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Cluster binding studies with two anti-Thomsen-Friedenreich (anti-core-1, CD176, TF) antibodies: Evidence for a multiple TF epitope

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ABSTRACT

Antibodies to carbohydrate epitopes are often of the IgM isotype and require multiple binding for sufficient avidity. Therefore clusters of epitopes are preferred antigenic sites in these cases. We have examined the type of clusters recognized by two anti-Thomsen-Friedenreich (TF, core-1, CD176) IgM antibodies, NM-TF1 and NM-TF2, using several different sets of TF-carrying synthetic glycoconjugates in ELISA experiments. To our surprise, the single most important factor determining binding strength was a close vicinity of several TF glycans at distances of ≤1 nm. Considering the known dimensions of IgM antibodies, our data strongly suggest that a cluster of up to four TF moieties, presenting as a "multiple epitope", is required to attach to a single combining site in order to result in adequate binding strength. This effect can also be achieved by "surrogate-multiple epitopes" consisting of separate TF-carrying molecules in close vicinity. In addition, it was found that serine-linked TFs are stronger bound than threonine-linked TFs by both antibodies. This peculiar type of cluster recognition may contribute to improved avidity and explicit tumor specificity.

1. Introduction

Monoclonal antibodies have become increasingly important drugs in the treatment of cancer and other diseases. Most of them target protein antigens. However, many important tumor markers are, in fact, carbohydrate structures [1,2]. One of the most specific tumor antigens is the Thomsen-Friedenreich (TF) disaccharide (Galβ1-3GalNAcα1-) [3–8], also known as core-1 or CD176, an intermediate in *O*-glycan biosynthesis which is atypically exposed on many types of cancer cells. We have generated several highly specific monoclonal antibodies to this structure [6,9]. Since they are IgM antibodies, they are expected to recognize TF clusters because of the generally weak affinities of single carbohydrate-antibody interactions. However, the exact nature of the clusters recognized by these antibodies was up to now unknown, especially with respect to the number of binding sites involved and the spatial requirements between TF units for optimal binding. In order to better understand these interactions, we started a number of ELISA experiments with two anti-TF antibodies, NM-TF1 and NM-TF2, and different sets of synthetic glycopeptides imitating either native TF-carrying structures (asialoglycophorin, anti-freeze glycoproteins of Antarctic fish), or polyacrylamide (PAA) conjugates of different TF densities. In addition, we have examined the influence of the amino acid carrying TF (serine versus threonine) on antibody binding. In both cases, the results were at first sight rather unexpected.

2. Materials and methods

2.1. Antibodies and antigens

The antibodies examined in this study were NM-TF1 and NM-TF2 [6]. Both are mouse anti-TF IgM (κ) antibodies, generated against human asialoglycophorin A (aGP) as immunogen. Both are specific for TFα (Galβ1-3GalNAcα1-) with slight cross-reactivity toward TFβ (Galβ1-3GalNAcβ1-). NM-TF2 shows, in common with most other known anti-TF antibodies, some cross-reactivity to core-2 (Galβ1-3[GlcNAcβ1-6]GalNAcα1-), whereas NM-TF1 does not [6]. (In the

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Table 1
Primary antibodies employed in this study.

Antibody	Isotype	Immunogen/Specificity	Preparation	Source
NM-TF1	Mouse IgM	aGP/TF	Supernatant or purified	Glycotope [6]
NM-TF2	Mouse IgM	aGP/TF	Supernatant or purified	Glycotope [6]
hIgG	Humanized NM-TF2 IgG	aGP/TF	Supernatant or purified	Glycotope
hIgM	Humanized NM-TF2 IgM	aGP/TF	Supernatant or purified	Glycotope
A63-C/A9	Mouse IgM	aGP/ TF on aGP (mixed epitope)	Supernatant	Glycotope [10]
LCA	Mouse IgG1 (mixture of 2B11 + PD7/26)	Leukemic cells/CD45	Concentrated	Immunotech Marseille, France
A89-B/D1	Mouse IgM	*Rhodococcus fascians* (isotype control)	Supernatant	Glycotope [11]

Table 2
Synthetic glycopeptides used as antigens.

