Antitumor Vaccines

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Self-Adjuvanting Synthetic Antitumor Vaccines from MUC1 Glycopeptides Conjugated to T-Cell Epitopes from Tetanus Toxoid**

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Dedicated to Professor Gunter Fischer on the occasion of his 70th birthday

Tumor-associated MUC1 is considered a most promising target for the development of a cancer immunotherapy. However, this endogenous glycoprotein expressed on many epithelial tumors exhibits low immunogenicity.[1] In order to elicit sufficiently strong immune responses, MUC1 glycopeptide antigens have been conjugated with T-cell epitope peptides,[2] Toll-like receptor 2 lipopeptide ligands,[3] or with both of these immunostimulating components.[4] Remarkable progress has been achieved using these synthetic vaccines for the induction of immune responses selectively directed against tumor-associated MUC1 antigen structures,[2c,e] also when the MUC antigen is expressed on tumor cells in culture,[2d,4] and protective against tumor growth in transgenic mice.[4c] In many cases, the induced immune reaction remained moderate (endpoint titers $< 30\,000$) and the recognition of tumor cells was incomplete. Since strong immune responses overriding the natural tolerance of the immune system are considered essential for a therapeutic vaccine, we focused on glycopeptide vaccines derived from the MUC1 tandem repeat sequence HGVTSAPDTRPAPGSTAPPA covalently conjugated to bovine serum albumin (BSA) or tetanus toxoid (TTox).[5] Administering these vaccines with Freund's adjuvant to Balb/c mice induced strong immune responses against tumor-associated MUC1 expressed on epithelial tumor cells. Mainly IgG antibodies were elicited. Particularly high titers of MUC1-binding IgG antibodies were induced by TTox-conjugated vaccines (endpoint titers 1 000 000).[5e] The antibodies showed almost complete recognition of breast tumor cells of cell lines MCF-7 and T-47D, and also binding to tumor cells in mammary carcinoma tissues.[5f] In addition, tetanus toxoid conjugated vaccines have been proven safe in human vaccination. However, the protein contains peptide epitopes, which are unnecessary for an immune activation, but may exhibit interferences during vaccination.[6] Moreover, the effect of Freund's adjuvant in humans still is unclear. Therefore, we are interested in simplifying the vaccine structure but retaining the essential stimulating effects of both the tetanus toxoid and the adjuvant required for eliciting a strong, selective immune response.

According to this concept, the MUC1 tandem repeat glycopeptide sequence HGVTSAPDTRPAPGSTAPPA was coupled to three known universal T-helper cell epitope peptides **P4** ($TT_{1273–1284}$: GQIGNDPNRDIL), **P2** ($TT_{830–843}$: QYIKANSKFIGITE), and **P30** ($TT_{947–967}$: FNNFTVSFWLRVPKVSASHLE) derived from the tetanus toxoid.[7] Basic immune mechanisms suggest that these T-helper cell peptides can replace the parent tetanus toxoid to stimulate the murine and human immune system. During the immune reaction, the MUC1 glycopeptide is recognized by the receptor of a B cell or another antigen-presenting cell (APC). The whole vaccine subsequently internalized by APC is subjected to proteolytic processing. Thus, the T-helper peptide within the major histocompatibility complex II (MHC II) can be presented by these cells to receptors of $CD4^+$ T-helper cells. Assisted by other cell-to-cell interactions and the release of cytokines, the activated $CD4^+$ T cells stimulate the B cell to differentiate into a plasma cell, and to proliferate and secrete antibodies against the MUC1 glycopeptide antigen.[8]

Within the MUC1 tandem repeat, the PDTRP and GSTAP regions are immunodominant motifs.[9,10] It was found that antibodies recognizing MCF-7 tumor cells as well as autoantibodies from sera of patients showed binding to these peptide segments.[1,10] As the conformation of glycopeptides is apparently influenced by the O-glycosylation,[11] the immunogenicity of MUC1 glycopeptides carrying Tn and T antigen (Scheme 1) at T9 in the PDTRP and at S15 in the GSTAP region are of particular interest. These glycopeptides were synthesized on solid phase (SPPS)[12] with Tn/Tn (**Peptide-1**) or T/Tn (**Peptide-2**) at sites of T9/S15. After assembly of the MUC1 glycopeptide, a triethylene glycol derived spacer amino acid was coupled at the N-terminus. Subsequently, the **P4**, **P2**, or **P30** T-cell peptide sequences were added by stepwise SPPS.[4e] The (glyco)peptides were detached from resin using trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O with concomitant removal of all acid-sensitive side-chain protecting groups.

