ABSTRACT

Sialyl Tn (sTn) is a mucin-associated carbohydrate antigen expressed in most types of human adenocarcinoma. Defining the configuration of tumor cell surface sTn recognized by antibodies is important for understanding the basis for the cancer cell specificity of sTn-reactive mAbs, for the development of more effective mAbs, and for designing cancer vaccines against sTn. In this study, we compared the immunogenicity of synthetic single sTn disaccharide epitopes and clusters of sTn-C) of 3 sTn epitopes covalently linked via serine to keyhole limpet hemocyanin (KLH; sTn-KLH and sTn(C)-KLH, respectively). The cell surface sTn configurations were analyzed with the use of sera from mice immunized with these neoglycoproteins and a panel of sTn-reactive mAbs. Sera from mice immunized with sTn-KLH reacted in direct and inhibition assays with sTn-human serum albumin (HSA) but only weakly with sTn(C)-HSA, whereas sera from mice immunized with sTn(C)-KLH reacted with sTn(C)-HSA but not with sTn-HSA. Both anti-sTn and anti-sTn(C) sera reacted with ovine submaxillary mucin (a natural source of sTn) and with sTn-positive human tumor cell line LS-C but not with sTn-negative LS-B cells. With regard to the sTn-reactive mAbs, B72.3 reacted exclusively with clustered sTn, whereas mAb B195.3R11 reacted preferentially with unclustered sTn. Results with mAbs TKH2, B239.1, and CC49 were less clear, although all reacted more strongly with clustered sTn than with unclustered sTn. These results suggest that sTn is recognized at the tumor cell surface in at least two quite distinct configurations, as clustered and nonclustered arrays.

INTRODUCTION

The sTn* epitope is a mucin-associated disaccharide linked to serine/threonine [NeuAc(2–6)GalNAc-O-Ser/Thr] and expressed in many types of human adenocarcinoma, including carcinomas of the colon, breast, prostate, pancreas, ovary, stomach and lung, with absent or limited expression on the corresponding normal tissues (1–5). Sialyl Tn is the target for mAb B72.3 (6, 7), and this structure has served as a target for a series of diagnostic and therapeutic trials with radioiodinated mAb B72.3 (8–11). In addition, several studies with cancer vaccines containing natural or synthetic sTn antigens have been completed recently (12–15) or are ongoing at this time. Defining the configurations of sTn expressed on the tumor cell surface is important for understanding the basis for the cancer cell specificity of B72.3 and related mAbs, for developing more effective mAbs, and for designing cancer vaccines against sTn. Although the chemical structure of sTn disaccharide has been known for some time, the precise nature of the epitopes recognized by B72.3 and the second generation anti-mucin mAbs such as CC49 and B195.3R11, have remained a mystery. In addition, although vaccination with sTn disaccharide covalently attached to KLH induced high-titer IgG antibodies against the disaccharide conjugates, these have not always reacted as well with purified mucins or cancer cells (13).

It has been proposed for the closely related Tn antigen, expressed on desialylated OSM, that the essential immunogenic structure is a cluster of 3–4 consecutive Tn monosaccharides (16). On the basis of these findings we developed neoglycoproteins containing sTn clusters. In this study, we compared the specificity of the antibody response after immunization with neoglycoproteins consisting of (a) synthetic single sTn disaccharide epitopes (sTn-); and (b) clusters of three sTn epitopes covalently linked through a triple serine backbone [sTn(C)-], conjugated to KLH (Fig. 1). With the use of sera from mice immunized with the single or clustered sTn antigens and a series of mAbs, we find that sTn is recognized on the tumor cell surface in at least two quite distinct configurations.

