SialylTn-mAb17-1A Carbohydrate–Protein Conjugate Vaccine: Effect of Coupling Density and Presentation of SialylTn

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Carbohydrate antigens resulting from aberrant glycosylation of tumor cells, such as SialylTn, represent attractive targets for cancer vaccination. However, T-cell-independent carbohydrate antigens are poorly immunogenic and fail to induce memory and IgG class switch. Clustered expression patterns of some carbohydrates on the cell surface add further complexity to the design of carbohydrate-based vaccines. We describe here a vaccine consisting of SialylTn carbohydrate epitopes coupled to a highly immunogenic carrier molecule, mAb17-1A, adsorbed on alhydrogel and coformulated with a strong adjuvant, QS-21. The SialylTn–mAb17-1A conjugate vaccine was administered in Rhesus monkeys, and the immune responses against mAb17-1A, SialylTn, ovine submaxillary mucin, and tumor cells were analyzed. The data demonstrate that the density of carbohydrate epitopes on the carrier is an essential parameter for induction of anti-carbohydrate specific memory IgG immune responses. Furthermore, the influence of different types of presentation of SialylTn (monomeric vs trimers vs clustered via a branched polyethylenimine linker) on antibody titers and specificity was studied. High-density coupling of SialylTn epitopes to mAb17-1A induced the strongest immune response against synthetic SialylTn and showed also the highest reactivity against natural targets, such as OSM and tumor cells.

INTRODUCTION

Accumulating data indicate that tumor-recognizing antibodies and immune effector cells may play a deciding role for the long-term benefit of cancer therapy. Elimination of circulating tumor cells and eradication of micrometastases which remain after surgery or radiotherapy are considered primary targets for immune cancer therapy. Therefore, identification and selection of the appropriate target antigen(s) on the tumor cells is essential for therapy efficacy. Cell surface-exposed carbohydrate and mucin antigens resulting from aberrant glycosylation of tumor cells may provide attractive targets. In animal models (1–3) as well as cancer patients the presence of natural or vaccine-induced antibodies against carbohydrates such as GM2 and SialylTn was found to correlate with prolonged survival (4–6). The mucin-derived SialylTn is expressed in more than 80% of cancers of breast, colorectal, prostate, and ovarian origin showing no or very limited expression on the corresponding normal tissues (7–9). SialylTn expression by various epithelial cancers correlates with a more aggressive phenotype and poor prognosis (10, 11). Recent data indicate that mucin-derived O-glycosylated truncated carbohydrates, such as the monosaccharide Tn or the disaccharides TF or SialylTn, are predominantly expressed as molecular clusters on the surface of tumor cells (9, 12–17). The exact pattern of these clusters is still under debate; clustering in trimers has been suggested as one favorable pattern for antibody recognition found on ovine submaxillary mucin (OSM) and on the surface of some tumor cells (14–17). However, it is rather likely that in different tumors slightly different predominant expression patterns exist, and coexpression of monomeric and clustered forms (13–15) as well as heteroclusters consisting of different types of carbohydrates are likely (18).

Our strategy consists of coupling tumor-associated carbohydrate epitopes to a highly immunogenic murine antibody with intrinsic antitumor activity (19) in order to (i) use an immunogenic carrier protein to increase the immunogenicity of the carbohydrate antigen and (ii) benefit from the antitumor immune response induced by the carrier molecule itself (20, 21). IGN402 is a first candidate of this type of conjugate vaccine consisting of SialylTn carbohydrate epitopes chemically coupled to mAb17-1A. The murine 17-1A antibody, a monoclonal antibody recognizing EpCAM (19–21), adsorbed on aluminum hydroxide has been used as vaccine antigen in cancer patients IGN101 and has recently been reported to prolong survival in metastatic colorectal cancer patients (22). Recently we have shown that the SialylTn carbohydrate conjugate vaccine IGN402, coformulated with QS-21 adjuvant, can induce an IgG response against the SialylTn moiety (23).

In the present study we investigated the effect of the SialylTn coupling density on the immune response. Furthermore, different types of presentation of SialylTn (monomeric vs trimers vs clustered via a branched polyethyleneimine linker) were studied. High-density coupling of SialylTn epitopes to mAb17-1A induced the strongest immune response against synthetic SialylTn as well as against natural targets such as OSM and tumor cells. IGN402 may provide an attractive prototype for further development toward multi-epitope vaccines with multiple carbohydrate epitopes coupled to an immunogenic murine antibody.