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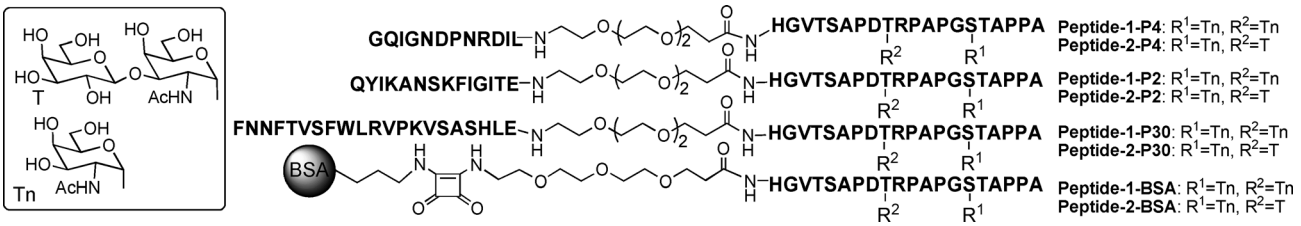


Scheme 1. Structures of MUC1 glycopeptides (**Peptide-1** and **Peptide-2**) conjugated with T-helper epitope peptides (**P4**, **P2** and **P30**) and BSA.

After HPLC purification, the *O*-acetyl groups were removed from carbohydrates using MeONa/MeOH at pH 9 without affecting the structures. The target glycopeptide conjugates **Peptide-1** and **Peptide-2** linked to **P4**, **P2**, and **P30**, respectively, were isolated by RP-HPLC (Scheme 1). In addition, BSA conjugates **Peptide-1-BSA** and **Peptide-2-BSA** were prepared according to the described procedures.[5b]

Each fully synthetic vaccine was intraperitoneally administered to four wild-type Balb/c mice in five immunizations at intervals of 14 days. The first immunizations were performed with complete (CFA), the others with incomplete Freund's adjuvant (IFA). One week after the fifth immunization, the mixed sera from each group of mice were analyzed by enzyme-linked immunosorbent assay (ELISA) for antibody detection. ELISA tests were performed by coating microtiter plates with BSA-conjugated antigens.[5c] **P4** and **P2** epitope-MUC1 vaccines induced weak immune responses, while **Peptide-1**- and **Peptide-2-P30** vaccines elicited much stronger immune reactions (Figure 1). Logically, further investigations focused on vaccines containing the **P30** epitope peptide.

In order to study the immunogenicity of different glycoforms of MUC1 glycopeptides, the three glycosylation sites (T9, S15, and T16) within the immunodominant motifs were

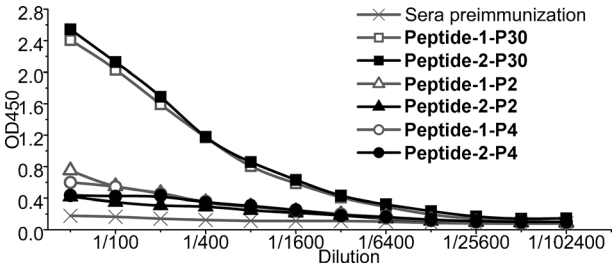
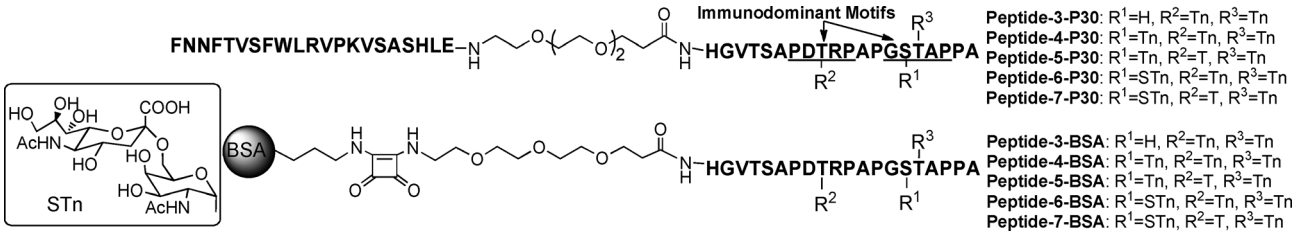