MATERIALS AND METHODS

Antigens, mAbs, and Adjuvants. sTn, sTn(C), and Tn(C) were synthesized with crotly linker arms and conjugated to KLH or HSA by ozonolysis, as shown in Fig. 1, by Biomira, Inc. (Edmonton, Alberta, Canada). The sTn-KLH immunogen contains sTn disaccharide without serine whereas sTn(C)-KLH immunogen contains sTn disaccharide linked to serine in a clustered structure. KLH was purchased from Cal-Biochem (La Jolla, CA), and HSA was from Sigma Chemical Co. (St. Louis, MO). The sTn-KLH and sTn-HSA ratios were 3000:1 and 30:1, respectively, as determined by standard sialic acid and protein determinations. The ratios of sTn(C) and Tn(C) to KLH were 30 to 1 and to HSA, 4 to 1 (i.e., 90 and 12, respectively, of sTn disaccharides/KLH molecule). sTn-S was synthesized by Biomira, Inc. and conjugated to BSA (Sigma) at Memorial Sloan-Kettering Cancer Center at a ratio of 10:1 by a heterofunctional reagent N-succinimidyl-3-(2-pyridyldithio)propionate (18). OSM was purchased from BioCarb (Lund, Sweden), and desialylated OSM was prepared by heating OSM in 0.1 m trifluoroacetic acid at 100°C for 1 h. α-Carboxylycoprotein was purchased from Sigma.

sTn-reactive mAbs B72.3 (18), TKH2 (7), and CC49 (19) were supplied by Dr. K. O. Lloyd and B239.1 and B195.3R11 by Biomira, Inc. Adjuvant QS-21 containing a purified Quil A saponin fraction (20) was obtained from Cambridge Bioscience (Worcester, MA).

Animals and Vaccine Administration. Female BALB/c-C57BL/6 F1 mice, 6 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, Maine). Immunization was begun within 2–6 weeks. Ten mice from each group were immunized with 20 μg sTn-KLH or sTn(C)-KLH plus 10 μg QS-21 three times at 1-week intervals. Mice were bled 10 days after the third vaccination.

Antisera Titer and Cell Reactivity by ELISA. ELISAs were performed as described previously (21). For testing the neoglycoproteins, sTn-HSA, sTn(C)-HSA, and OSM were coated on ELISA plates at 0.1 μg/well. A series of antiserum dilutions was incubated with the coated antigen for 1 h at room temperature. Secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse IgG or IgM at a dilution of 1:200 (Zymed, San Francisco, CA). The ELISA titer is defined as the highest dilution yielding an absorbance of 0.10 or greater than that of normal mouse sera.

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3 The abbreviations used are: sTn, sialyl Tn; KLH, keyhole limpet hemocyanin; sTn(C), the sum of sTn epitopes; HSA, human serum albumin; OSM, ovine submaxillary mucin; sTn-S, sTn-serine; NeuAc, N-acetylmuramic acid; GalNAc, N-acetylgalactosamine; Ser/Thr, serine/threonine.

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Fig. 1. Structures of sTn-KLH, sTn-S-KLH, and sTn(C)-KLH vaccines. sTn epitope, NeuAcα(2→6) GalNAcβ(1→1) Ser/Thr; Tn epitope, GalNAcβ(1→1) Ser/Thr.

Fig. 2. Reactions of sera from mice immunized with sTn-KLH (R1-R5) and sTn(C)-KLH (C1-C5) plus immunological adjuvant QS-21 on human colon tumor cell lines LS-B (sTn-negative) and LS-C (sTn-positive) tested with a cellular ELISA assay. NS was a mixture of 5 unprimed mouse sera. M1 and M2, and D1 and D2 were sera from mice immunized with MUC1 peptide-KLH or GD3 ganglioside-KLH, respectively. Antisera were used at 1:160, and mAbs TKH2 and B7.3 were at 1:10 dilutions of the supernatants. Reactions were detected with the use of goat anti-mouse IgG-AP at 1:200. All the test values were the average of triplicate determinations.

