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Specificities of anti-sialyl-Tn and anti-Tn monoclonal antibodies generated using novel clustered synthetic glycopeptide epitopes

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The fine specificities of MAbs generated using novel synthetic clustered STn and Tn glycopeptides as immunogens were compared with the anti-TAG-72 antibodies B72.3 and CC49. Hapten inhibition experiments demonstrated the specificity of several of the MAbs for STn and Tn expressed on ovine submaxillary mucin and tumor derived MUC-1 mucin. Amongst the STn specific MAbs only the B195.3 MAb shows absolute dependence on the presence of sialic acid and specificity to the simple disaccharide NANAAα2-6-GalNAc. Identification of tumor associated carbohydrate epitopes in cluster and monomer configurations are possible using MAbs detecting the defined structure specificities described herein.

***Keywords*: sialyl-Tn, Tn, MUC-1, cancer mucin**

Introduction

The tumor associated glycoprotein epitope defined by the monoclonal antibody B72.3 is expressed on most human adenocarcinomas and is minimally expressed on normal tissues [1]. The epitope for MAb B72.3 has been identified as sialyl-Tn (STn), i.e. αsialyl 2-6αGalNAc, O-linked to Ser/Thr on mucin-type glycoproteins [2]. Interest in this epitope over the past several years is due to the fact that its expression is considered to be an independent predictor of poor prognosis in a variety of cancer types including breast, ovarian, gastric, and colorectal cancer [3–6]. Because of its association with cancer aggression, it has been suggested that STn epitopes are actively involved in the cell adhesion and metastatic spread of tumor cells to endothelial cell surfaces [3, 7]. Other sialo-oligosaccharide epitopes such as sialyl-Lewis^x and sialyl-Lewis^a have been shown to function in adhesion of leukocytes and tumor cells to endothelial cell surfaces [8, 9] via selectin receptors. B72.3 was the first anti-STn specific monoclonal antibody described [10, 11]. It was derived following immunization of mice with human breast carcinoma cells and was subsequently found to react with the sialyl-Tn epitope on TAG-72 mucin molecules. The second generation antibody CC49 [12] was generated following immunization of mice with B72.3 affinity purified TAG-72. TKH2 is another anti-STn MAb [2] which was generated following immunization with ovine submaxillary mucin (OSM), a well characterized mucin that displays clusters of STn and Tn epitopes [13].

There are three important clinical applications of the STn epitope: in vitro diagnostics, in vivo diagnostics, and active specific immunotherapy. The MAbs B72.3 and CC49 have been used clinically for radioimmunoimaging of adenocarcinomas [14–19] and might have immunotherapeutic applications [20]. TKH2, B72.3 and CC49 are components of in vitro diagnostic kits used for the monitoring of circulating mucins bearing STn epitopes in cancer patients [5, 21]. The third clinical application of the STn epitope structure is the use of synthetic STn conjugated to KLH for active specific immunotherapy (ASI) of cancer. We have shown that active immunization of breast, ovarian and colorectal cancer patients with a synthetic STn epitope conjugated to KLH will induce the production of antibodies which react against both synthetic and natural sources of STn [22–24]. Furthermore, we have recently found that patients who make higher titres of STn specific antibodies following ASI treatment with STn-KLH plus DETOX™ have prolonged survival compared to patients who make lower anti-STn antibody titres [35].

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While it is known that the STn epitope is expressed on TAG-72, a mucin-like molecule expressed both on the cancer cell surface and shed into the circulation, to our knowledge, the expression of STn on the mucin encoded by the human MUC-1 gene has not been previously reported in the literature. Furthermore, the precise structural feature of the epitopes recognized by various anti-STn MAbs have not been fully defined. Based on the analysis of the specificities of polyclonal and monoclonal antibodies, we have recently provided evidence [25] that STn can be expressed as both isolated structures as well as clusters on a succession of serines and/or threonines of cancer-associated mucins. This is not a unique observation as the monoclonal antibody MLS128 [26] (anti-Tn Ser/Thr) was previously reported to be specific for a cluster of three adjacent serine or threonine residues, each with GalNAc [27]. Indeed this clustering effect appears to be a key to the mucin glycoprotein specificity of these monoclonal reagents because mucins typically present multiple tandems of serine/threonine in the peptide core sequences. The biological role of clustered versus single STn epitopes on cancer cells is still not understood. A study comparing the binding specificities of various anti-STn monoclonal antibodies might enhance our understanding of the importance of STn epitopes in the metastatic process leading to the design of novel diagnostic and therapeutic agents. In the present communication, we report the use of novel synthetic STn carbohydrate and glycopeptide antigens to analyse STn and Tn related cancer associated epitopes on MUC-1 mucin.

Materials and methods

All peptides, glycopeptides, cluster peptides and conjugates were synthesized by Biomira Inc. (Edmonton, Alberta, Canada) as described below.

Peptides

All peptides were synthesized using NovaSyn® TGA resin (Novabiochem, San Diego, CA) as solid phase and model 9050 automated peptide synthesizer (PerSeptive Biosystems, Farmingham, MA) utilizing Fmoc (9-Flurenylmethoxycarbonyl) chemistry. Following the synthesis, the crude peptides were cleaved using a cocktail of scavenging reagents in trifluoroacetic acid. The crude peptides were purified on reverse phase HPLC using a gradient between water and acetonitrile containing 0.05% trifluoroacetic acid to obtain the desired peptide at 95–98% purity. Amino acid ratio analysis and electrospray mass spectral analysis were done to ascertain the integrity of the peptide.

Glycosylated amino acids and glycopeptides

Glycosylated amino acids were synthesized using processes developed at Biomira and reported elsewhere [28]. Tn-serine, Tn-threonine, STn-serine and STn-threonine, made in a suitably protected form for use in the glycopeptide synthesis, were used as part of the sequence in the same way as described above for the solid phase synthesis of the peptides.

Monomeric, dimeric and trimeric clusters of Tn-Ser and STn-Ser

All of the clusters were synthesized in solution phase using classical coupling techniques. A C-terminal linker arm (2-crotyloxy ethylamine) was attached to the Fmoc protected glycosylated (Tn or STn) serine carboxyl to obtain the monomer (which may be completely deblocked and N-acetylated to obtain structures 1 or 4, Figure 1). The amino protecting Fmoc group was deblocked and another Tn or STn serine was coupled to the above protected monomer to obtain the protected dimer (structures 2 or 5 may be obtained at this stage by complete deblocking and N-acetylation). A trimeric structure was synthesized similarly by coupling another glycosylated serine to the above Fmoc deblocked dimer followed by total deblocking and N-acetylation to obtain structures 3 or 6.

Protein conjugates of clusters

The cluster haptens 3 and 6 and STn-crotyl were conjugated to Keyhole Limpet Hemocyanin (KLH) by reductive amination. The hapten was subjected to ozonolysis to cleave the double bond of the linker to generate an aldehyde. The 'hapten aldehyde' was then added to the KLH (10 mg ml^{-1}) in a ratio of 1:1 (by weight) in phosphate buffer at pH 7.5 followed by the addition of sodium cyanoborohydride ($NaBH_3CN$) at a 2 to 3 molar excess of the hapten. The mixture was stirred for about 72 h at room temperature and dialysed against phosphate buffer. The conjugate concentration was determined using colorimetric protein analysis. The hapten/protein ratio was determined by acid hydrolysis of the conjugate to cleave the individual carbohydrate hexoses and determining either sialic acid or N-acetylgalactosamine by pulsed amperometric detector (PAD) on HPLC.