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**EXPERIMENTAL PROCEDURES**

**Coupling of SialylTn Carbohydrate to mAb17-1A at Different Ratios.** The SialylTn carbohydrate antigen was coupled to the murine monoclonal antibody 17-1A (mlgG2a) at different molar ratios (i.e. 4.5:1; 18:1; and 36:1) to produce SialylTn–mAb17-1A carbohydrate-protein conjugates vaccines with low, medium, and high SialylTn density, respectively. Coupling was performed by reacting 4.5, 18, or 36 mol of nitrophosphated spacer SialylTn, Neu5Ac2-6GalNAca-O(CH2)6NHCOCOCH2-\(\text{NO}_2\text{C}_6\text{H}_4\)) (mw 819 g/mol, Lectinity, Finland), to 1 mol of mAb17-1A. Briefly, 70 mg of mAb17-1A was dialyzed twice against coupling buffer (0.1 M NaPO4, 0.15 M NaCl, pH 8.5) using a Slide-A-Lyzer dialysis cassette MWCO 10K (Pierce). Concentration of mAb17-1A was determined by size exclusion chromatography (SEC) on a Zorbax GF-250. Dialyzed mAb17-1A was diluted to a final concentration of 4 mg/mL and stored on ice. The following amounts were transferred to 15 mL vials (Falcon): 3.5 mL for IGN402 ‘high SialylTn’, 4.6 mL for IGN402 ‘low SialylTn’, and 3.5 mL for IGN402 ‘medium SialylTn’, 4.6 mL for IGN402 ‘low SialylTn’. In parallel, 5 mg of SialylTn-O(CH2)4NHCOO-(\(\text{NO}_2\text{C}_6\text{H}_4\)) (Falcon): 3.5 mL for IGN402 ‘high SialylTn’, 4.6 mL for IGN402 ‘low SialylTn’, 4.6 mL for IGN402 ‘medium SialylTn’, and 4.6 mL for IGN402 ‘low SialylTn’. The reaction mixture was incubated at 100 °C for 1 h. The optical density (OD) of the coupling product was dialyzed twice against formulation buffer (0.1 M NaPO4, 0.15 M NaCl, pH 8.5) using a Slide-A-Lyzer dialysis cassette MWCO 10K (Pierce). Concentration of mAb17-1A was determined by size exclusion chromatography (SEC) on a Zorbax GF-250. Dialyzed mAb17-1A was diluted to a final concentration of 4 mg/mL and stored on ice. The following amounts were transferred to 15 mL vials (Falcon): 3.5 mL for IGN402 ‘high SialylTn’, 4.6 mL for IGN402 ‘low SialylTn’, 4.6 mL for IGN402 ‘medium SialylTn’, and 4.6 mL for IGN402 ‘low SialylTn’. In parallel, 5 mg of SialylTn-O(CH2)4NHCOO-(\(\text{NO}_2\text{C}_6\text{H}_4\)) (Falcon): 3.5 mL for IGN402 ‘high SialylTn’, 4.6 mL for IGN402 ‘low SialylTn’, 4.6 mL for IGN402 ‘medium SialylTn’, and 4.6 mL for IGN402 ‘low SialylTn’.

**Coupling of Branched PEI Linker to mAb17-1A.** The coupling procedure consisted of coupling the branched polyethylenimine (PEI) linker molecule to mAb17-1A, followed by the coupling of nitrophosphated spacer SialylTn to the mAb17-1A–PEI conjugate. Briefly, 20 mg of SPDP (20 mM in DMSO) for 60 min at RT. The SPDP conjugates were dialyzed against PBS. PEI–SPDP (4.2 mg in 2.1 mL PBS) was reduced with 55 mg of DTT for 30 min at RT. The reduced PEI–SPDP was dialyzed against PBS.

**Reaction of mAb17-1A–SPDP with Reduced PEI–SPDP.** Reduced PEI–SPDP (3.95 mg) and mAb17-1A–SPDP (24.4 mg) were reacted at a molar ratio of 1:1 for 24 h at +4 °C. The reaction mixture was dialyzed against PBS using SpectraPor Float-A-Lyzer (MWCO 60 kDa). The mAb17-1–PEI coupling product was confirmed by LDS–PAGE, WesternBlot, BioRAD protein assay, and ninhydrin assay.

