α {4.59}, CH_2O squarate {4.59}, H^α {4.58}, R^α {4.53}, $T^{T*\alpha}$ {4.53}, $A^{3\alpha}$ {4.47}, $A^{2\alpha}$ {4.47}, $S^{2\alpha}$ {4.40}, $A^{4\alpha}$ {4.35}, $S^{1\alpha}$ {4.32}, 1′-H {4.29}, $P^{1-5\alpha}$ {4.30, 4.29, 4.27, 4.25, 4.23}, V^α {4.19}, $T^{T*\beta}$ {4.14}, $A^{1\alpha}$ {4.12}, $T^{1\alpha}$ {4.09}, $T^{2\alpha}$ {4.09}, $T^{2\beta}$ {4.07}, $T^{1\beta}$ {4.06}); 2.80 (m, 3H, $D^{\beta b}$, 12-CH_2 spacer), 2.58 (m, 2H, CH_2 spacer); 1.14 (d, 3H, $J_{T\gamma,T\beta}=6.3$ Hz, $T^{T*\gamma}$), 1.08 (d, 3H, $J_{T\gamma,T\beta}=6.6$ Hz, $T^{2\gamma}$), 1.06 (d, 3H, $J_{T\gamma,T\beta}=7.2$ Hz, $T^{1\gamma}$), 0.84 ppm (t, 6H, $J_{V\gamma,V\beta}=8.3$ Hz, V^γ). ^{13}C NMR

(100.6 MHz, D2O, ^{13}C, HSQC): $\delta = 188.83$ (C=O squarate), 176.19, 174.94, 174.89, 174.84, 174.44, 174.20, 173.99, 173.77, 173.67, 173.53, 173.51, 173.11, 172.58, 172.42, 172.02, 171.96, 171.51, 171.35, 171.22, 171.14, 170.93, 170.74 (C=O, C=O acetyl), 156.70 (C=NH, O-*C*=C squarate), 133.41 (H^{ε}), 128.47 (H^{γ}), 117.29 (H^{δ}), 104.63 (C1′), 99.13 (C1); 18.80 ($T^{1\gamma}$), 18.72 ($T^{2\gamma}$), 18.47 ($V^{\gamma a}$), 18.35 ($T^{T*\gamma}$), 17.83 ($V^{\gamma b}$), 16.20, 15.18, 15.06, 15.02 ($A^{1-4\beta}$), 14.03 ppm (CH_3 squarate).

8: 20 mg (73 %), colorless lyophylisate, $[\alpha]_D^{23} = -82.9$ ($c = 1.0$, H_2O), $t_R = 25.1$ min (Phenomenex Luna, gradient: MeCN/H_2O + 0.1 % TFA (10:90)→(25:75), 40 min, →(70:30) 60 min). ESI-MS: *m*/*z*: 874.70 ($[M+K+2H]^{3+}$, calcd: 874.92), 1292.59 ($[M+2H]^{2+}$, calcd: 1292.84). ^{1}H NMR (400 MHz, D_2O, COSY, HMQC) $\delta = 8.61$ (d, $J_{H\varepsilon,H\delta} = 1.4$ Hz, 1H, H^{ε}), 7.31 (d, $J_{H\delta,H\varepsilon} = 1.1$ Hz, 1H, H^{δ}), 5.01 (d, $J_{H1,H2} = 3.7$, 1H, 1-H), 4.75–4.50 (m, 15H, D^{α} {4.72}, CH_2O squarate {4.71}, H^{α} {4.69}, 6a′-H {4.68}, 6a-H {4.67}, T^{α}* {4.65}, R^{α} {4.65}, $S_1{}^{\alpha}$ {4.52}, $A_3{}^{\alpha}$ {4.58}, 6b′-H {4.58}, 6b′-H {4.56} $A_2{}^{\alpha}$ {4.53}), 4.48–4.19 (m, 18H, $A_4{}^{\alpha}$ {4.45}, $S_2{}^{\alpha}$ {4.46}, 1′-H {4.45}, $P_{1-5}{}^{\alpha}$ {4.39, 4.38, 4.37, 4.35, 4.33}, $T_{1-2}{}^{\alpha}$ {4.34, 4.30}, T^{β}* (4.31}, V^{α} {4.29}, 5-H {4.30}, $A_1{}^{\alpha}$ {4.23}, 4-H {4.21}, $T_{1-2}{}^{\beta}$ {4.20, 4.19}, 2-H {4.18}); 2.88 (dd, $J_{D\beta b,D\alpha}$ 6.9, $J_{D\beta b,D\beta a} = 17.1$, 1H, $D^{\beta b}$), 2.77–2.61 (m, 2H, CH_2-amide spacer); 1.24 (d, $J_{T\gamma*,T\beta*} = 6.3$ Hz, 3H, T^{γ}*), 1.19 (d, $J_{T\gamma,T\beta} = 6.5$ Hz, 3H, $T_1{}^{\gamma}$), 1.17 (d, $J_{T\gamma,T\beta} = 6.4$ Hz, 3H, $T_2{}^{\gamma}$), 0.96 (d, $J_{V\gamma,V\beta} = 4.4$ Hz, 3H, V^{γ}), 0.95 ppm (d, $J_{V\gamma,V\beta} = 4.3$ Hz, 3H, V^{γ}). ^{13}C NMR (100.6 MHz, D_2O, DEPT): $\delta = 188.8$, 183.3 (C=O (squarate)), 177.1, 176.0, 174.9, 174.9, 174.0, 173.7, 173.5, 173.1, 172.5, 172.0, 171.5, 171.5, 171.4, 171.2, 171.1, 171.0, 170.8, 170.7 (C=O, C=O acetyl, C=C squarate), 156.7 (C=NH), 133.4 (H^{ε}), 128.4 (H^{γ}), 117.3 (H^{δ}), 104.5 (C1′), 99.2 (C1); 18.8, 18.7 (T^{γ}), 18.5 (V^{γ}), 18.2 (T^{γ}*), 17.8 (V^{γ}), 16. 2, 15.2, 15.1, 15.0 ($A_{1-4}{}^{\beta}$), 14.9 ppm (CH_3 squarate). ^{19}F NMR (376.4 Hz, D_2O): $\delta = -229.1$–-229.4 ppm (m, 2F, {−229.3 (dt, $J_{F,H5} = 13.7$ Hz, $J_{F,H6} = 47.1$ Hz}).

[22] S. Dziadek, C. Griesinger, H. Kunz, U. M. Reinscheid, *Chem. Eur. J.* **2006**, *12*, 4981.

[23] S. Dziadek, D. Kowalczyk, H. Kunz, *Angew. Chem.* **2005**, *117*, 7798; *Angew. Chem. Int. Ed.* **2005**, *44*, 7624.

[24] H. D. Soule, J. Vazguez, A. Long, S. Albert, M. Brennan, *J. Natl. Cancer Inst.* **1973**, *51*, 1409.