Sandwich radioimmunoassay

Polystyrene tubes were coated with capture monoclonal antibodies at saturating conditions overnight prior to blocking with 1% BSA for 2 h. Human ovarian and breast adenocarcinoma derived ascites samples were diluted 1:20 in pH 5.0 acetate buffer and incubated on prepared solid phases for 2 h at room temperature on a circulatory shaker at 250 rpm. Tubes were washed prior to addition of ^{125}I-labeled tracers B27.29 (anti-MUC-1) or B43.13 (anti-CA125) (10 ng) and similarly incubated for 2 h before washing and counting of bound activity.

ELISA hapten inhibition studies

Synthetic carbohydrate, peptide or glycopeptide structures were pre-incubated at the stated molar concentrations with

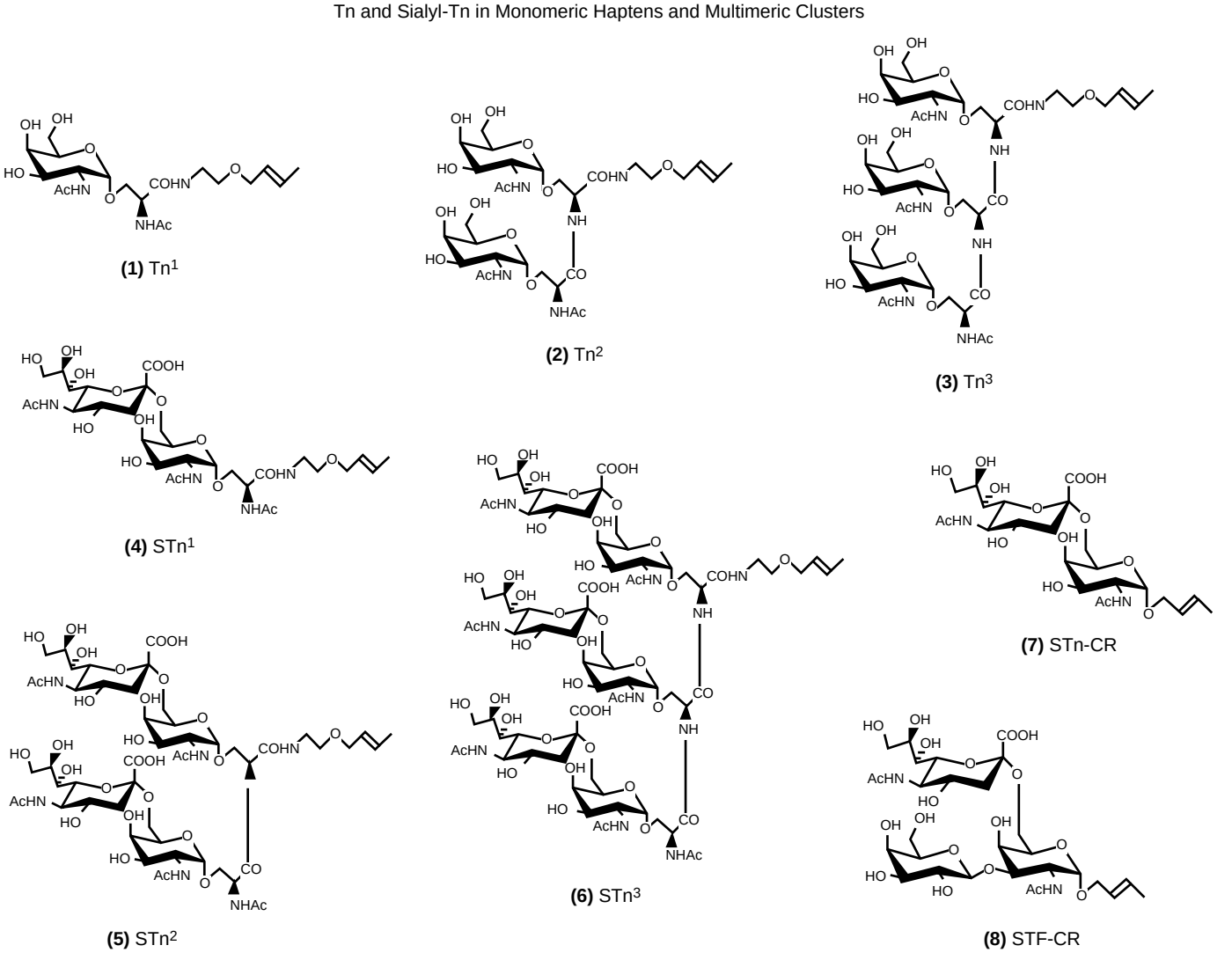

Figure 1. Structures 1, 2 and 3 are Tn-serine as monomeric, dimeric and trimeric clusters, respectively. Structures 4, 5 and 6 represent sialyl-Tn-serine as monomeric, dimeric and trimeric clusters, respectively. Structures 1–6 are glycopeptides with an attached C-terminal linker. Structure 7 represents sialyl-Tn with a crotyl linker and Structure 8 represents sialyl-TF with a crotyl linker.

the test monoclonal antibodies (MAbs) for 1 h prior to adding to microwells previously coated at saturation with ovine submaxillary mucin. Plates were incubated 90 min before washing and addition of goat anti-mouse IgG peroxidase conjugate (Jackson Immunoresearch, West Grove, PA), which was incubated a further 90 min. Plates were extensively washed prior to addition of ABTS substrate (Kirkegaard and Perry, Gaithersburg, MD). Plates were read in the kinetic mode using a Thermomax plate reader (Molecular Devices, Menlo Park, CA). Data presented are the percentages of inhibition relative to the no inhibitor controls and represent the mean of three experiments, each conducted in triplicate. Standard deviations of the triplicate samples were less than 10% of the mean.

Monoclonal antibodies

MAbs CC49 and B72.3 were generously donated by Dr Jeffrey Schlom of the National Cancer Institute, Bethesda, MD. MAb B195.3 (see Table 2) was derived from an SP2.0 fusion with spleen cells from a CAF1 mouse that had been immunized three times with 50 μg of a synthetic sialyl-Tn antigen linked to a KLH carrier emulsified in Ribi adjuvant (Ribi Immunochem Research Inc., Hamilton, MT). This STn-KLH antigen is the experimental adenocarcinoma therapeutic vaccine THERATOPE® that has been extensively tested in phase I and II clinical trials [22–24, 29]. This MAb has been shown to be similar in specificity to a subset of human [23, 29] and murine [25] polyclonal antibodies

generated in response to this vaccine construct. The monoclonal antibodies B230.9 and B239.6 were similarly derived from fusions of splenic cells from CAF1 mice that had been immunized with trimeric Tn-serine-KLH (B230.9) or trimeric STn-serine-KLH (B239.6) conjugates, again emulsified in Ribi adjuvant. The monoclonal antibodies B72.3 [10, 11] and CC49 [12] have been previously described.