**Coupling of SialylTn to mAb17-1A–PEI.** Ten milligrams of nitrophosphated spacer SialylTn, Neu5Ac2-6GalNAca-O(CH2)6NHCOCOCH2-\(\text{NO}_2\text{C}_6\text{H}_4\)) (Falcon) were dialyzed against PBS. PEI coupling product was confirmed by LDS–PAGE, WesternBlot, BioRAD protein assay, and ninhydrin assay.

**Analysis of Final Coupling Products.** Size Exclusion Chromatography. Concentrations of SialylTn–mAb17-1A coupling products were quantified by size exclusion chromatography (SEC) on a ZORBAX GF-250 column in a Dionex system and on a TSKgel G3000SW column in a HP1100 system.

**IF, LDS–PAGE, and Western Blots.** SialylTn–mAb17-1A coupling products were analyzed by SERVALYT PRECOTES horizontal flatbed IEF electrophoresis pH 3–10 (SERVA) followed by Coomassie blue staining (Invisitrogen) and by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS–PAGE, NuPAGE Electrophoresis System, Bis-Tris-Gel, 4–12%) under both nonreducing and reducing conditions (50 mM DTT, 1,4-dithiothreitol) followed by SilverXpress staining (Invitrigen).

**For Western blot analysis, samples following LDS–PAGE were transferred** (25 V, 1.1 W, 1.5 h) to Immobilon membranes (PVDF 0.45 μm, Millipore). Membranes were blocked with 3% skim milk and stained with rabbit anti-mouse IgG (H+L)–HRP (1:1000, Zymed) or alternatively with anti-SialylTn CD175s (IgG1) (10 μg/mL, DAKO) and rat anti-mouse IgG1–HRP (1:1000, Becton Dickinson).

**Quantification of Sialic Acid by the Resorcinol Assay.** Amount of sialic acid in the coupling products was quantified by the resorcinol–HCl reaction in the presence of CuSO4 and spectrophotometric measurement of absorbance at 562 nm. Briefly, 0.15 mL of samples and 0.15 mL of resorcinol reagent (2% resorcinol, 2.5 mM CuSO4, HCl (37%)) were mixed in tightly closed glass vials and incubated at 100 °C for 1 h. The optical density (OD) of the developing color was measured by adsorption at 562 nm (Spectrophotometer Ultrospec II, LKB Biochrom). As a standard, a ‘SialylTn mix’ (consisting of sialic acid (i.e. N-acetylsaccharinamide) and GalNAc at a molar ratio 1:1), 1.25–25 μg/mL, was used for calibration.
Quantification was done by measuring the peak area of N-acetylneuraminic acid (NANA). HPLC profile of SialylTn–mAb17-1A conjugate (upper panel) and nonconjugated mAb17-1A (lower panel) are shown. The arrowhead points to the peak corresponding to NANA in the sample. Synthetic NANA (Sigma), 1.25–25 µg/mL, was used for calibration.

initial immunizations on days (d) 1, 15, 29, and 57. Blood samples were taken before (d −15, −11, −6) and after immunization (d 15, 22, 29, 36, 57; 71 and 85).

In a second Rhesus monkey study, four animals per group were vaccinated with four initial immunizations on days (d) 1, 21, 49, and 76. Blood samples were taken before (d −13 and −6) and after immunization (d 27, 49, 52, 70, 79, and 91). All immunizations were well tolerated by the animals with no signs of systemic or local toxicity related to immunization. One animal (no. 83 from the SialylTn[mAb17-1A group), however, had to be euthanized during the course of the study because of moribund conditions. The pathological and histopathological examinations of the animal showed pathologies in blood coagulation (thrombus in liver vein, embolus in lungs) most probably not related to the vaccination.

ELISA for Immune Reactivity against mAb17-1A.

Presera and immune sera were analyzed regarding the induced immune response against mAb17-1A by ELISA. Briefly, ELISA plates (F96 Maxisorp, NUNC) were coated with 10 µg/mL mAb17-1A. ELISA plates were blocked with 2% HSA in PBS (1 h, 37 °C), and samples were prediluted in PBS with 0.5% HSA and were incubated for 1.5 h at 37 °C. A positive control serum with known reactivity against mAb17-1A was tested in parallel and used for normalization between different ELISA plates. For detection, plates were incubated with a sheep anti-human IgG(γ-chain)–HRP conjugate (1:2000, Chemicon) for 30 min at 37 °C. Staining with substrate OPD (10 mg OPD dissolved in 25 mL + 10 µL 30% H2O2) was stopped by adding 50 µL of H2SO4 (30%) and measured.
at 492/620 nm. The titer was defined as reciprocal serum dilution yielding an absorbance of OD \(1.0\) on a titration curve. Curve fitting was done using GraphPad Prism program version 4.0.

