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Cancer Therapy

Synthetic Antitumor Vaccines Containing MUC1 Glycopeptides with Two Immunodominant Domains—Induction of a Strong Immune Response against Breast Tumor Tissues**

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Dedicated to Professor Dieter Hoppe on the occasion of his 70th birthday

An active immunization against human tumor tissues can be achieved only through vaccines that induce selective immune reactions directed towards membrane structures of tumor cells. The tumor-associated mucin MUC1 apparently is a promising target structure for antitumor vaccines.[1–3] MUC1 occurs on nearly all epithelial tissues. It contains a large, highly glycosylated domain in the extracellular part, which is composed of numerous tandem repeat sequences, and it is strongly overexpressed on epithelial tumor cells.[4] Tumor-associated MUC1 is markedly different from MUC1 of normal epithelial cells with regards to the glycosylation profile.[1–3,5] The changed activities of glycosyltransferases result in short, prematurely sialylated glycans on tumor-associated MUC1, such as the Thomson–Friedenreich (T-) antigen, the T_N antigen, and their sialylated forms 2,6-sialyl-T_N, 2,6-sialyl-T, and 2,3-sialyl-T antigens.[1,5,6] As a result of the predominant short glycans, peptide epitopes,[1] which are masked in MUC1 on normal cells through the long carbohydrates, are accessible for the immune system in tumor-associated MUC1. However, vaccines designed with non-glycosylated MUC1 tandem repeat peptides or tumor-associated saccharide antigens conjugated to carrier proteins, for example, KLH (keyhole limpet hemocyanin), effected no satisfactory immune reactions.[1c,7] Yet it could be shown that fully synthetic vaccines consisting of MUC1 glycopeptide antigens combined with a T-cell epitope peptide induce highly selective immune responses. The induced antibodies selectively recognized the MUC1 glycopeptide, but neither the nonglycosylated MUC1 peptide of identical sequence nor the saccharide antigen linked to a different peptide.[8] The immunogenicity of MUC1 antitumor vaccines generated so far has been too low to break the natural tolerance against tumor-associated glycoprotein structures. Recently, we could solve this problem by developing vaccines that contain MUC1 glycopeptide antigens coupled to tetanus toxoid (TTox).[9] Extraordinarily high titers of antibodies (1/50000–1/500000) were induced in wild-type mice. These antibodies bind to MCF-7 breast tumor cells, and their binding can be neutralized by synthetic tumor-associated glycopeptide antigens from MUC1.

Binding studies with MUC1 glycopeptides bound to microchips[10] showed that the recognition profile of the antibodies induced by the aforementioned vaccines is different from that found for the biologically optimized, tumor-selective antibody SM3[11]. It also differs from those of autoantibodies isolated from serum of tumor patients.[12] These divergent recognition selectivities probably can be traced back to different glycosylation positions in the MUC1 tandem repeat sequence **1**:

Pro-Ala-His-Gly-Val-Thr^{6}-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser^{17}-Thr^{18}-Ala-Pro-Pro-Ala **1**

The MUC1 tetanus toxoid vaccines[9] described above carried the T antigen or the sialyl-T_N antigen side chains at threonine-6 of **1**, whereas the SM3 antibody[10,11] as well as the autoantibodies in the sera of patients[12] showed intensive binding to MUC1 glycopeptides glycosylated in the GSTA region (Ser^{17}, Thr^{18}). NMR spectroscopic analyses had shown that glycan side chains in the STAPPA peptide sequence of MUC1 influence the conformation of this peptide segment.[13] As the conformation is apparently decisive for the tumor selectivity of the MUC1 glycopeptides,[14] the investigation of MUC1 tetanus toxoid vaccines glycosylated at serine-17 or threonine-18 is of particular interest. To include the conformational influence of the STAPPA sequence, the peptide sequence was extended to the 22mer glycopeptides displayed in **1** incorporating both the APDTRP motif[11] and the second