Neuraminidase digestion

Microtitre wells were coated overnight with saturating concentrations of ovine submaxillary mucin (OSM) prior to washing and blocking with 2% BSA for 2 h. Washed plates were then digested with neuraminidase (Sigma Chemical Co., St. Louis, MO) from *Vibrio cholerae* at 0.002 U ml^{-1} for 0–120 min prior to washing and addition of test MAb. Plates were then incubated and developed as above.

Results

Fine specificity of MAb B239.6 generated following immunization with synthetic serine-STn trimers conjugated to KLH

Various synthetic carbohydrates and glycopeptides were used (see Figure 1 and Table 1) for hapten inhibition studies. Figure 2 demonstrates dose dependent hapten inhibition of B239.6 binding to OSM solid phases with various synthetic cluster structures. No inhibition of binding was noted with the BP24-MUC-1 peptide, and weak inhibition was obtained with BP24-Tn^2-MUC-1. Comparably strong inhibition of binding was obtained with the di-STn-serine containing glycopeptide BP24-STn^2-MUC-1 and with the STn-serine dimers and trimers (STn^2 and STn^3 structures in Figure 1). Further hapten inhibition experiments were conducted with a 'saturating' hapten concentration (100 nmol ml^{-1}). Figure 3 confirms that the STn-serine-dimer and STn-serine-trimer completely inhibited binding of B239.6 to OSM, while STn-serine-monomer was considerably less effective, and STn-crotyl-monomer did not produce significant inhibition. Furthermore, the cross-reactivity of B239.6 with Tn-serine clusters is confirmed. Thus, MAb B239.6 reacts with STn-serine clusters with strong cross-reactivity with Tn-serine containing clusters.

Table 1. Tn and STn MUC-1 synthetic glycopeptides used in this study.

Name	*Sequence*
BP-24	TAPPAHGVTSAPDTRPAPGSTAPP
BP-24 Tn^2	TAPPAHGVTSAPDTRPAPGS(Tn)T(Tn)APP
BP-24 STn^2	TAPPAHGVTSAPDTRPAPGS(STn)T(STn)APP

Fine specificity of MAb B195.3 generated following immunization with STn-Crotyl-KLH

Various synthetic carbohydrates and glycopeptides were also used (see Figure 1) for hapten-inhibition of binding of MAb B195.3 to an OSM solid phase in order to determine the fine specificity of the antibody. Purified MAb B195.3 was used at a concentration of 37.5 ng ml^{-1} and all haptens were used at an excess of the saturating concentration for the optimal structures (250 nmol ml^{-1}). Similar to the dose curve shown in Figure 2, these concentrations of other haptens are at least an order of magnitude higher than that required for maximum inhibition when STn haptens were used as inhibitors (data not shown). Figure 4 demonstrates that all STn containing haptens, whether clusters, or serine or crotyl monomer, completely inhibited the binding of B195.3 to OSM but there was no significant inhibition of binding of B195.3 by Tn or STF haptens. These data demonstrate that serine is not involved in the recognition of the STn disaccharide by MAb B195.3. The absolute requirement for the sialic acid as part of the B195.3 epitope is confirmed in Figure 5 which demonstrates that *Vibrio cholerae* neuraminidase treatment of the OSM solid phase abolishes binding of B195.3. In contrast, MAbs B72.3 and CC49 remain reactive with the neuraminidase-treated OSM solid phase. These results are consistent with the hapten inhibition studies using the synthetic mucin core structures that

Table 2. STn and Tn reactive MAbs used in these studies.

MAb	*Immunogen*	*Mouse Ig class*	*Reference*	*Summary of specificity*
B72.3	Human breast carcinoma	IgG_1	9	STn-serine clusters with cross-reactivity for Tn-serine clusters
CC49	MAb B72.3-Reactive TAG-72 glycoprotein	IgG_1	11	Strong cross-reactivity with STn-serine clusters with less cross-reactivity for Tn-serine clusters; probably has a higher affinity for an undefined carbohydrate structure
B195.3	STn-KLH	IgG_1	23, 25, 27	STn monomers without the involvement of core O-glycoside bond to serine
B230.9	Tn-Trimer-KLH	IgG_1	This Paper	Tn clusters, limited cross-reactivity with STn clusters
B239.6	STn-Trimer-KLH	IgG_1	This Paper	STn clusters, cross-reactivity with Tn clusters

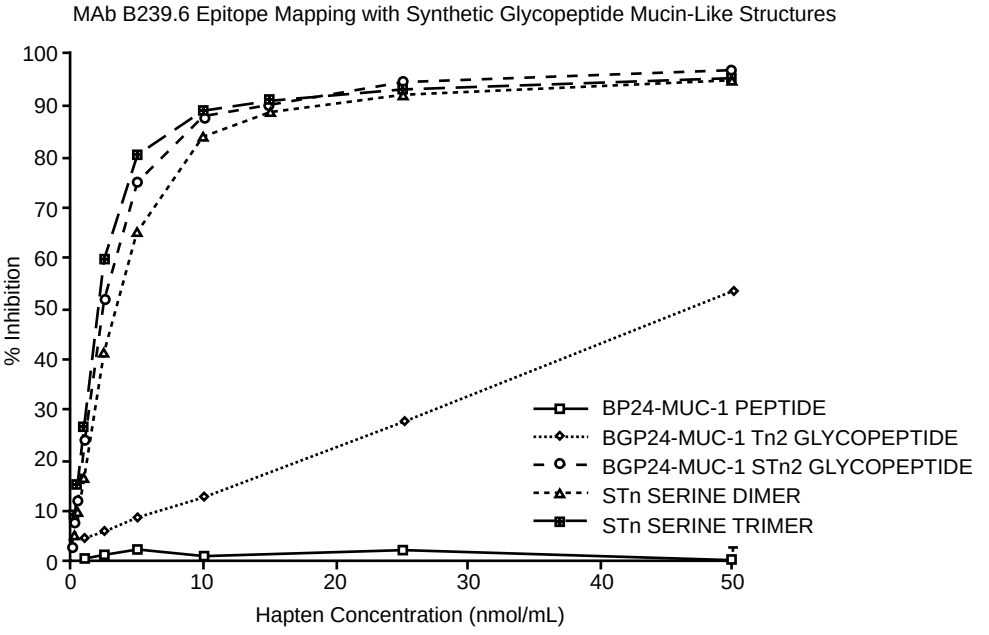

Figure 2. Results of synthetic glycopeptide binding inhibition studies with MAb B239.6 on an ovine submaxillary mucin solid phase as described in Materials and methods. The synthetic MUC-1 peptide sequence was substituted with Tn and STn groups at the threonine-serine site for comparison to the serine di- and trimeric antigen clusters. The 24 amino acid glycopeptide (BGP24STn^2, open circle) demonstrates comparable binding inhibition characteristics to the di- and tri-serine STn clusters, indicating the recognition of the STn cluster within the context of the larger synthetic mucin sequence. Cross reactivity to the MUC-1 Tn^2 glycopeptide is also notable.

contain Tn-serine clusters, which show that peptide core is involved in the specificities of these MAbs (see below). Thus only the MAb B195.3 is strictly reactive with the NANAα2-6GalNAc without influence of the O-glycosidic bond and the core amino acid. In addition, only B195.3 lacks cross-reactivity to the non-sialylated Tn antigen as either a monomer or a cluster.