**SialylTn-PAA ELISA.** Preserum and immune sera were analyzed concerning the immune response against the synthetic SialylTn carbohydrate antigen (coupled to polyacrylamide) by SialylTn-PAA ELISA. Briefly, ELISA plates (F96 Maxisorp, NUNC) were coated 10 \(\mu\)g/mL SialylTn-PAA (Lectinity). ELISA plates were blocked with PBS containing 2% HSA for 1 h at 37 °C, followed next by a washing step. Samples were prediluted in PBS with 0.5% HSA and 5% glucose and incubated for 2 h at 37 °C. A positive control serum with known reactivity against SialylTn was used for normalization between different ELISA plates. For detection, plates were incubated with mouse anti-human IgM-HRP conjugate (1:2000, SB, Southern Biotechnology) or sheep anti-human IgG-(\(\gamma\)-chain)-HRP conjugate (1:2000, Chemicon), respectively, for 30 min at 37 °C. Staining with substrate OPD (10 mg OPD dissolved in 25 mL + 10 \(\mu\)L of 30% \(\text{H}_2\text{O}_2\) was stopped by adding 50 \(\mu\)L of \(\text{H}_2\text{SO}_4\) (30%) and measured at 492/620 nm. Titers were defined as the reciprocal of serum dilutions yielding an absorbance of OD = 1.0 and OD = 0.5 for IgM and IgG, respectively. Curve fitting was done using GraphPad Prism program version 4.0.

**Ovine Submaxillary Mucin (OSM) ELISA.** Presera and immune sera were analyzed regarding the immune response to OSM which is a natural substrate highly expressing SialylTn. Briefly, ELISA plates (F96 Maxisorp, NUNC) were coated with 10 \(\mu\)g/mL OSM (Accurate Chemical, Westbury, NY). ELISA plates were blocked with 2% HSA for 1 h at 37 °C, followed next by a washing step. Samples were prediluted in PBS with 0.5% HSA and incubated for 2 h at 37 °C. A positive control serum with known reactivity against OSM was tested in parallel and used for normalization between different ELISA plates. For detection, plates were incubated with mouse anti-human IgM-HRP conjugate (1:2000, SB, Southern Biotechnology) or sheep anti-human IgG-(\(\gamma\)-chain)-HRP conjugate (1:2000, Chemicon), respectively, for 30 min at 37 °C. Staining with substrate OPD (10 mg OPD dissolved in 25 mL + 10 \(\mu\)L of 30% \(\text{H}_2\text{O}_2\) was stopped by adding 50 \(\mu\)L of \(\text{H}_2\text{SO}_4\) (30%) and measured at 492/620 nm. Titers were defined as reciprocal of serum dilutions yielding an absorbance of OD = 1.0 and OD = 0.5 for IgM and IgG, respectively. Curve fitting was done using GraphPad Prism program version 4.0.

**Table 1. Molar Ratios of SialylTn to mAb17-1A**

<table>
<thead>
<tr>
<th>coupling product</th>
<th>molar ratio SialylTn to mAb17-1A</th>
<th>resorcinol</th>
<th>HPLC (NANA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb17-1A</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SialylTn-mAb17-1A (high)</td>
<td>16.2 (0.5)</td>
<td>12.9</td>
<td>1.9</td>
</tr>
<tr>
<td>SialylTn-mAb17-1A (medium)</td>
<td>9.1 (0.4)</td>
<td>7.1</td>
<td>1.9</td>
</tr>
<tr>
<td>SialylTn-mAb17-1A (low)</td>
<td>2.8 (0.1)</td>
<td>2.8</td>
<td>1.9</td>
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</table>

Quantification of sialic acid in the different SialylTn-mAb17-1A coupling products were estimated by the resorcinol assay. The resorcinol assay is based on the resorcinol-HCl reaction in the presence of \(\text{CuSO}_4\) and spectrophotometric measurement of absorbance at OD562 nm. Mean and standard deviation (SD) are shown.