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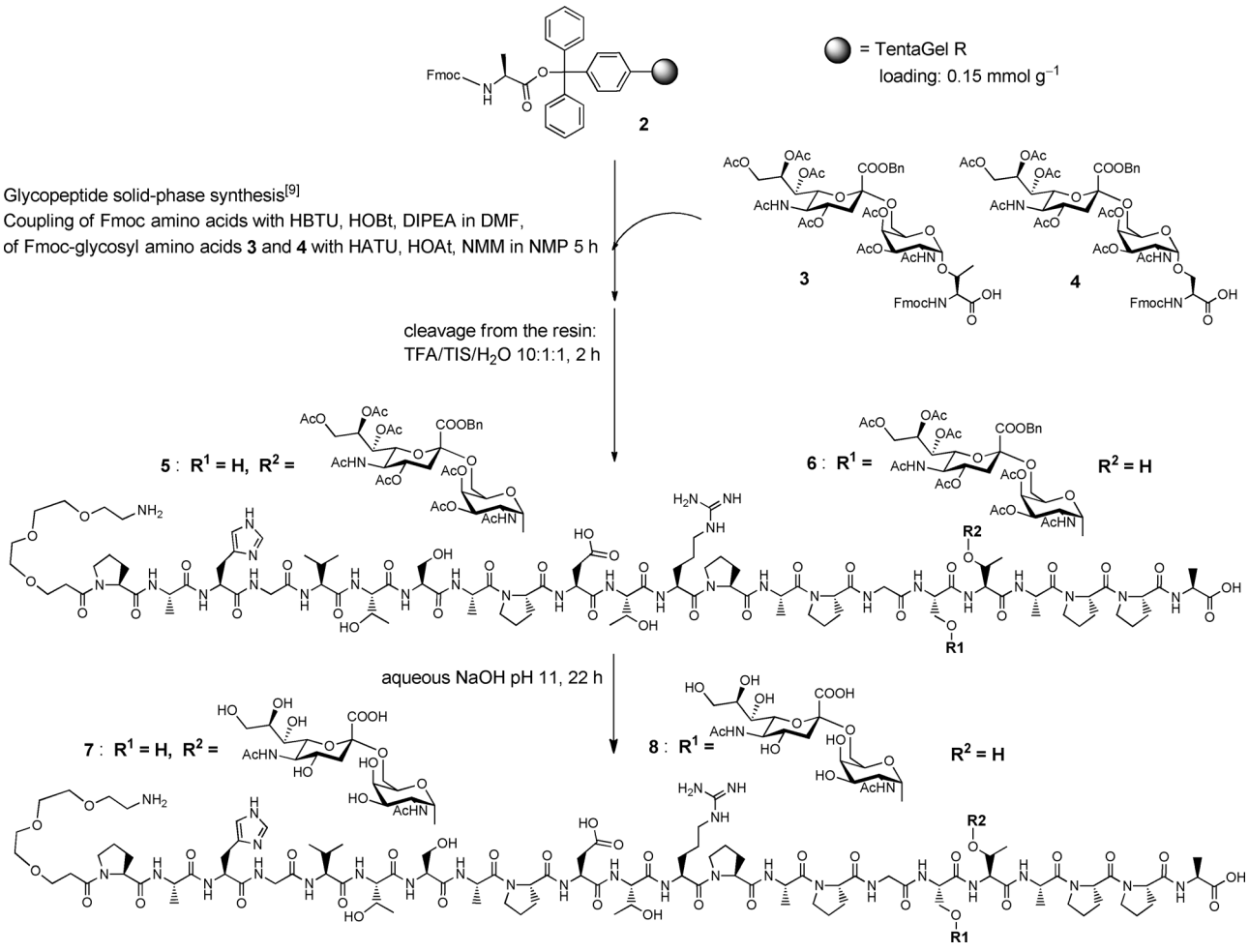
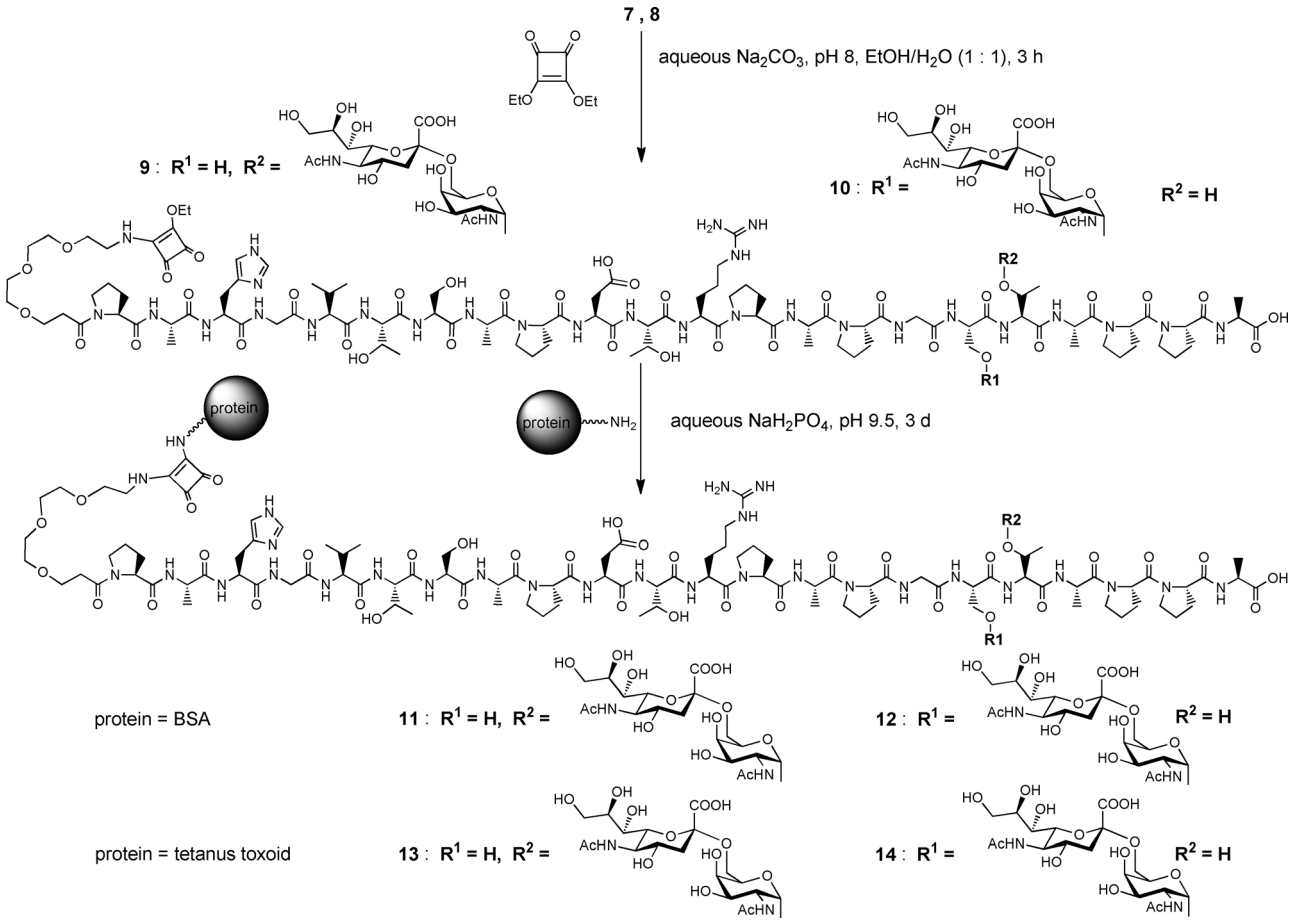
binding epitope[10,11] of the tumor-selective anti-MUC1 antibody.