Fine specificity of MAb B230.9 generated following immunization of mice with Tn-Serine trimers conjugated to KLH

Figure 6 demonstrates the hapten inhibition of binding of B230.9 to neuraminidase treated OSM. Tn-serine dimeric and trimeric structures completely inhibited binding while the Tn monomers produced only about 15–30% inhibition. Even under these conditions of hapten excess, only modest inhibition (20–40%) was noted with STn trimer and dimer clusters, demonstrating the preferential reactivity of this MAb for Tn clusters. This MAb shows minimal reactivity with OSM native mucin that is not neuraminidase digested (data not shown).

Fine specificity studies with MAb B72.3

Figure 7 demonstrates the hapten inhibition of binding of B72.3 to an OSM solid phase. Complete inhibition of binding was achieved with both STn-serine clusters (dimers and trimers), and partial inhibition was noted with STn-serine-monomer. In contrast, no inhibition of binding was noted with STn-crotyl-monomer, demonstrating the involvement of the mucin core in the specificity of MAb B72.3, including the O-glycosidic bond and the core peptide amino acid. Figure 7 also demonstrates the cross-reactivity of B72.3 with Tn-serine clusters and no demonstrable reactivity with either Tn-serine-monomer or the TF crotyl monomeric structure. This observation is further supported by the significant correlation noted between this MAb and the Tn-trimer specific MAb B230.9 when used in capture RIA assays with human ascites samples (Figure 9), and the noted reactivity with neuraminidase digested OSM. We conclude that the minimal epitope for MAb B72.3 is, therefore, the STn-O-serine structure, with optimal binding to STn-serine clusters.

Fine specificity studies with MAb CC49

Figure 8 demonstrates the dose related hapten inhibition binding of MAb CC49 to an OSM solid phase. No significant inhibition of binding was found with the Tn or STn serine monomers (data not shown). Significant inhibition of binding was noted with the STn-serine dimers or trimers and less inhibition was noted with the Tn-serine dimers or trimers (data not shown). The strongest inhibition of binding (approximately 80%) was noted with the BP24-STn$_2$-MUC-1 glycopeptide indicating strong reactivity to the dimeric STn structure within the context of a larger

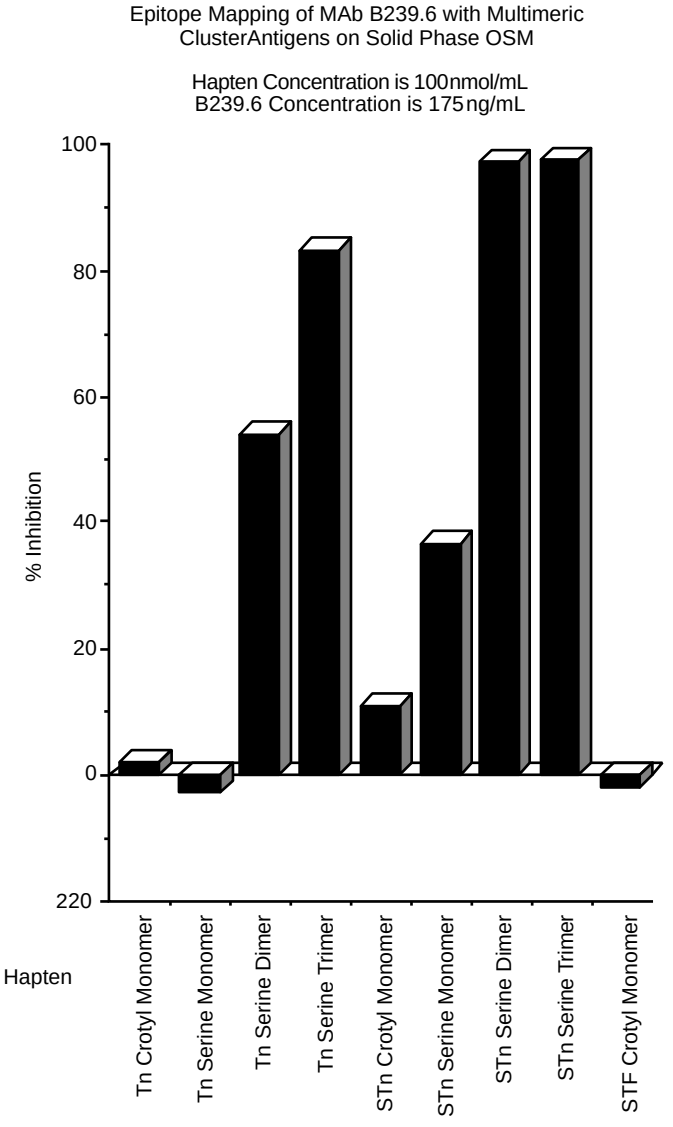

Figure 3. Results of hapten inhibition testing with MAb B239.6. The solid phase antigen is the native ovine submaxillary mucin (OSM), a mucin with repetitive STn epitopes. MAb B239.6 (175 ng ml^{-1}) was pre-incubated (1 h) with the described Tn and STn hapten constructs (100 nM ml^{-1}) prior to incubation (1 h) with the mucin solid phase. Bound antibody was detected after washing by incubating with peroxidase labeled goat anti-mouse IgG and substrate. Data are presented as the percent inhibition relative to the no inhibitor controls and represent the mean of two experiments each conducted in triplicate. Reactivity with di- and trimeric STn serines is notable as is cross-reactivity to the di- and trimeric Tn serine clusters.