Figure 1. ELISA of the mixed antisera induced by the MUC1 glycopeptides conjugated to **P4**, **P2**, and **P30** T-helper epitope peptides. Rabbit anti-mouse antibody conjugated to horseradish peroxidase was used as the second antibody. Optical density was measured at $\lambda = 450$ nm using *O*-phenylenediamine (OPD) and H_2O_2 as substrates.

glycosylated with Tn, T, and STn antigens. Glycopeptides **Peptide-3**, **-4**, **-5**, **-6**, and **-7** were prepared by SPPS and extended with the spacer and the **P30** epitope sequence to give glycopeptide-**P30** conjugates **Peptide-3-P30**, **Peptide-4-P30**, **Peptide-5-P30**, **Peptide-6-P30**, and **Peptide-7-P30** after release from resin and purification by preparative HPLC (Scheme 2). Benzyl esters of sialic acid in **Peptide-6-P30** and **Peptide-7-P30** were reduced prior to removal of the *O*-acetyl groups using MeONa/MeOH. **Peptides-3**, **-4**, **-5**, **-6**, and **-7** were also coupled with BSA for comparison of the immune reactions induced by **P30**- and BSA-conjugated vaccines. **Peptide-3-**, **-4-**, **-5-**, **-6-**, and **-7-P30** and **Peptide–3-**, **-4-**, **-5-**, **-6-**, **7-BSA** vaccines were applied together with CFA or IFA as described above. For BSA conjugates, only three immunizations were performed. To study the influence of Freund's adjuvant, glycopeptide-**P30** vaccines were also administered without adjuvant by direct injection after dissolving in phosphate-buffered saline (PBS). The immune responses were evaluated by ELISA. Surprisingly, all **P30** epitope peptide-conjugated glycopeptide vaccines induced much stronger immune responses when administered in PBS than when they were injected with CFA (Figure 2a–e). The immune responses were also stronger than those induced by similar three-component vaccines containing a lipopeptide and a **P2** or **P4** peptide.[4e] These results disclose two important effects: Freund's adjuvant can suppress the immunogenicity of glycopeptide vaccines, and the tetanus toxoid **P30** peptide acts as both a T-helper cell epitope and an adjuvant, apparently due to its amphiphilic nature. The titers of the antisera induced by **Peptide-4-P30** and **Peptide-5-P30** were much higher than that of **Peptide-3-P30** (Figure 2a–c) indicating that the second glycosylation within the GSTAP region can enhance the immunogenicity. Compared with antisera elicited by the BSA-conjugated vaccines, the titers induced by the **P30** T-helper epitope conjugates were lower except for sera induced with **Peptide-4-P30** (Figure 2b). It is considered particularly remarkable that immunization with the fully synthetic vaccine **Peptide-4-P30** administered with-



Scheme 2. Vaccines from MUC1 glycopeptides (**Peptide-3**, **-4**, **-5**, **-6**, **-7**) conjugated with **P30** T-helper epitope peptides and with BSA.