The solid-phase syntheses of the MUC1 glycopeptides were carried out in a peptide synthesizer on Tentagel R-resin **2**[16] preloaded with Fmoc-alanine through a trityl linker according to a previously described method[9] (Scheme 1). The couplings of the Fmoc-modified amino acids (10 equiv) were performed with HBTU/HOBt.[17] The consecutive prolines at the C terminus and the early building in of the *O*-glycosyl amino acids result in particular demands on the coupling reactions. Fmoc-protected sialyl-T_N-threonine (**3**)[18] as well as Fmoc-sialyl-T_N-serine (**4**)[19] were activated with HATU/HOAt[20] and coupled in 5 h under vigorous shaking (Vortex). The two subsequent Fmoc-modified amino acids were coupled following the standard protocol, but the reaction had to be repeated in order to achieve adequate conversion. The remaining Fmoc amino acids and the Fmoc spacer amino acid were coupled according to the standard procedure,[9,18] whereby another drop in yield occurred during the coupling of Fmoc-alanine-8 to proline-9. Instead of acetylation after removal of the N-terminal Fmoc group, MUC1(22) glycopeptides **5** and **6** were detached from the resin using trifluoroacetic acid (TFA)/triisopropylsilane (TIS) and water. Simultaneously, all acid-sensitive protecting groups were cleaved. After purification by semipreparative HPLC, **5** was obtained in 30% and **6** in 29% overall yield. These relatively[9] moderate yields reflect the above-mentioned difficulties in these syntheses, and are presumably a consequence of the influence of the saccharide on the conformation[13,19,21] of the resin-bound peptide in the sense of back-folding. Finally, the protecting groups of the carbohydrate portions of **5** and **6** were removed through treatment with aqueous NaOH solution at pH 11, and the pure sialyl-T_N MUC1(22) peptides **7** and **8** were obtained by semipreparative HPLC.