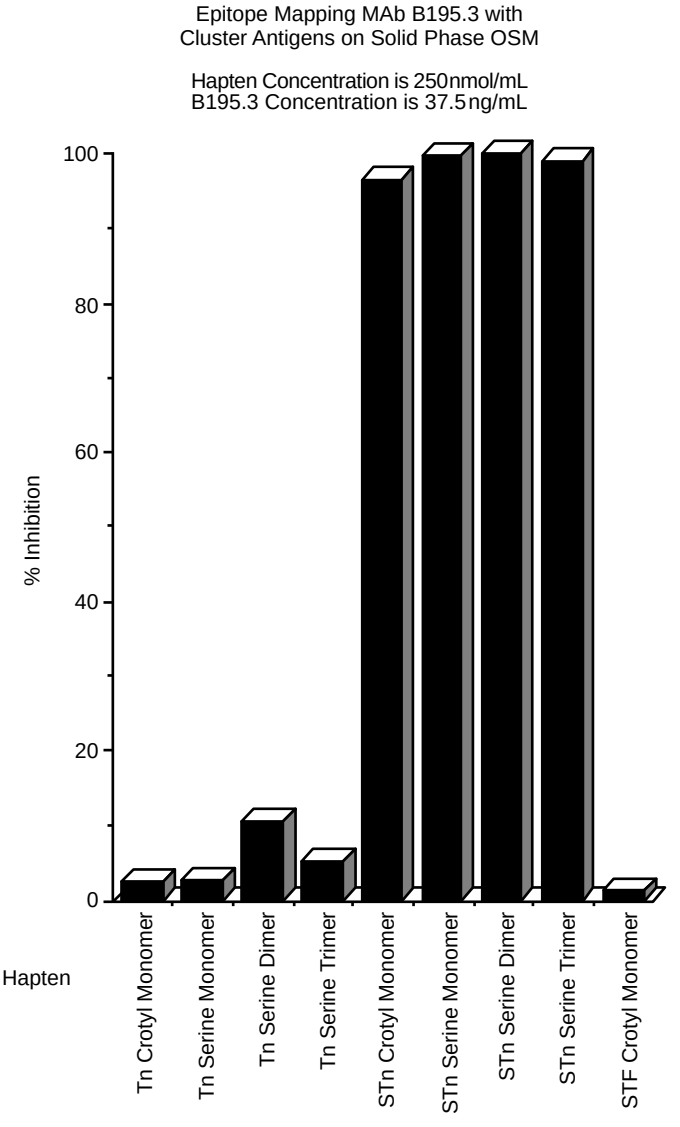

Figure 4. Results of synthetic glycopeptide hapten inhibition studies with MAb B195.3, performed as outlined in Materials and Methods. Results with the synthetic STn monomers, dimers and trimers are all equivalent and capable of complete inhibition of binding to the OSM solid phase. Of particular note is the ability of the MAb to recognize the simple disaccharide in the absence of the O-glycosidic serine core. In addition, this MAb shows no reactivity to the Tn antigen as either a monomer or multimer.

synthetic glycopeptide mucin analog. This dimeric STn cluster is a mixed cluster, STn-(threonine), STn-(serine), within the larger synthetic mucin analog.

Evidence for the presence of STn epitopes on MUC-1 mucin

MAb B27.29 has been shown to specifically react with MUC-1 core peptide sequences and is used as the tracer antibody in a commercial competitive inhibition RIA for serum MUC-1 [30]. A double determinant sandwich RIA was used to determine whether the epitopes detected by MAbs CC49, B72.3, B195.3, B230.9 and B239.9 were expressed on MUC-1 mucin derived from human cancer patients. The MAbs listed above were used as solid phase capture reagents and anti-MUC-1 core peptide specific ^{125}I-labeled MAb B27.29 [30] was used as a tracer. As a control for non-specific binding, anti-CA125 ^{125}I-labeled MAb B43.13 [31] was also used as a tracer in parallel

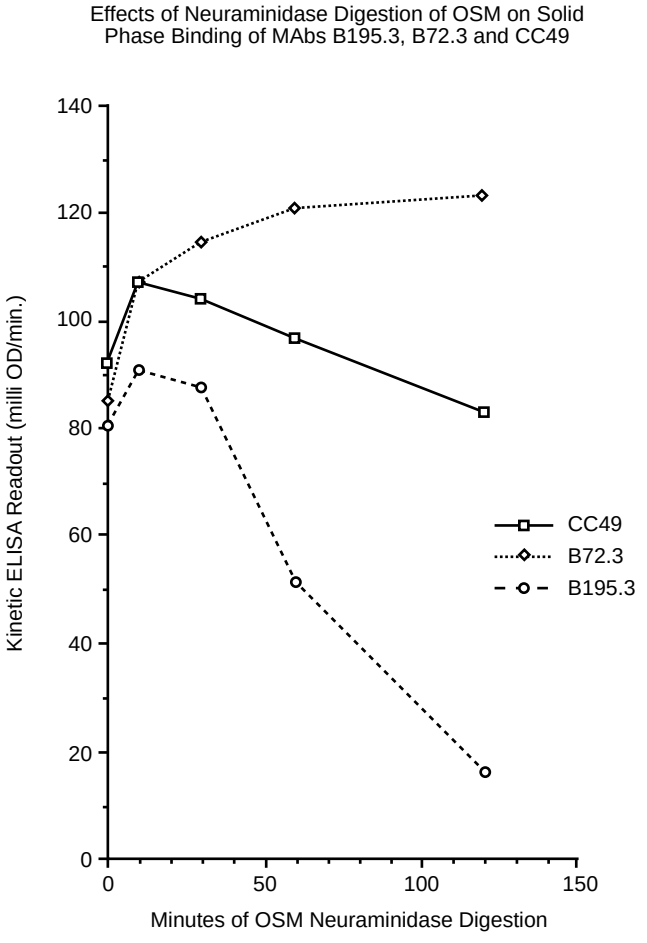

Figure 5. Results of a neuraminidase digestion time course experiment on the OSM solid phase using the MAbs B72.3, CC49, and B195.3. *Vibrio cholerae* neuraminidase (0.002 U ml^{-1}) was used to digest sialic acid from the mucin for the indicated time course prior to the addition of the specific MAbs. Rapid loss of binding for B195.3 is evident while MAbs B72.3 and CC49 retain binding activity for the digested solid phase.

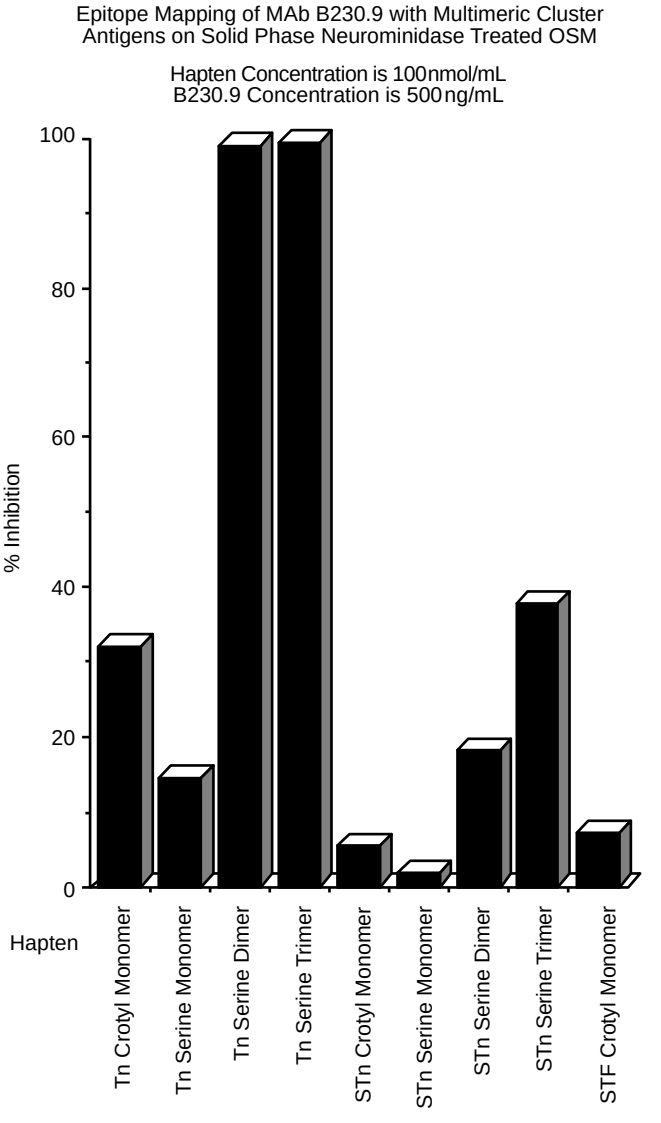

Figure 6. Results of synthetic carbohydrate and glycopeptide binding inhibition studies with MAb B230.9 on a neuraminidase digested OSM solid phase as described in Materials and methods. The various cluster and monomer constructs of Tn and STn epitopes are tested for hapten inhibition to demonstrate the specificity of this MAb that was derived from CAF1 mice immunized with the Tn-serine tripeptide cluster. Both the dimeric and trimeric Tn haptens show strong binding inhibition while the sialated structures are minimally cross-reactive, even under these hapten excess conditions.