**Figure 3.** Effect of SialylTn coupling density on immune responses induced against SialylTn, mAb17-1A, and OSM. Rhesus monkeys were immunized with SialylTn-mAb17-1A plus QS-21 vaccines containing high, medium, or low SialylTn coupling density. Preserum and immune sera at a time kinetic were analyzed for immune response against mAb17-1A carrier protein and SialylTn carbohydrate antigen and ovine submaxillary mucin by ELISA. Antibody titers (geomean and Scatter factor) against SialylTn (IgG) (A), IgM (B), mAb17-1A (IgG) (C), and OSM (IgM) (D) are shown. Statistics: *\(p < 0.05\) vs preserum (one-tailed, paired \(t\)-test).
Immune Response against Tumor Cells (FACS analysis). Binding of immune sera to tumor cells was measured by cell surface staining using a FACScan (Becton Dickinson). OVCAR-3 human ovary adenocarcinoma cells (ATCC, HTB-161) were incubated with serum (diluted 1:40 in PBS with 2% FCS) for 2 h on ice. For detection, a goat F(ab′)2 anti-human IgG (H+L)–PE conjugate (1:100, Immunotech, Marseille, France) was used. Mean fluorescence intensities (MFI) values obtained for binding of preserum were compared to binding of the corresponding immune serum. For better comparison, the binding of preserum of each individual animal was set at 10% positive cells.

Antibody-Dependent Cellular Cytotoxicity, ADCC. Preserum and immune serum was tested for ADCC against SialylTn positive OVCAR-3 cells. Human PBMCs were used as effector cells and incubated with the 51Cr-labeled target cells at different E:T ratios, i.e., 60:1 and 20:1 for 14 h. 51Cr-release was measured by a γ-counter.

RESULTS

Effect of SialylTn Coupling Density on Immune Responses Induced against mAb17-1A, SialylTn, and OSM. Nitrophenyl-activated SialylTn carbohydrates were coupled to mAb17-1A at three different molar ratios of SialylTn to mAb17-1A (4.5:1; 18:1; and 36:1) resulting in three SialylTn–mAb17-1A conjugate vaccines with low, medium, and high SialylTn density, respectively. SialylTn–mAb17-1A coupling products analyzed by LDS–PAGE under reducing conditions followed by Silver staining are shown in Figure 1A. LDS–PAGE showed an increase in the molecular weight of the heavy chains (50 kDa) and the light chains (25 kDa) in the coupling products (lanes 3–5, compared to uncoupled mAb17-1A, lane 2) indicating that SialylTn has been coupled to both heavy and light chains of mAb17-1A. Western blot analysis of the SialylTn–mAb17-1A coupling products with either anti-SialylTn CD175s (mIgG1) and rat anti-mouse IgG1–HRP (Figure 1B) or alternatively with rabbit anti-mouse IgG (H+L)–HRP (Figure 1D) demonstrated that SialylTn was detectable in the SialylTn–mAb17-1A coupling products but not in the mAb17-1A (Figure 1B vs 1D). The amount of SialylTn detectable in the coupling product correlated with the coupling ratio (lane 3: high, lane 4: medium, lane 5: low). Coupling of the negatively charged SialylTn to mAb17-1A resulted also in a shift of the isoelectric point (pI) in the coupling products as shown by IEF (Figure 1C). The pI shift correlated with the coupling SialylTn ratio (lane 3: high; lane 4: medium; lane 5: low SialylTn).

The molar ratios of SialylTn to mAb17-1A in the coupling products were quantified by Recorcinol assay or HPLC of N-Acetyleneuraminic acid (NANA). In the resorcinol assay the amount of sialic acid (as part of SialylTn) in the coupling products is quantified by the resorcinol-HCl reaction. Molar ratios of SialylTn to mAb17-1A for the high, medium, and low ratio coupling product as determined by the resorcinol-HCl reaction are shown in Table 1. Quantification of amount of sialic acid in the coupling products was also performed by reverse phase HPLC followed by fluorescence detection of DMB derivatives of sialic acid. Quantification is based on the peak area of N-acetyleneuraminic acid (Figure 2). Molar ratios of SialylTn to mAb17-1A for the high, medium, and low ratio coupling product as determined by HPLC of NANA correlate well with those estimated by the resorcinol assay (Table 1).

The SialylTn–mAb17-1A coupling products (i.e. with final SialylTn to mAb17-1A ratios of 3:1, 9:1, and 16:1,
respectively, as determined by the resorcinol assay) were adsorbed on aluminum hydroxide (i.e. 500 μg coupling product coupled to 1.67 mg aluminum hydroxide) and coformulated with 100 μg QS-21 adjuvant. Addition of strong adjuvants, such as QS-21, was previously found to be essential for induction of switching carbohydrate specific IgM antibodies to the IgG isotype (23).