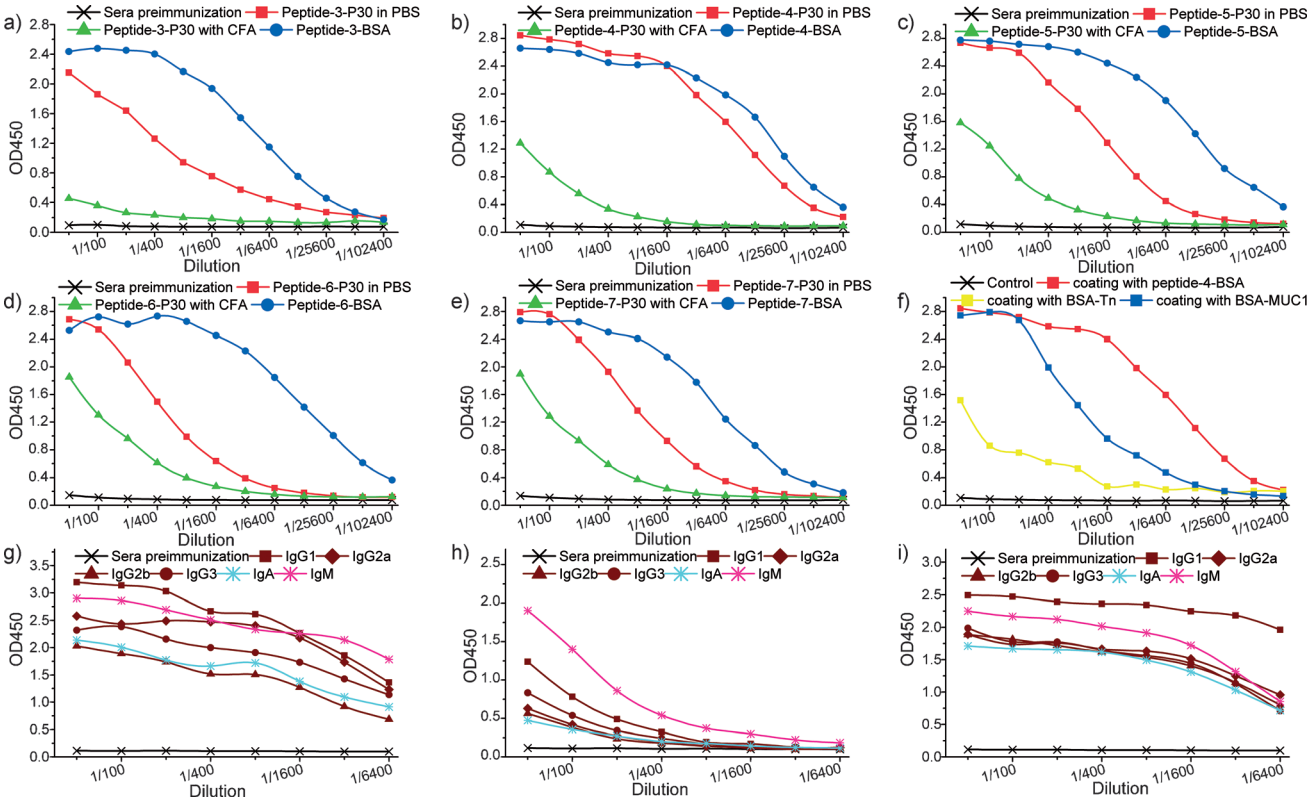


Figure 2. a–e) ELISA of the antisera induced by vaccines **Peptide-3**, **-4**, **-5**, **-6**, **-7** linked to **P30** T-cell epitope or BSA. The microtiter plates for the analyses of the sera induced by BSA-conjugated vaccines were coated with the corresponding nonconjugated glycopeptides, and with the BSA-conjugated glycopeptides for the other ELISA analyses. f) Binding of antibodies induced by vaccine **Peptide-4-P30** to different antigens coated on the microtiter plates. BSA-Tn is the conjugate of Tn-Thr to BSA; BSA-MUC1 is the conjugate of the unglycosylated MUC1 tandem repeat peptide with BSA. g–i) Antibody isotype analysis of the antisera induced by **Peptide-4-P30** (g, h) and **Peptide-4-BSA** (i); g: administered in PBS, h: administered with CFA.

out support by any covalently linked lipopeptide immunostimulant or external adjuvant almost reaches the level of immunogenicity as induced by the analogous glycopeptide conjugate to an immunogenic protein. The affinity of antibodies induced by **Peptide-4-P30** to different antigens was also evaluated by ELISA (Figure 2 f). Compared to the binding to **Peptide-4-BSA** (red), the affinity to BSA-MUC1 containing the unglycosylated MUC1 peptide (blue) is distinctly reduced. The antibodies show low binding to Tn-Thr conjugated to BSA (yellow) although there are three copies of Tn antigen in **Peptide-4** vaccine. These results demonstrate that both the peptide and the carbohydrate contribute to the efficient epitope of MUC1.

The antibody isotypes of the antisera induced by vaccine **Peptide-4-P30** were also characterized by ELISA using goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA isotyping reagents as the secondary antibodies and horseradish peroxidase conjugated rabbit anti-goat IgG as the third antibody. As shown in Figure 2 g, when vaccine **Peptide-4-P30** was administered in PBS, prevailing IgG_1 and IgM are induced, along with considerable amounts of IgG_3 typical for an anti-carbohydrate response and $IgG_{2a/b}$ antibodies indicating cell-mediated (MHC1) immune response.