assays. Ascites fluids from eight breast or ovarian cancer patients were tested with each capture MAb combined with each of the two tracer MAbs. Table 3 demonstrates that all five solid phase MAbs captured molecules from all eight cancer ascites fluids that reacted significantly in the sandwich RIA with anti-MUC-1 tracer MAb B27.29, with little or no reactivity with the CA125 specific control MAb B43.13. Table 4 lists the correlations of the RIA results with each capture MAb with those of every other capture MAb. We were particularly interested in comparing MAbs B72.3 and CC49 with the various MAbs derived using the synthetic Tn and STn synthetic mucin analogs. For example, Figure 9 demonstrates the highly significant correlation between the RIA results with MAb B72.3 and the anti-Tn cluster specific MAb B230.9. MAb B72.3 also showed significant correlations with MAbs B195.3 and B239.6 which also correlated with one another and B230.9. CC49 capture RIA results showed no significant correlation with any of the other capture MAbs.

Detection of STn-epitope bearing MUC-1 mucin in the sera of human cancer patients using a B195.3 heterosandwich assay with ^{125}I-labeled B27.29

Figure 10 shows the distribution of assay results with serum samples from normal Red Cross blood donors using B195.3 as the catcher antibody and ^{125}I-labeled anti-MUC-1 core

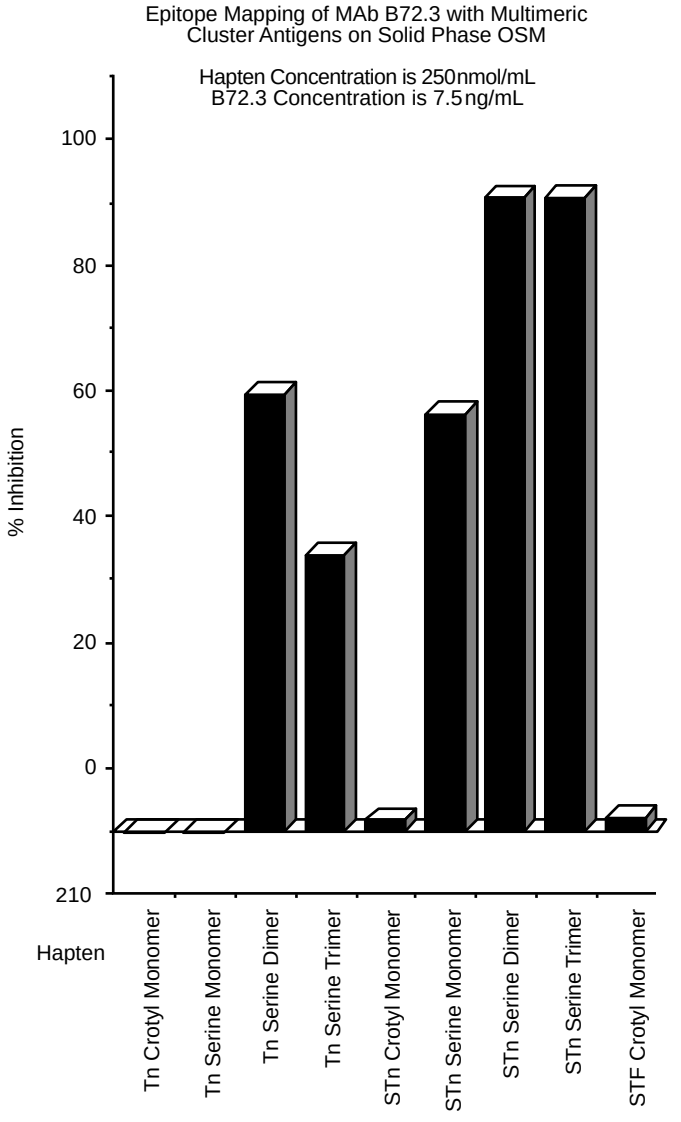

Figure 7. Results of synthetic glycopeptide binding inhibition studies with the TAG-72 specific MAb B72.3 on an OSM solid phase as described in Materials and methods. The various cluster and monomer constructs of the Tn and STn epitopes are tested for hapten inhibition to demonstrate the specificity and cross-reactivities of this mucin core specific MAb. Under these hapten excess conditions, total inhibition of binding is noted with the STn di- and tri-serine structures. Considerable cross-reactivity is noted to the dimeric and trimeric Tn antigens. The monomeric STn-O-serine gives significant inhibition while the STn crotyl disaccharide monomer does not, an indication that the O-glycosidic bond and mucin core amino acid contribute to the binding recognition of this antibody.

peptide MAb B27.29 as the tracer. A group of 30 stage III and IV ovarian cancer patient samples that had been selected for elevated CA27.29 values were compared with this normal sample distribution. Using 2 SD above the normal mean as a cut-off, 29 out of 30 samples were significantly elevated. These data indicate that a majority of ovarian cancer derived MUC-1 mucins show evidence of expression of the STn epitope on this mucin backbone. Investigation of this hetero-capture assay as a prognostic for long term survival in breast and ovarian cancinoma patients is ongoing.

Discussion

It is well established that the STn disaccharide epitope is an important tumor antigen which is associated with a poor prognosis of a variety of cancer types [3–6]. Recent studies establish that the expression of STn is related to local metastatic spread and aggressiveness of human cancer [3]. Because of the potential importance of the expression of STn in the metastatic process as well as the potential of using STn as a target for immunotherapy, further definition of the exact nature of the epitope on cancer-associated mucins is important. Our approach has been to use defined synthetic carbohydrate and glycopeptide antigens as immunogens for the derivation of new anti-STn monoclonal antibodies and to use novel synthetic peptide and glycopeptide haptens to more precisely define the specificities of anti-STn MAbs. In our previous studies [23, 29], we demonstrated that a monomeric STn-crotyl linked to KLH can be used to immunize mice for the induction of high titres of anti-STn IgG and IgM antibodies that react with natural sources of STn including STn expressed on OSM as well as on human cancer cells. These mouse studies have been extended into humans, demonstrating that the monomer STn-crotyl conjugated to KLH can induce similar populations of antibodies in human cancer patients [22, 24]. This establishes that synthetic STn molecules can be used to induce populations of antibodies that react with natural sources of the STn epitope.