Rhesus monkeys, four animals per group, were immunized with SialylTn−mAb17-1A vaccines with high, medium, or low SialylTn coupling density, respectively. The time kinetics of the immune responses against mAb17-1A carrier protein, the SialylTn carbohydrate antigen, and OSM, a natural substrate with high SialylTn expression, were analyzed by ELISA (Figure 3, Panels A to D). The induced immune responses against SialylTn (Panel A: IgG, Panel B: IgM) and OSM (Panel D: IgM) were found to correlate with the density of coupled SialylTn. Highest responses against SialylTn (IgM, IgG) and OSM were induced at the highest coupling density (i.e. 16:1), while only low IgG titers against OSM were found (data not shown). With the low SialylTn density (i.e. 3:1) vaccine formulation, generally only marginal reactivity against SialylTn and no reactivity with OSM were found. In contrast, all three vaccines induced comparable IgG responses against the mAb17-1A carrier protein (Panel C). The data demonstrate a clear correlation between SialylTn ligand density and the efficacy to induce an anti-carbohydrate immune response, with the highest tested density showing highest efficacy of immune response against SialylTn or OSM.

**Mimic of Clustered Presentation of SialylTn.** Recent data from the literature indicate that mucin-derived, truncated carbohydrates, such as Tn or SialylTn, are recognized by antibodies preferably as clusters on the surface of tumor cells rather than as single molecule epitopes (12-17). To address this issue, the following variants of vaccines with different presentations of SialylTn were designed: monomeric SialylTn (Figure 4A) coupled at a ratio of 33:1 (corresponding to the highest ratio of the previous experiment) was compared with a conjugate coupled at very high SialylTn to mAb17-1A ratio, i.e. 100:1. Furthermore, a trimer SialylTn with three SialylTn moieties attached to one linker, SialylTn[tri], was coupled to mAb17-1A at a ratio of 33:1 (trimer-to-carrier ratio) (Figure 4B). Finally SialylTn was coupled to mAb17-1A−PEI conjugate at a coupling ratio of 100:1. The branched polyethyleneimine linker molecule, PEI 25 kDa, is a polycation with a high density of primary amino groups, and coupling of SialylTn to the mAb17-1A−PEI conjugate aimed to mimic highly clustered SialylTn presentation (Figure 4C). SialylTn−mAb17-1A coupling products were analyzed using LDS−PAGE under reducing conditions (Figure 5A). An increase in molecular weight of the heavy and the light chains was found in all coupling products. The SialylTn[tri]−mAb17-1A conjugate (lane 5) showed a peculiar ladder pattern with several distinct bands for both heavy and light chains. Western blot analysis with anti-SialylTn CD175s (mIgG1) x rat-anti-mouse IgG1−HRP (B), or subjected to IEF−PAGE and stained with Coomassie blue (C). Heavy and light chains of mAb17-1A or conjugated SialylTn−mAb17-1A under reducing conditions (A, B) can be seen.

**Table 2. Molar Ratio of SialylTn to mAb in Different Coupling Products**

<table>
<thead>
<tr>
<th>Coupling product</th>
<th>molar ratio SialylTn to mAb17-1A (resorcinol)</th>
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<tbody>
<tr>
<td>SialylTn-mAb17-1A 33:1</td>
<td>19</td>
</tr>
<tr>
<td>SialylTn-mAb17-1A 100:1</td>
<td>75</td>
</tr>
<tr>
<td>SialylTn-mAb17-1A 33[tri]:1</td>
<td>65</td>
</tr>
<tr>
<td>SialylTn-mAb17-1A 100:1</td>
<td>27</td>
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</table>

Quantification of sialic acid in the different SialylTn−mAb17-1A coupling products was estimated by the resorcinol assay. The resorcinol assay is based on the resorcinol-HCl reaction in the presence of CuSO4 and spectrophotometric measurement of absorbance at OD562 nm.