Reaction of the glycopeptide antigens **7** and **8** with diethyl squarate[22] at pH 8 afforded the corresponding squaric acid monoamides **9**[23] and **10**[24] after purification by semipreparative HPLC (Scheme 2). These monoamides were conjugated with bovine serum albumin (BSA) in phosphate buffer solution at pH 9.5 to form the neoglycoproteins **10** and **11** used for coating the microtiter plates or with tetanus toxoid (TTox) to give the synthetic vaccines **13** and **14**. All protein



Scheme 1. Solid-phase synthesis of the sialyl-T_N MUC1 glycopeptides **7** and **8**: Fmoc = fluorenyl-9-methoxycarbonyl; HBTU = *O*-benzotriazole-1-yl *N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate; HOBt = 1-hydroxybenzotriazole; DIPEA = diisopropylethylamine (Hünig base); NMM = *N*-methylmorpholine; NMP = *N*-methylpyrrolidone; TFA = trifluoroacetic acid; TIS = triisopropylsilane.



Scheme 2. Conjugation of the synthetic sialyl-T_N MUC1 glycopeptide antigens with the carrier proteins BSA and tetanus toxoid to give vaccines; BSA = bovine serum albumin.

conjugates **11**–**14** were isolated as colorless lyophilisates after ultrafiltration (30 kDa membrane).

The MALDI-TOF mass spectra of the BSA conjugates show that in **11** on average at least four and in **12** at least three molecules of glycopeptide are bound to one molecule of protein (see the Supporting information). One should keep in mind that for glycoprotein conjugates such as **11** and **12** containing sialic acid only the lighter ones can be detected; the higher ones are not amenable to MALDI-TOF analysis. As had been shown already for other examples,[9a] the glycopeptide loading of TTox vaccines **13** and **14** can only be estimated through comparison of ELISA binding studies and corresponds to approximately 20 molecules glyopeptide per molecule protein.

Three wild-type (Balb/cJ) mice were immunized with vaccine **13** (mice 1–3) and **14** (mice 4–6) together with complete Freund's adjuvant for biological evaluation. At intervals of 21 days two booster immunizations were performed with the same vaccines. Five days after the third immunization, blood was drawn and the sera were analyzed for the binding of the induced antibodies to the BSA conjugates **11** and **12** in ELISA binding studies (Figure 1). Very strong immune responses breaking the natural tolerance (titers approximately 1/30000) were determined for all six mice.

Characterization of the isotype showed that IgG_1 antibodies were generated by **13** as well as by **14**. The binding of the antibodies induced by vaccines **13** and **14** to breast tumor cells of the cell line MCF-7[25] was determined by FACS (fluorescent-activated cell-sorter) analysis (Figure 2).[9b] MCF-7 tumor cells that had been treated only with buffer solution (Figure 2a) were counted by laser light scattering; they show no binding of the fluorescence-labeled (Alexafluor 488) goat-anti-mouse antibody and appear in the left field. In contrast, all MCF-7 cells incubated with the serum of mouse 2 (Figure 2b), which had been immunized with vaccine **13**, show fluorescence (right field) and thus that they are recognized by the induced antibodies. The serum of a mouse immunized with pure tetanus toxoid (Figure 2c) barely contains antibodies that bind to MCF-7 cells. In contrast, antibodies induced by the MUC1(22) tetanus toxoid vaccine **14** in the serum of mouse 5 (Figure 2d) recognized the membrane glycoproteins on the MCF-7 tumor cells. This binding is selective, because it was neutralized through incubation of the antibodies with sialyl-T_N MUC1 glycopeptide **8** (25 $\mu g\,mL^{-1}$; Figure 2e). Figure 2f shows that the SM3 antibody, which was optimized by cloning, binds clearly less selectively to MCF-7 breast tumor cells than the antibodies induced by the synthetic vaccines **13** (Figure 2b) and **14** (Figure 2d). In addition, the binding of the antibodies elicited