MAb B195.3 was generated following immunization of mice with monomer STn-crotyl conjugated to KLH. This MAb was shown to bind to OSM, and to be specifically inhibited by STn hapten in its binding to OSM [29]. In addition, it was demonstrated that this antibody binds to human tumor cells expressing STn. We have recently presented preliminary data [25] suggesting that MAb B195.3 reacts preferentially with unclustered STn glycoconjugates. In this paper, we do in fact confirm that MAb B195.3 is specific for the monomer STn disaccharide without the need of O-serine as part of the epitope. To our knowledge MAb B195.3 is the only antibody that has excellent specificity for STn disaccharide as an isolated epitope and is non-reactive with Tn core antigen monomer or clusters. Furthermore, in the present study, we provide evidence that B195.3 reacts with STn monomers expressed on shed MUC-1 mucin from cancer patients. Though it reacts equally well with STn clusters, its binding domain is clearly defined by a single sialyl Tn epitope.

We have recently demonstrated [25] that immunization of mice with clustered STn neoglycoconjugates induces preferentially polyclonal and monoclonal antibodies which bind

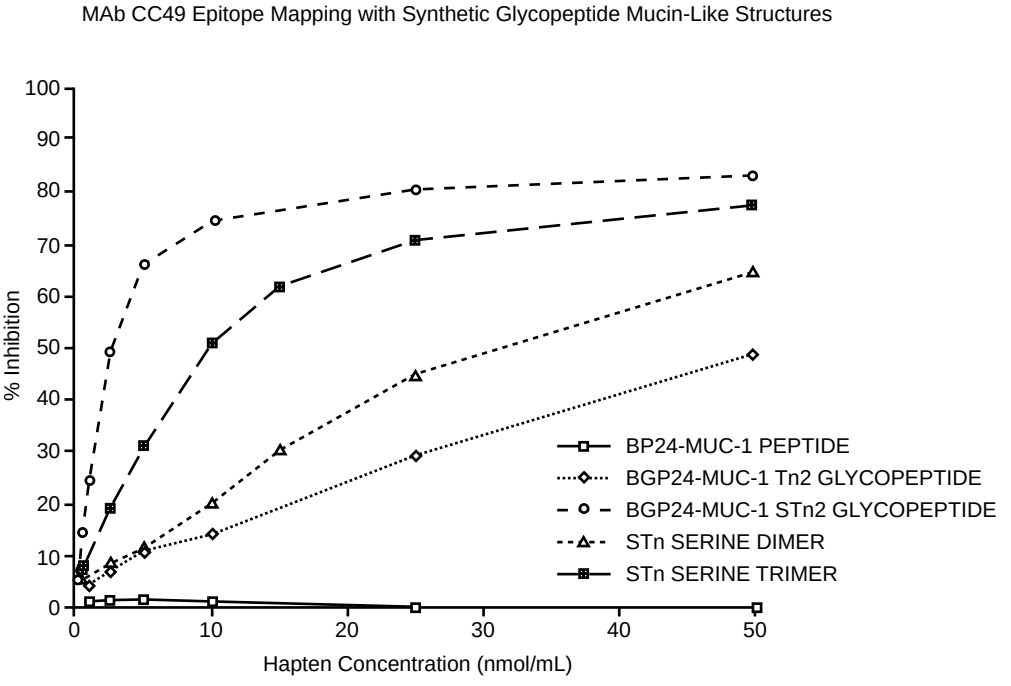

Figure 8. Results of the synthetic glycopeptide binding inhibition studies with MAb CC49 on an ovine submaxillary mucin solid phase. Monoclonal antibody was pre-mixed with the indicated concentrations of the synthetic haptens for one hour before transfer to the OSM solid phase as described in Materials and methods. Data are presented as the percentage inhibition relative to no inhibitor controls and represent the mean of three experiments, each conducted in duplicate. This monoclonal antibody shows enhanced binding inhibition with the mixed STn-threonine STn-serine cluster when presented within the 24 amino acid MUC-1 glycopeptide (BGP-24 STn^2). Some cross-reactivity to the Tn^2 24 amino acid glycopeptide is also noted. The non-glycosylated 24 amino acid peptide is non-reactive.

to clustered STn epitopes with little cross-reactive binding to monomer STn epitopes. We provided preliminary evidence that MAbs B72.3, TKH2 and B239.1 react preferentially with clustered STn epitopes [25]. Thus, these 'STn-specific' antibodies appear similar to the previously described anti-Tn MAb MLS 128 which clearly also requires the type of Tn clustering that is found on cancer-associated mucins [27]. These results suggested that STn is recognized at the tumor cell surface in at least two quite distinct configurations. In the present study, we confirm the binding of several MAbs to STn clusters. For example, MAb B239.6 was found to bind to both STn dimers and trimers while the STn serine-monomer was considerably less effective and STn-crotyl monomer did not produce any significant hapten inhibition. This establishes the specificity of MAb B239.6 for STn clusters which involve serine as part of the epitope. Furthermore, there was some cross-reaction of MAb B239.6 with Tn-serine clusters. In the case of MAb B72.3, complete inhibition of binding to OSM was achieved with both STn-serine dimers and trimers, with partial inhibition with STn-serine monomers, and no inhibition with STn-crotyl monomers, all similar to MAb B239.6. The specificity of MAb B72.3 is similar to the STn-serine cluster MAbs generated in these studies. Also similar to MAb B239.6, B72.3 did have cross-reactivity for Tn-serine clusters suggesting that B72.3, which was generated using a natural source of STn, is very similar in its specificity to MAb 239.6 which was generated with synthetic STn-serine trimers conjugated to KLH.

These results are consistent with the data of Springer et al. [32], who reported that B72.3 was capable of agglutinating red blood cells expressing Tn. Also, previous hapten studies by Johnson et al. with MAb B72.3 [33] demonstrated OSM binding inhibition with Tn monomeric structures and STn monomers under similar assay conditions. In these studies, however, millimolar concentrations of the monomeric haptens were required to achieve significant binding inhibition. In this report, we show that only micromolar concentrations are required for comparable inhibition when the serine dimeric or trimeric antigen clusters are used.

In the case of MAb CC49, which is a second generation TAG-72 antibody, the STn-serine dimers and trimers inhibited the binding of CC49 to OSM by about 80%, while the STn-serine monomer did not produce a significant inhibition, demonstrating the preferential reactivity of MAb CC49 with clustered STn-serines as well. In addition, some cross-reactivity for CC49 for Tn-serine clusters was noted. Since only 80% inhibition of binding was noted and the reactivity of CC49 with MUC-1 mucin did not correlate with the reactivity of the other MAbs in this study, additional possible specificities of this antibody need to be

investigated. We were unable, however, to confirm the inhibition of binding of this antibody by monomeric sialyl-TF [34].

Our data suggests that MAb B195.3 strongly reacts with STn monomer with no detectable cross-reactivity to Tn, TF or STF. As yet, we have no evidence that any of the antibodies we tested are truly specific for STn clusters as all of the anti-cluster antibodies show some cross-reactivity with the Tn clusters. This suggests that immunohistological data based on the binding of putative anti-STn antibodies in assessing the degree of expression of STn on tumors has to be interpreted with a degree of caution since all of the anti-STn antibodies in current use for immunohistology studies, including B72.3, CC49, and TKH2, are STn cluster reactive with cross-reactivity to Tn clusters. The generation of a MAb with true specificity for STn clusters without cross-reactivity to Tn would be a valuable reagent to estimate the expression of STn clusters on tumor cells and for

Table 3. Heterosandwich RIA results with eight (8) human ascites samples selected for elevated concentrations of CA5.3 antigen. Various STn or Tn specific MAbs served as solid phase capture antibodies with anti-MUC-1 (B27.29) or anti-CA125 (B43.13) radiolabeled MAbs as tracers. Data presented as percentage of input bound over total input.