Immunization with **Peptide-4-P30** together with CFA resulted in a much lower overall immune response and a prevalence of less specific IgM antibodies (Figure 2 h). Immunization with vaccine **Peptide-4-BSA** (Figure 2 i) resulted in an antibody isotype pattern quite similar to that induced by **Peptide-4-P30.** The antisera induced by vaccines **Peptide-3-**, **-5-**, **-6-**, and **-7-P30** administered in PBS showed similar isotype spectra with prevailing IgG_1/IgG_3 subtype indicating a bias towards the Th2 immune reaction.[13]

Similar to the different immunogenicity and antibody subtype pattern, the binding of the induced antibodies to MCF-7 breast tumor cells was also dependent on the mode of application of the MUC1 glycopeptide-**P30** vaccines. Flow cytometry analyses (FACS) with fluorescein-isothiocyanate (FITC)-labeled rabbit–antimouse IgG brought to light that antisera induced by **Peptide-3-**, **-4-**, **-5-**, **-6-**, and **-7-P30** without CFA exhibited stronger binding to MCF-7 cells than those elicited by the same vaccines using CFA as the adjuvant (see the Supporting Information). This difference is particularly significant for **Peptide-6-P30**.

In general, the sera induced by BSA-conjugated vaccines showed stronger binding to MCF-7 cells. The difference in this binding seems to be larger when the MUC1 glycopeptide antigen is more hydrophilic. Although these effects are not yet understood, they may be traced back to the varying aggregation and conformation of the glycopeptide antigens in the different preparations. The induced antibodies not only

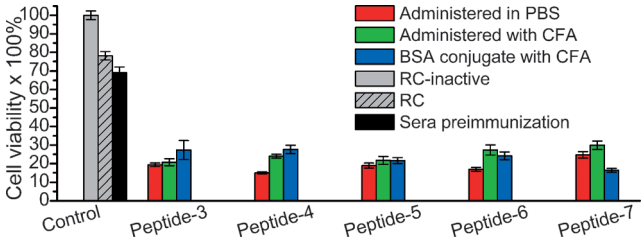


Figure 3. Complement-dependent cytotoxicity killing of MCF-7 tumor cells. Peptide-3–7 corresponds to the vaccines with **P30** (red and green bars) or BSA (blue) attached. RC-inactive: the cell viability of inactivated rabbit complement (RC) was taken as 100% cell viability; cell viability was measured after incubation for 2 h. Data are mean of five experiments.

bound to tumor cells, but also initiated killing of the recognized MCF-7 tumor cells by activation of complement-dependent cytotoxicity (CDC). The tetrazolium bromide (MTT) assays of the survival rate showed (Figure 3) that the cytotoxicity of induced antisera using rabbit complement (RC) as the source of complement is stronger when the **P-30**-conjugated vaccines were administered in PBS solution than those induced with CFA.

In conclusion, novel and efficient two-component antitumor vaccines were obtained by conjugation of MUC1 tandem repeat glycopeptides with the **P30** T-helper cell peptide derived from tetanus toxoid. These vaccines surprisingly induced much stronger immune responses when not administered with Freund's adjuvant, but in plain buffer solution. The **P30** T-cell epitope obviously is of superior efficiency compared to the **P2** and **P4** epitopes because it comprises three epitopes. Each epitope can be recognized in association with a different MHC II molecule.[11] In addition, this **P30** T-cell peptide concomitantly exhibits the functions of a built-in adjuvant, rendering the application of an external adjuvant as well as a covalently linked lipopeptide immunostimulant superfluous. It should be kept in mind, that lipopeptide conjugates are difficult to synthesize and to purify, and that Freund's adjuvant is not recommended for application to humans. Moreover, the results for the first time show that Freund's adjuvant may reduce the immunogenicity of a synthetic glycopeptide vaccine as well as the ability of the induced antibodies to recognize tumor cells. The results also give evidence that the efficiency of the fully synthetic vaccines depends on the glycosylation pattern. Three glycan side chains in the PDTRP and GSTAP regions enhanced the immunogenicity of the vaccines. Three Tn saccharide antigens linked to the glycosylation sites led to a vaccine which, without support by an adjuvant, elicited the strongest immune response (endpoint titer 100000) and antibodies binding to tumor cells. Thus, it is considered a most promising lead for the development of fully synthetic antitumor vaccines.

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