Sera were also tested by ELISA with LS-C (sTn-positive) and LS-B (sTn-negative) cells that had been cloned from the parental human colon cancer cell line LS174T/C22. In cellular ELISA, 5 × 10^4 cells/well were seeded on the plate and mouse antisera were used at 1:160. All the data were the average of triplicates.

Inhibition Assay. Antisera at 1:500 dilution or mAbs at 0.4 μg/ml were mixed with different concentrations of inhibitor (i.e., sTn-HSA, sTn-S-BSA, sTn(C)-HSA, and Tn(C)-HSA) for 30 min at room temperature. The mixtures were transferred to a plate coated with OSM (0.1 μg/well), and an ELISA was performed. Percentage inhibition was calculated as

\[
\% \text{ of inhibition} = \frac{A \text{ without inhibitor} - A \text{ with inhibitor}}{A \text{ without inhibitor}} \times 100
\]

where A is absorbance. Tables 1 and 2 and Figs. 3 and 4 show ELISA results from single complete experiments performed for this publication. All the results had been confirmed previously in multiple smaller experiments.

RESULTS

Serological Responses after Immunizations with sTn Neoglycoproteins. Sera from ten mice immunized with sTn-KLH and ten mice immunized with sTn(C)-KLH were examined. All the sera from these mice contained IgM and IgG antibodies against the immunizing antigens and against OSM. In several sera from mice immunized with sTn-KLH, IgM and IgG responses against clustered sTn were

| Table 1 Tities of sera from mice immunized with sTn-KLH or sTn(C)-KLH on sTn-HSA, sTn(C)-HSA, and OSM |

<table>
<thead>
<tr>
<th>Mice</th>
<th>sTn-HSA</th>
<th>sTn(C)-HSA</th>
<th>OSM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>R1</td>
<td>1,280</td>
<td>10,240</td>
<td>640</td>
</tr>
<tr>
<td>R2</td>
<td>160</td>
<td>5,120</td>
<td>10</td>
</tr>
<tr>
<td>R3</td>
<td>1,280</td>
<td>10,240</td>
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<td>320</td>
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</tr>
<tr>
<td>R10</td>
<td>320</td>
<td>1,280</td>
<td>640</td>
</tr>
<tr>
<td>Median</td>
<td>480</td>
<td>5,120</td>
<td>640</td>
</tr>
</tbody>
</table>

a Reciprocal titers by ELISA, all from a single experiment, are shown.
detected, although neither IgM nor IgG in the sera from mice immunized with sTn(C)-KLH reacted with sTn-HSA. Antibody titers against OSM were roughly comparable in the two groups of animals (Table 1).

IgG antibodies from mice immunized with sTn-KLH and sTn(C)-KLH reacted with the sTn-positive cell line LS-C by ELISA but did not react with the sTn-negative cell line LS-B (Fig. 2). Pooled normal mouse serum and sera from mice immunized with the negative controls, peptide-KLH plus QS21 (M1 and M2) and GD3 ganglioside-KLH plus QS21 (D1 and D2), reacted with neither LS-B nor LS-C.

**Table 2: Inhibition (percentage) of immune mouse serum IgG reactivity against OSM by various sTn-related structures**

<table>
<thead>
<tr>
<th>sTn(C)-HSA</th>
<th>sTn-S-BSA</th>
<th>sTn(C)-HSA</th>
<th>sTn(C)-HSA</th>
<th>sTn(C)-HSA</th>
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<td>sTn-KLH</td>
<td>84</td>
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<td>sTn-S-BSA</td>
<td>2</td>
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<td>2</td>
</tr>
<tr>
<td>sTn(C)-HSA</td>
<td>17</td>
<td>7</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>sTn(C)-HSA</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>R1</td>
<td>R2</td>
<td>R3</td>
<td>R4</td>
<td>R5</td>
</tr>
<tr>
<td>R6</td>
<td>R7</td>
<td>R8</td>
<td>R9</td>
<td>R10</td>
</tr>
<tr>
<td>Median</td>
<td>69</td>
<td>62</td>
<td>29</td>
<td>14</td>
</tr>
</tbody>
</table>