SialylTn to mAb17-1A ratios in the final coupling products were quantified by the resorcinol assay, and the following ratios (calculated on monomeric SialylTn) were estimated: 19:1, 75:1, 65:1, and 27:1 for the monomeric SialylTn−mAb17-1A conjugates, monomeric at coupling ratio 33:1 (lane 3), monomeric at coupling ratio 100:1 (lane 4), SialylTn[tri] at coupling ratio 33:1 (lane 5), and SialylTn−PEI-mAb17-1A (lane 6) were subjected to LDS−PAGE under reducing conditions followed by silver staining (A), or blotted and stained with anti-SiaTn CD175s (mIgG1) x rat-anti-mouse IgG1−HRP (B), or subjected to IEF−PAGE and stained with Coomassie blue (C). Heavy and light chains of mAb17-1A or conjugated SialylTn−mAb17-1A under reducing conditions (A, B) can be seen.
ratio compared to the 33:1 coupled conjugate (final ratio 19:1); a lower ratio (27:1) was measured in the SialylTn-PEI-mAb17-1A coupling product. The SialylTn-mAb17-1A conjugates were adsorbed on aluminum hydroxide, coformulated with QS-21 adjuvant and tested in a Rhesus monkey vaccination study. Presera and the corresponding immune sera were tested for reactivity against SialylTn-HSA and OSM by ELISA (Figure 6). The time kinetics for the immune responses against SialylTn (Panel A: IgM, Panel B: IgG) and OSM (Panel C: IgM, Panel D: IgG) are shown. Again, a strong correlation between density of coupled SialylTn and the immune responses against both synthetic SialylTn and OSM was found. Regarding induced titers, the high coupling product of monomeric SialylTn to mAb17-1A (coupling ratio at 100:1, i.e. final ratio 75:1) was found superior to all other coupling products. The SialylTn-PEI-mAb17-1A conjugates were significantly more efficient than the monomeric SialylTn (33:1, final 19:1), but were inferior when compared to the 75:1 monomeric coupling product. The superior efficacy of the high density coupling product was particularly dramatic concerning the response against OSM, a natural substrate with high SialylTn content (Figure 6C,D).

**Immune Response against SialylTn Positive Tumor Cells.** Finally, the immune response against the natural target, SialylTn positive tumor cells, was tested. Preserum and immune sera of Rhesus monkeys immunized with SialylTn-mAb17-1A plus QS-21 were analyzed for binding to SialylTn positive OVCAR-3 cells by FACS analysis. Histograms of IgG and IgM binding to OVCAR-3 cells are shown for the 75:1 high-density SialylTn-mAb17-1A vaccine (Figure 7A,B). A statistically significant increase in cell binding to OVCAR-3 cells (both IgG and IgM) was found for the high coupling product ($p < 0.05$, paired t-test). A less consistent reactivity was found with the other coupling products (data not shown).

Finally, presera and immune sera of animals immunized with the high-density SialylTn-mAb17-1A vaccine were tested for ADCC against SialylTn positive OVCAR-3 cells. Human PBMCs were used as effector cells and incubated with the target cells at two E:T ratios, i.e., 60:1 and 20:1. While different initial levels of lytic activity were found in the different animals, a clear increase in ADCC activity was found in the immune sera of all animals in comparison to the corresponding presera (Figure 7C).

**DISCUSSION**

A panel of synthetic cancer vaccine formulations based on SialylTn carbohydrate antigens chemically coupled to an immunogenic protein carrier molecule were designed and tested in Rhesus monkeys. Coupling of the SialylTn carbohydrate epitopes to mAb17-1A aims to increase the immunogenicity of the SialylTn carbohydrate antigen and to provide with the mAb17-1A carrier an additional antigen that is thought to mediate an immune response against epithelial cancer. The murine 17-1A antibody, a monoclonal antibody (mAb) recognizing the epithelial cell
adhesion molecule (EpCAM), has been used for passive cancer therapy in patients with epithelial carcinomas (19) whereby part of the observed efficacy has been attributed to the induction of anti-idiotypic and anti-anti-idiotypic antibodies (20, 21). In the cancer vaccine candidate IGN101 mAb17-1A adsorbed on aluminum hydroxide is being used as vaccine antigen to induce an immune response against epithelial cancer. IGN101 has demonstrated an excellent safety profile and has recently been shown to improve survival in metastatic colorectal cancer patients (22).