by vaccine **14** in mouse 5 to human breast tumor cells of the line T-47D[26] was investigated. Whereas T-47D cells treated with buffer showed no fluorescence labeling in FACS analysis after addition of Alexafluor 488-goat-anti-mouse antibodies (Figure 2g), all of the cells incubated with the serum of mouse 5 were fluorescence-labeled in FACS analysis after addition of the secondary antibody (Figure 2h). This binding was completely neutralized through incubation of the antibodies with glycopeptide **8** (6 μg mL^{-1}; Figure 2i), which proves the structure selectivity of the immune reaction initiated by **14**.

This selective binding can be observed not only with cultured tumor cells but also in native tumor tissues. Figure 3 shows three examples of mammary carcinoma tissue sections, which were fixed with formalin and embedded in paraffin, in a light microscope (magnification 1/100). An isotype-control antibody (IgG_1) was used as negative control (Figure 3a–c). In contrast, the tissue sections shown in Figure 3d–f were incubated with the serum of mouse 5, which had been immunized with vaccine **14**. Bound tumor-selective antibodies were detected with a biotinylated goat-anti-mouse/anti-rabbit antibody, whose adhesion was displayed with a streptavidin horseradish peroxidase conjugate, which cata-

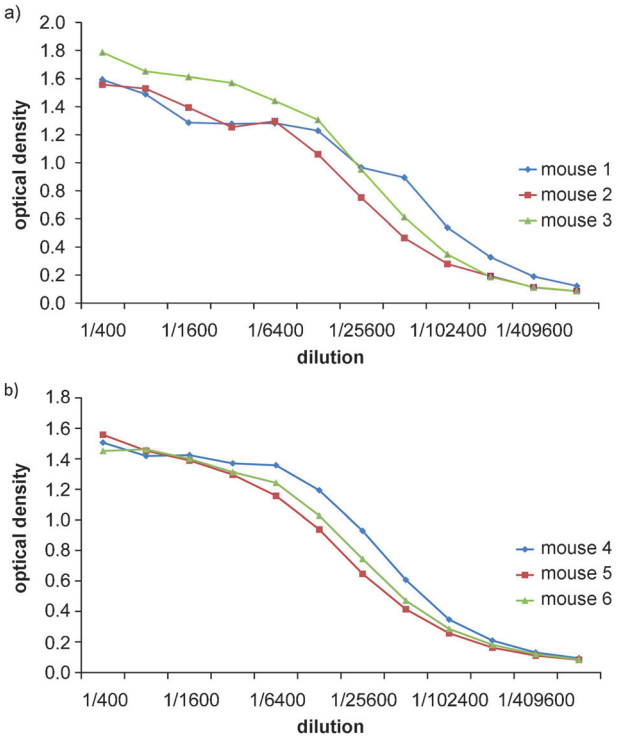


Figure 1. ELISA binding studies of the antisera induced by vaccine **13** (a) and **14** (b); binding to BSA conjugates **11** (a) and **12** (b).

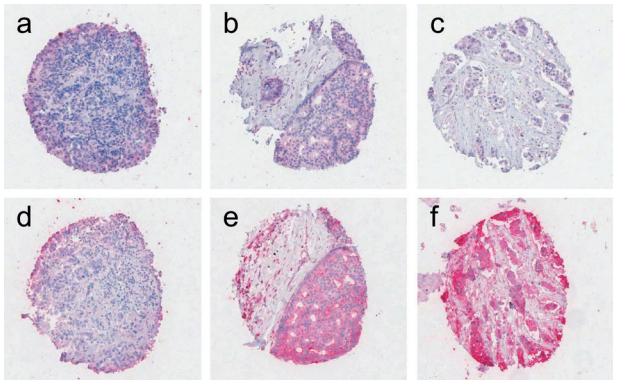


Figure 3. Sections of breast tumor tissues of three patients: a–c) tissues were fixed with formalin and embedded in paraffin in a light microscope (1:100); d–f) tissues were incubated with the serum from mouse 5, which had been immunized with the synthetic vaccine **14**. The detection was carried out with a biotinylated goat-anti-mouse/anti-rabbit antibody (ChemMate Detection Kit, Dakocytomation, Glostrup, Denmark), horse radish peroxidase bound to streptavidin, which catalyzes oxidation of 3-amino-9-ethylcarbazole.