a ELISA plates were coated with OSM at 0.1 µg/well; antisera to sTn-KLH and sTn(C)-KLH were used at 1:1000; inhibitors sTn-HSA, sTn-S-BSA, Tn(C)-HSA, and sTn(C)-HSA were used at 25 µM; secondary antibody was goat anti-mouse IgG-AP. All test values were obtained from a single experiment and were the average of duplicates. Compared with the inhibition of sTn-HSA or sTn-S-BSA by Mann-Whitney nonparametric statistics; P < 0.01.

b Compared with the inhibition of sTn-HSA, sTn-S-BSA or Tn(C)-HSA by Mann-Whitney/Wilcoxon nonparametric statistics; P < 0.01.

d ELISA plates were coated with OSM at 0.1 µg/well; antisera to sTn-KLH and sTn(C)-KLH were used at 1:1000; inhibitors sTn-HSA, sTn-S-BSA, Tn(C)-HSA, and sTn(C)-HSA were used at 25 µM; secondary antibody was goat anti-mouse IgG-AP. All test values were obtained from a single experiment and were the average of duplicates. Compared with the inhibition of sTn-HSA or sTn-S-BSA by Mann-Whitney/Wilcoxon nonparametric statistics; P < 0.01.

e All the targets were coated on the plates at 0.2 µg/well. mAbs B72.3, CC49, B239.3, and B195.3R11 were used at 0.4 µg/ml; TKH2 was supernatant and used at 1:4. Absorbance at 405 nm after 20 min is shown. All the test values were obtained from a single experiment and the average of duplicates.

**Specificity of Serum Antibodies Determined by Inhibition Assays.** The specificity of the antibodies was analyzed by inhibition tests. The reactivity for OSM of IgG antibodies from mice immunized with sTn-KLH was inhibited with sTn(HSA (median inhibition, 69%) and sTn-S-BSA (62%) but only weakly by sTn(C)-HSA (29%) and not by Tn(C)-HSA (14%) (Table 2; Fig. 3A). The differences in inhibition between sTn-HSA or sTn-S-BSA groups and the sTn(C)-HSA group were statistically significant (P < 0.01). The reactivity of IgG antibodies from mice immunized with sTn(C)-KLH was inhibited with sTn(C)-HSA (median inhibition, 72%) but only weakly by sTn-HSA (22%), sTn-S-BSA (18%), and Tn(C)-HSA (19%) (P < 0.01; Table 2; Fig. 3B). Although sera from sTn-KLH and sTn(C)-KLH immunized mice reacted equally with LS-C tumor cells and with OSM, the specificity of these reactions was clearly different.

**Specificity Analysis of mAbs.** A number of mAbs reported to react with sTn or Tn-like structures (Table 3) was also tested with mucin and the neoglycoprotein antigens. All the mAbs reacted with OSM, and mAb B239.1 also reacted with desialylated OSM, suggesting cross-reactivity with the Tn antigen. MAb B72.3 and B239.1 reacted in a direct ELISA with sTn(C)-HSA but not or weakly with sTn-HSA, whereas B195.3R11 reacted with sTn-HSA and at a lower level with sTn(C)-HSA (Table 4). The reaction of B72.3 and B239.1 with OSM was inhibited by sTn(C)-HSA but not by sTn-HSA, sTn-S-BSA, or Tn(C)-HSA (Fig. 4). The reaction of B195.3R11 with OSM was inhibited by sTn-HSA and sTn-S-BSA but not by sTn(C)-HSA or Tn(C)-HSA (Fig. 4). It was concluded that mAb B72.3 and the sTn(C) immune sera react exclusively with clustered sTn, whereas B239.1 reacts preferentially with clustered sTn. On the other hand, B195.3R11, like sTn immune sera, reacts preferentially with unclustered sTn. CC49 reacted strongly with OSM, weakly with the clustered sTn, and not at all with the unclustered in both direct and inhibition assays (Fig. 4). TKH2 reacted strongly with OSM and sTn(C)-HSA in the direct assay but was not inhibited by sTn or sTn(C)-HSA (Fig. 4).