In the present study, we investigated the effect of various SialylTn coupling densities and different ways of carbohydrate presentation on the carrier molecule on the induced immune response. Recent data have indicated that the monosaccharide Tn or the disaccharide SialylTn are predominantly present as molecular clusters on the surface of tumor cells (9, 12, 17). Therefore, increasing the coupling density of SialylTn on the carrier molecule and mimicking various types of clustered presentation was tested in this study. Our results demonstrate that at least for this type of carbohydrate–protein conjugate vaccine, the coupling density of SialylTn is an essential parameter determining the efficacy of the induced immune response against the carbohydrate antigen. While only marginal anti-SialylTn responses were generated at a very low carbohydrate to mAb17-1A carrier ratio of 3:1, a ratio of ~16:1 resulted in significant anti-SialylTn IgG titers. At the highest coupling density achieved in this study of 75:1, the highest IgG titers against SialylTn were induced. In contrast, the immune response against mAb17-1A was not significantly affected by the SialylTn to mAb17-1A ratio. However, the magnitude of the immune response against OSM was again strongly dependent on the SialylTn coupling density in the vaccine. In particular IgG antibodies (indicative for an isotype switch from IgM to IgG) specific for OSM were found at significant levels only with high SialylTn coupling densities in the vaccine. Induction of a significant IgG response against OSM and tumor cells indicates that SialylTn in its natural presentation is also recognized by the induced immune response, although to a lesser degree than the synthetic SialylTn immunogen. Alternatively designed clustered presentation of SialylTn as trimers or on a branched PEI linker did not further improve the induced immune response against SialylTn, OSM, or tumor cells compared to the highest monomeric SialylTn coupling density. This result was somewhat unexpected as trimeric presentation of SialylTn on an immunogenic carrier has been shown by others to be a better mimic of the natural presentation (9, 12, 16). One explanation may be that the recognition of the trimer SialylTn in the present study is negatively influenced by the use of longer and more flexible linker molecules in the trimer conjugate, thus perhaps placing the three SialylTn moieties at an unfavorably large distance to each other for recognition. Furthermore, recent data indicate that it is not only the clustered carbohydrate but also the context with the peptide anchor which can affect the immune response (16, 17). It is currently still under investigation whether the native mucin glycopeptide architecture (16) or rather the more immunogenic non-native carbohydrate–peptide linkage (17) would be better suited for induction of therapeutic efficacy in tumor patients. Finally, it is known that different tumor cell lines, depending on the growth conditions, but also primary tumors and metastatic cells can differ in their carbohydrate clusters. A trimeric cluster may be typical for certain cells; for others different types of clusters may prevail (16, 17, 18).

On the basis of its amino acid sequence, the mAb17-1A carrier, a murine monoclonal IgG2a antibody, contains a total of 98 lysine residues which present potential primary amino groups for coupling, depending on the accessibility. Lysines, due to their positive charge and extended, rather rigid structure, are generally surface exposed (25). To get insight in the three-dimensional distribution of the lysine residues within the murine

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**Figure 7.** Rhesus monkeys were immunized with the SialylTn–mAb17-1A vaccines. Preserum (grey line filled) and immune sera (bold blank line) (both diluted 1:40 in PBS + lylTn) were tested for ADCC against SialylTn positive OVCAR-3 cells. Human PBMCs were used as effector cells and incubated with the ^51^Cr-labeled target cells at E:T ratio of 60:1 and 20:1 for 14h. ^51^Cr-release was measured using a γ-counter (C).
sequence of the heavy chain constant regions reveals 100% homology whereas for the variable regions the homology is, as expected, lower due to the unique complementary determining regions.

Coupling of carbohydrate epitopes to mAb17-1A may provide the advantage of creating different types of presentation for the carbohydrate epitopes, both monomeric as well as different kinds of clusters. Furthermore, the data of the present study demonstrate that it is feasible to design a synthetic cancer vaccine which is adjustable to a controlled and reproducible, large scale manufacturing process. The SialylTn–mAb17-1A conjugate vaccine may provide a prototype for development of vaccines with several different carbohydrate epitopes coupled to the same immunogenic carrier molecule. The need for high density coupling may limit the number of different types of carbohydrate epitopes which can be coupled to the carrier. However, immune recognition of clustered carbohydrates has been shown so far only for a selected group of carbohydrates such as Tn and SialylTn and is probably not typical for recognition of larger carbohydrates, such as GM2 and GloboH. Therefore, combining carbohydrate epitopes which are recognized as monomers with epitopes with clustered presentation on one carrier molecule should be feasible (18). Alternatively, using a mixture of different types of carbohydrate–carrier conjugates coformulated in one vaccine formulation might be another pragmatic solution for the generation of a sufficiently broad panel of tumor-associated carbohydrate recognizing IgG antibodies. Strategies for cancer vaccines which target multiple tumor antigens (18, 24) can be expected to provide higher therapeutic efficacy and to minimize the risk for tumor escape, two key parameters for the successful treatment of cancer patients.

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LITERATURE CITED

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