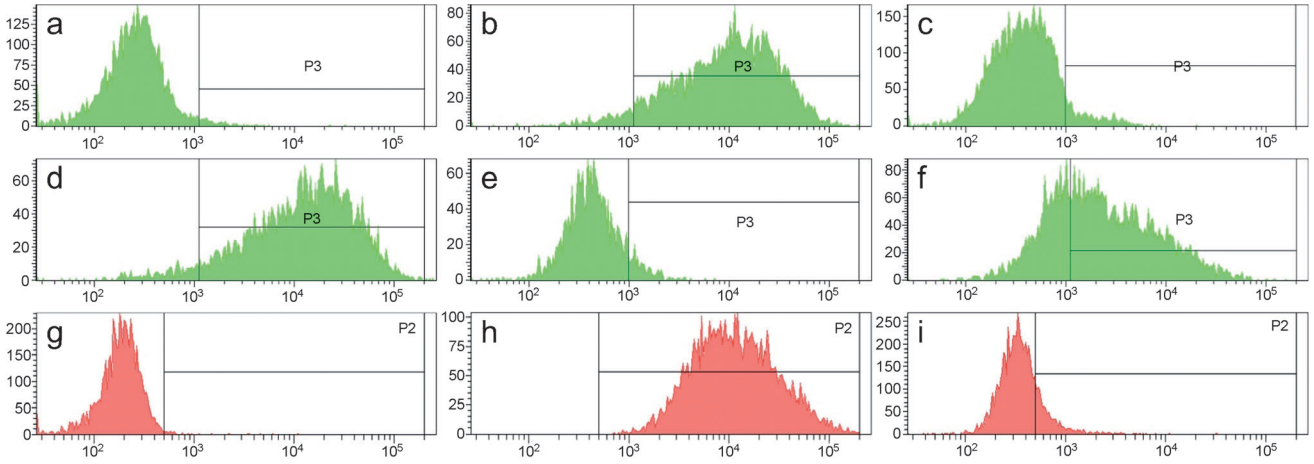


Figure 2. FACS analysis of the binding of the antisera induced by vaccines **13** and **14**: a) MCF-7 tumor cells treated with PBS buffer; b) MCF-7 cells treated with antiserum (1:1000) of mouse 2, which had been immunized with **13**; c) MCF-7 cells treated with serum of a control mouse, which had been immunized only with tetanus toxoid; d) MCF-7 cells treated with antiserum (1:1000) of mouse 5, which had been immunized with **14**; e) the binding shown in (d) is neutralized by the addition of MUC1 glycopeptide **8** (25 μg mL^{-1}); f) binding of the SM3 antibody to MCF-7 tumor cells; g) T-47D breast tumor cells treated with PBS buffer; h) T-47D cells treated with antiserum (1:1000) of mouse 5, which had been immunized with **14**; i) the binding shown in (h) is neutralized by the addition of MUC1 glycopeptide **8** (6 μg mL^{-1}).

lyzes the oxidation of 3-amino-9-ethyl-carbazole to a rose-colored dye. Figure 3a shows a barely dedifferentiated tumor tissue (early phase, grade G1, less than 1% reacting tumor cells), Figure 3b a tissue, in which between 1 and 9% of the cells react (grade G2), and Figure 3c an advanced tumor tissue containing 10–50% reacting cells.

After incubation with serum from mouse 5, which had been immunized with the synthetic vaccine **14**, the rose coloring indicates that the IgG_1 antibodies induced by **14** bind only weakly to the tumor tissue in the early phase (Figure 3d, G1), but bind considerably to the tumor in G2-phase (Figure 3e). The connective tissue seen in the picture is not marked. The tumor tissue in the advanced G3 phase (Figure 3f) is very strongly marked by the antibodies, which were induced in mice by the synthetic vaccine **14**.