**DISCUSSION**

In the past, we have immunized patients with vaccines containing OSM (12) and sTn-KLH (13) plus immunological adjuvants DETOX...
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Fig. 4. Inhibition of mAb B72.3 (A), B195.3R11 (B), B239.1 (C), CC49 (D), and TKH2 (E) reactivity against OSM. Inhibitory antigens were sTn-HSA, sTn-S-BSA, Tn(C)-HSA, and sTn(C)-HSA. OSM was coated on ELISA plates at 0.1 μg/well. The mAbs were used at 0.4 μg/ml. Results from a single experiment are shown.

or Bacillus Calmette-Guérin. Only low-titer antibodies were induced with OSM vaccines, although these antibodies reacted equally with synthetic sTn and OSM. Although high-titer IgG antibodies against synthetic sTn resulted from clinical trials with sTn-KLH vaccines, these reacted at much lower titers with sTn expressed on OSM or tumor cells. Antibody titers were further increased by the use of immunological adjuvant QS-21, but the IgG antibodies that were produced continued to react more strongly with the synthetic sTn than with sTn naturally expressed on mucins (13). These data and the low reactivity of mAb B72.3 on synthetic sTn, despite its strong reactivity with mucins expressing sTn (13), suggested to us that the immune system might detect sTn in more than one configuration. Nakada et al. (16) have reported that the essential epitopic structure of the Tn antigen recognized by mAb MLS 128 is a cluster composed of three or four consecutive sequences of GalNAc-Ser/Thr. For the sTn antigen, a similar cluster structure has also been proposed, but the essential epitopic structure has not yet been identified (23). In addition, mucins such as MUC-2 and MUC-3 expressed in various carcinomas have consecutive 3-5 serine or threonine residues which may carry sTn disaccharides (24, 25). We therefore explored the possibility that one sTn epitope on mucin might consist of clusters of sTn.

The availability of neoglycoproteins containing sTn(C) structures sTn(C)-KLH and sTn(C)-HSA has permitted us to test this possibility in the context of active immunization studies. Both sTn-KLH and sTn(C)-KLH were able to induce moderate-titer IgM and high-titer IgG antibodies against the immunizing synthetic epitopes and against natural sTn expressed on OSM and human tumor cells. The mice were immunized with equal amounts of sTn-KLH or sTn(C)-KLH, but because the sTn-KLH epitope ratio (3000:1) was higher than that of sTn(C)-KLH (30:1), mice vaccinated with sTn(C)-KLH actually received a 30-fold lower dose of sTn. The finding that sera from mice immunized with both preparations reacted with OSM and tumor cells at a comparable level suggests that the sTn(C) structure may act as a particularly effective immunogen. Inhibition assays demonstrated that sera from mice immunized with sTn-KLH reacted strongly with sTn-HSA and sTn-S-BSA, weakly with sTn(C)-HSA, and least of all with Tn(C)-HSA. Conversely, sera from mice immunized with sTn(K)-KLH reacted strongly with sTn(C)-HSA but not with sTn-HSA, sTn-S-BSA, or Tn(C)-HSA. The fact that sera from sTn-KLH- and sTn(C)-KLH-immunized mice reacted with the immunizing epitopes, but also reacted equally with OSM and with LS-C tumor cells, suggests that the murine antisera are recognizing sTn in two different configurations on OSM and on LS-C tumor cells (i.e., in clustered and nonclustered arrays).