Ascites Number	*% B/T (Solid Phase MAb)*				
	CC49	*B72.3*	*B195.3*	*B230.9*	*B239.9*
A. B27.29 Tracer, 157038 cpm input					
None	1.3	1.2	1.4	0.9	1.3
151	19.0	11.5	26.1	13.0	26.3
160	38.6	23.8	48.7	25.2	39.0
161	46.5	40.8	51.2	42.2	44.1
164	47.0	43.2	51.0	35.9	42.1
196	30.8	53.3	49.0	41.5	48.4
313	39.4	30.8	36.3	29.4	43.8
325	45.0	13.3	29.4	5.4	25.4
328	31.3	13.2	30.5	27.7	43.9
B. B43.13 Tracer, 95590 cpm input					
None	0.3	0.2	0.5	0.3	0.4
151	0.3	0.3	0.4	0.4	0.6
160	5.3	2.4	4.8	2.2	4.4
161	6.3	2.6	5.3	1.8	4.8
164	7.1	3.2	6.3	2.1	5.6
196	0.5	0.4	0.7	0.5	0.5
313	4.8	2.2	4.4	1.0	5.5
325	2.0	1.1	2.8	0.7	2.3
328	0.3	0.5	0.5	0.6	0.5

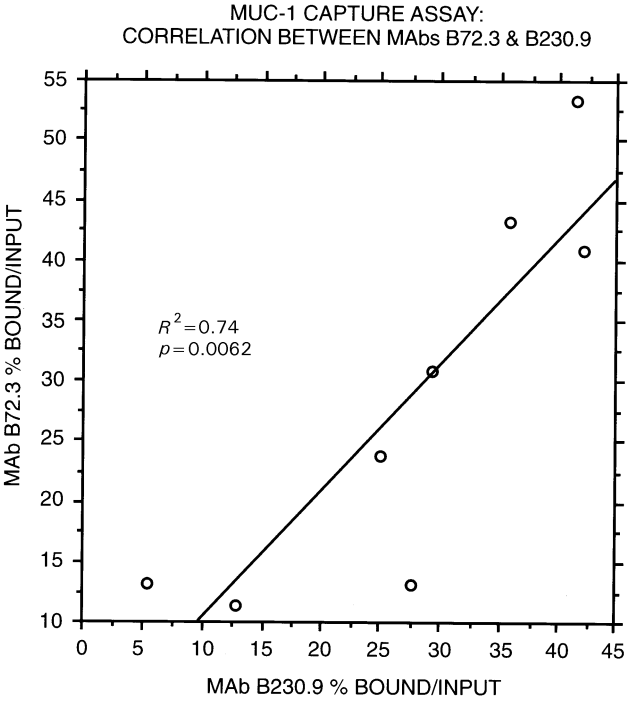

Figure 9. Linear regression analysis of data generated from heterosandwich capture RIA assays using either a B72.3/B27.29 I-^{125} combination assay or a B230.9/B27.29 I-^{125} combination assay. Samples (n = 8) are human ascites fluids collected from patients with adenocarcinomas of the breast or ovaries, and had been selected for elevated concentrations of the MUC-1 mucin as determined by the TRUQUANT® BR™ radioimmunoassay [28]. Samples were centrifuged and filtered to remove cell debris prior to 1:10 dilution in pH 5.0 acetate buffer for assay. A significant correlation is seen between results in the Tn-multimeric specific MAb based assay (B230.9) and the results generated by capture with the TAG-72 specific monoclonal B72.3

Table 4. Correlations among various MAbs used as solid phase 'catchers' for MUC-1 mucin from cancer ascites fluids.

MAbs	*CC49*		*B72.3*		*B195.3*		*B230.9*	
	R2	*p*	*R2*	*p*	*R2*	*p*	*R2*	*p*
B72.3	0.12	0.39	–	–	–	–	–	–
B195.3	0.28	0.18	0.72	0.008	–	–	–	–
B230.9	0.06	0.55	0.74	0.006	0.65	0.016	–	–
B239.6	0.05	0.61	0.54	0.039	0.44	0.071	0.86	0.001

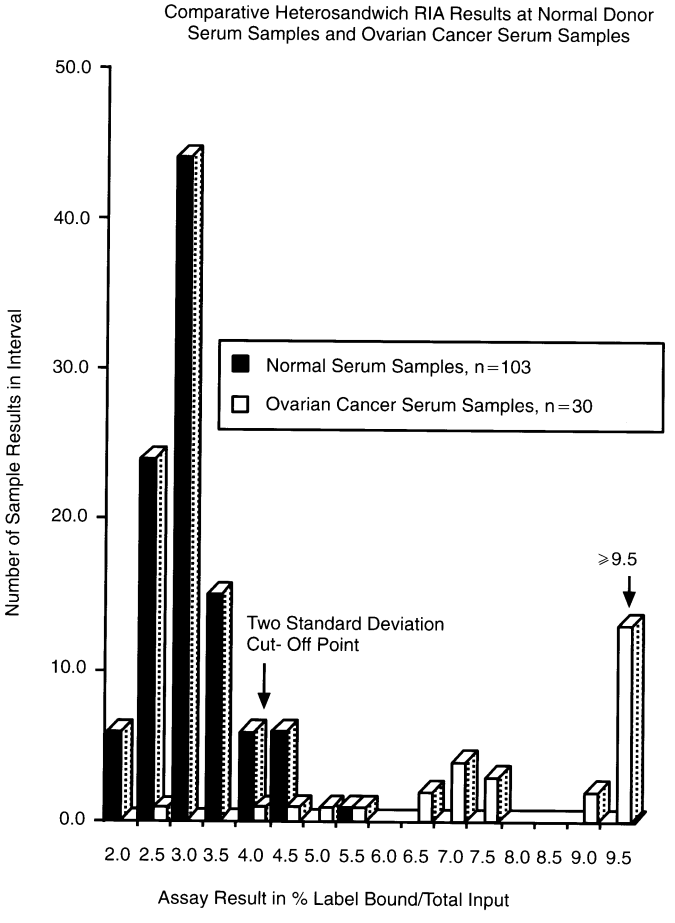

Figure 10. Results of the heterosandwich RIA capture assays with random normal blood donor serums (n = 103) and stage III/IV ovarian cancer serum samples. Malignant ovarian samples (n = 30) were pre-selected for elevated CA27.29 antigen levels as determined by TRUQUANT® BR™ RIA [28]. Heterosandwich assays utilized the STn monomer specific MAb B195.3r11 as the solid phase capture antibody and the MUC-1 peptide core specific MAb B27.29 serves as the I^{-125} labeled tracer antibody. Results are expressed as the percentage of total radioactive input that is bound (% B/T).

specific binding studies involving the putative role of clusters versus monomers in tumor metastases and/or adhesion to endothelial cells.

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This manuscript is dedicated to the memory of Dr Wu Hong.

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