These results give evidence for the first time of the diagnostic value of the antibodies induced by the synthetic MUC1 glycopeptide TTox vaccines. Moreover, if one keeps in mind that according to the immunological mechanisms, tumor cells recognized by the IgG antibodies should be catabolized through the immune system, then the results shown in Figures 2 and 3 suggest that an active immunization of patients against their own tumor tissues should be feasible with synthetic MUC1-glycopeptide vaccines such as **13** and **14**.

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[1] a) J. Arklie, J. Taylor-Papadimiriou, W. Bodmer, M. Egan, R. Millis, *Int. J. Cancer* **1981**, *28*, 23; b) S. Gendler, J. Taylor-Papadimitriou, T. Duhig, J. Rothbard, J. Burchell, *J. Biol. Chem.* **1988**, *263*, 12820; c) recent review: R. E. Beatson, J. Taylor-Papadimitriou, J. M. Burchell, *Immunotherapy* **2010**, *2*, 305, and references therein.

[2] a) F.-G. Hanisch, J. Peter-Katalinic, H. Egge, U. Dabrowski, G. Uhlenbruck, *Glycoconjugate J.* **1990**, *7*, 525; b) F.-G. Hanisch, T. Stadie, K. Boßlet, *Cancer Res.* **1995**, *55*, 4036; c) 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. Schwientek, R. Graham, J. Taylor-Papadimitriou, M. A. Hollingsworth, J. Burchell, H. Clausen, *Glycobiology* **2006**, *16*, 96.

[4] a) K. O. Lloyd, J. Burchell, V. Kudryashov, B. W. Tin, J. Taylor-Papadimitriou, *J. Biol. Chem.* **1996**, *271*, 33325; b) R. T. Greenlee, M. B. Hill-Harmon, T. Murray, M. Thun, *Ca-Cancer J. Clin.* **2001**, *51*, 15.

[5] a) S. Müller, F.-G. Hanisch, *J. Biol. Chem.* **2002**, *277*, 26103; b) S. Wilkie, G. Picco, J. Foster, D. M. Davies, S. Julien, L. Cooper, S. Arif, S. J. Mather, J. Taylor-Papadimitriou, J. M. Burchell, J. Maher, *J. Immunol.* **2008**, *180*, 4902.

[6] G. F. Springer, *Science* **1984**, *224*, 1198.

[7] S. F. Slovin, G. Ragupathi, C. Musselli, K. Olkiewicz, D. Verbel, S. D. Kuduk, J. B. Schwarz, D. Sames, S. Danishefsky, P. O. Livingston, H. I. Scher, *J. Clin. Oncol.* **2003**, *21*, 4292.

[8] a) S. Dziadek, A. Hobel, E. Schmitt, H. Kunz, *Angew. Chem.* **2005**, *117*, 7803; *Angew. Chem. Int. Ed.* **2005**, *44*, 7630; b) U. Westerlind, A. Hobel, N. Gaidzik, E. Schmitt, H. Kunz, *Angew. Chem.* **2008**, *120*, 7662; *Angew. Chem. Int. Ed.* **2008**, *47*, 7551.

[9] a) 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; b) A. Hoffmann-Röder, A. Kaiser, S. Wagner, N. Gaidzik, D. Kowalczyk, U. Westerlind, B. Gerlitzki, E. Schmitt, H. Kunz, *Angew. Chem.* **2010**, *122*, 8676; *Angew. Chem. Int. Ed.* **2010**, *49*, 8498.

[10] U. Westerlind, H. Schröder, A. Hobel, N. Gaidzik, A. Kaiser, C. M. Niemeyer, E. Schmitt, H. Waldmann, H. Kunz, *Angew. Chem.* **2009**, *121*, 8413; *Angew. Chem. Int. Ed.* **2009**, *48*, 8263.

[11] a) J. Burchell, J. Taylor-Papadimitriou, M. Boshell, S. Gendler, T. Duhig, *Int. J. Cancer* **1989**, *44*, 691; b) M. A. Tarp, A. L. Sørensen, U. Mandel, H. Paulsen, J. Burchell, J. Taylor-Papadimitriou, H. Clausen, *Glycobiology* **2007**, *17*, 209.

[12] O. Blixt, E. Cló, A. S. Nudelman, K. Kildegaard Sørensen, T. Clausen, H. H. Wandall, P. O. Livingston, H. Clausen, K. J. Jensen, *J. Proteome Res.* **2010**, *9*, 5250.