Our studies also demonstrate that some mouse mAbs thought to be reactive with sTn (Table 4) have the ability to distinguish between the two different epitope configurations. The epitope recognized by B72.3 has been reported to be sTn, based on its reactivity with a panel of mucins (6) and inhibition with unconjugated synthetic sTn disaccharides (7), but this result was complicated by the fact that B72.3 does not react well with conjugated synthetic sTn or sTn-S in direct tests. We describe here that mAb B72.3 reacts with synthetic sTn(C)-HSA but not sTn-HSA in direct tests, and that reactivity on OSM is inhibited by synthetic clusters present in sTn(C)-HSA but not in sTn-HSA. From these data it would seem that mAb B72.3 exclusively recognizes blocks of sTn epitopes present as clusters. On the other hand, mAb B195.3R11 was found to react preferentially with unclustered sTn structure; this reactivity is consistent with its derivation from mice immunized with sTn-KLH. Since CC49 fails to react strongly with the sTn or sTn(C) conjugates, no firm conclusion concerning the nature of the antigen recognized by CC49 is possible based on these studies. However, the fact that CC49 shows some reactivity with sTn(K)-KLH and sTn(C)-HSA in direct or inhibition assays but none with sTn-HSA and sTn-KLH suggests that the CC49 antigen may also require clustered epitopes.

The results obtained with mAbs TKH2 and B239.1, two other mAbs that react with sTn, in direct assays differed from those obtained with inhibition assays. MAB TKH2, which was raised against OSM (7), has a specificity very similar to mAb B72.3 in direct test, but reactivity with OSM was not inhibited by sTn(C)-HSA. MAB
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B23.9, which was raised against sTn(C)-KLH, had a broad pattern of reactivity in direct tests, reacting with both clustered and nonclustered structures and even with Tn structures. However, by inhibition assays, B23.9 reacted specifically with clustered sTn. We have no explanation for the differing results obtained with direct, as opposed to inhibition assays, for these two mAbs. Although they both appear to react preferentially with sTn(C)-epitopes, these differences make it difficult to come to firm conclusions about their specificities. However, the lack of reactivity against nonclustered sTn for the 3 mAbs raised against tumor cells or OSM (B27.2, CC49, and TKH2) suggests that the single sTn epitope is less immunogenic than the clustered sTn epitope. With regard to the single sTn epitopes, immune sera and mAbs reacted equally well with the sTn disaccharide and the sTn serine conjugates. Apparently the serine is not a part of the single sTn antigenic epitope. Little is known about the clustered sTn epitope on cells. The immunogenicity of clusters containing 2 or more than 3 sTn disaccharides and the need for serine as part of these epitopes remains to be determined.

Immunohistochimistry studies have demonstrated that B27.3 reacts with colon, breast, pancreatic, and other adenocarcinomas without significant reactivity with most adult normal tissues (4, 5, 26, 27). Although the glycosylation pattern of substitution on serine or threonine has not yet been elucidated for any mucin, the presence of 3 or 4 of adjacent serines and/or threonines in Muc-2 and Muc-3 mucins (24, 25) would be consistent with the presence of clustered carbohydrate epitopes in these mucins. Although the protein backbone of mucins on adenocarcinomas is thought to be the same as those on normal tissues (28), the glycosylation of carcinoma mucin backbones is less complete and could involve shorter and simpler carbohydrates such as sTn. Because sTn is also expressed on some normal tissues (7, 29), the unique specificity of B27.3 for adenocarcinomas may result from the preferential expression of sTn clusters on adenocarcinomas. They suggest that the cluster configuration of sTn (and perhaps cluster configuration of other antigens as well) is the most appropriate target for localization and therapy with mAbs and active immunization with cancer vaccines. Although there are other reports concerning the importance of clustered epitopes for maximal immunoreactivity, the present study is the first to show the recognition of two discrete types of sTn epitopes, clustered and nonclustered, on mucins and tumor cells.

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Immune Sera and Monoclonal Antibodies Define Two Configurations for the Sialyl Tn Tumor Antigen

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