[13] a) P. Braun, G. M. Davies, M. R. Price, P. M. Williams, S. J. Tendler, H. Kunz, *Bioorg. Med. Chem.* **1998**, *6*, 1531; b) F. Corzana, J. Busto, M. Garcia de Luis, J. Jimenez-Barbero, A. Avenoza, J. M. Peregrina, *Chem. Eur. J.* **2009**, *15*, 3863; c) F. Corzana, J. Busto, F. Marcelo, M. Garcia de Luis, J. C. Asenso, S. Martin Santamaria, J. Jimenez-Barbero, A. Avenoza, J. M. Peregrina, *Chem. Eur. J.* **2011**, *17*, 3105.

[14] a) M. J. Scanlon, S. D. Morley, D. E. Jackson, M. R. Price, S. J. B. Tendler, *Biochem. J.* **1992**, *284*, 137; b) J. D. Fontenot, N. Tjandra, B. Dawen, H. Chien, R. C. Montelaro, O. J. Finn, *Cancer Res.* **1993**, *53*, 5386; c) J. A. Mollick, F. S. Hodi, R. J. Soiffer, L. M. Nadler, G. Dranoff, *Cancer Immunity* **2003**, *3*, 3.

[15] J. M. J. Fréchet, K. E. Haque, *Tetrahedron Lett.* **1975**, *16*, 3055.

[16] Rapp Polymere, Tübingen, Deutschland.

[17] V. Dourtoglou, B. Gross, V. Lambropoulou, C. Zioudrou, *Synthesis* **1984**, 572.

[18] a) S. Keil, C. Claus, W. Dippold, H. Kunz, *Angew. Chem.* **2001**, *113*, 379; *Angew. Chem. Int. Ed.* **2001**, *40*, 366; b) C. Brocke, H. Kunz, *Synthesis* **2004**, 525.

[19] A. Kuhn, H. Kunz, *Angew. Chem.* **2007**, *119*, 458; *Angew. Chem. Int. Ed.* **2007**, *46*, 454.

[20] L. A. Carpino, *J. Am. Chem. Soc.* **1993**, *115*, 4397.

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

[22] L. F. Tietze, M. Arlt, M. Beller, K.-H. Glüsenkamp, E. Jähde, M. F. Rajewsky, *Chem. Ber.* **1991**, *124*, 1215.

[23] **9**: 5.5 mg (48%); $[\alpha]_D^{23} = -131.0$ ($c = 0.36$; H_2O). $R_t = 25.7$ min (Phenomenex Jupiter C18, acetonitrile/water with 0.1% TFA, grad.: (5:95)→(25:75), 30 min, $\lambda = 214$ nm). *MALDI-TOF-MS* (*dhb, positive*), *m/z:* 2877.3 ($[M+H]^+$, calc.: 2877.3), 2585.6 ($[M+H-NeuNAcCOOH]^+$, calc.: 2585.2).

[24] **10**: 14.5 mg, (72%); $[\alpha]_D^{23} = -144.4$ ($c = 0.72$, H_2O), $R_t = 26.5$ min (Phenomenex Jupiter C18, acetonitrile/water with 0.1% TFA, grad.: (10:90)→(25:75), 30 min, $\lambda = 214$ nm). ESI-MS (positive ion), *m/z*: 870.13 ($[M+3H+Na-NeuNAcCOOH]^{3+}$, calc.: 870.08). *HR-MS*, *m/z:* 1440.6854 ($[M+2H]^{2+}$, calc.: 1440.6769), 1440.1788 ($[M+2H]^{2+}$, calc.: 1440.1752), 1439.6710 ($[M+2H]^{2+}$, calc.: 1439.6736), 1439.1685 ($[M+2H]^{2+}$, calc.: 1439.1719), 1438.6707 ($[M+2H]^{2+}$, calc.: 1438.6702). MALDI-TOF-MS (dhb, positive ion), *m/z:* 2878.1 ($[M+2H]^+$, calc.: 2877.3), 2586.3 ($[M+2H-NeuNAcCOOH]^+$, calc.: 2586.2).

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

[26] I. Brockhausen, J.-M. Yang, J. Burchell, C. Whitehouse, J. Taylor-Papadimitriou, *Eur. J. Biochem.* **1995**, *233*, 607.