A. Synthetic glycopeptides related to anti-freeze glycoprotein (AFGP). Three sets were synthesized: unlabeled (I), labeled (II), and Thr replaced by Ser, also labeled (III). Each set consists of glycopeptides of different length (n = 1-6 or 1-5).

Set:	Set I	Set II	Set III
n (Number of repeats = number of **TF**s)	(-A-[TF]T-A-)$_n$ Unlabeled Hokkaido University MW [Da][1]	(-A-[TF]T-A-)$_n$-[Btn]K-amid Biotin-labeled Biosyntan MW [Da]	(-A-[TF]S-A-)$_n$-[Btn]K-amid Biotin-labeled Biosyntan MW [Da]
1	627	1051	1037
2	1235	1660	1632
3	1843	2269	2227
4	2451	2878	2821
5	3059	3486	3416
6	3668	4095	-

[1]Calculated as average mass.

B. Synthetic glycopeptides corresponding to two sections of the extracellular asialoglycophorin (aGP) domain [12]. All are biotinylated at the C-terminus.

Sequence of aGP	Synthetic aGP glycopeptides[1]	MW[2] [Da]	Number of TFs	Remarks
A aa[3] 9-25	**A**: HTST*S*SSVT*KSYIS*SQT-K(Btn)-amid	3616	4 [2 tight]	Native sequence, but less v
	A-: HTSTSSSVTKSYISSQT-K(Btn)-amid	2155	0	Native sequence, not glycosylated
B aa 34-55	**B**: YAAT*PRAHEVS*EIS*VRT*VYPPE-K(Btn)-amid	4286	4	Native sequence

[1] T* or S*: Thr or Ser *O*-glycosylated with TF.
[2] Calculated as average mass.
[3] aa: amino acids.

further context of this paper, TF always means TFα). Both antibodies bind to anti-freeze glycoprotein (AFGP), to TF-BSA, and to other TF-positive structures (data not shown). In addition, humanized derivatives from NM-TF2 were also employed in some experiments. Together with some further antibodies employed, they are shown in Table 1.

The synthesized glycoconjugate antigens are listed in Table 2. They consist *i.* of a series of polyacrylamide (PAA) conjugates of TF providing different densities of the glycan along the PAA spiral molecule, *ii.* glycopeptides corresponding to anti-freeze glycoprotein (AFGP), but of different length (Table 2A), *iii.* glycopeptides as in *ii.*, in which threonine was replaced by serine (Table 2A), *iv.* glycopeptides corresponding to certain sequences of native aGP (Table 2B), and finally, *v.* model glycopeptides imitating TF clusters of aGP (Table 2C). Most glycopeptides were synthesized by BIOSYNTAN GmbH, Berlin-Buch, Germany. AFGP glycopeptides were synthesized at two different laboratories (Table 2). PAA-conjugates were purchased from Lectinity, Moscow, Russia. Native glycophorin A (GP) and asialoglycophorin A (aGP) were purchased from Sigma-Aldrich, Taufkirchen, Germany. Native anti-freeze glycoprotein (AFGP) samples prepared from Antarctic fish were kindly provided by Dr. A. Wöhrmann (Boehringer, Mannheim, Germany); in this study we used the 30 kDa fraction.

2.2. Immunocytochemistry and immuno-electron microscopy

Test cells were KG-1 (acute myelogenous leukemia) [13]. Immunocytochemistry was performed as immunofluorescence assays on 10-well multitest slides (Menzel, Braunschweig, Germany), on which the cells were suspended and allowed to settle in a CO_2 incubator for 1 h. Thereafter medium was carefully sucked off, and the slides were air-dried. Cells were fixed with 5% formaldehyde (histology grade, Merck, Darmstadt, Germany; 5 min), washed, and incubated with the primary antibody (1 h), followed by DTAF (5-[4,6-dichlorotriazinyl] aminofluorescein)-labeled anti-mouse IgG or Cy3-labeled anti-mouse IgM secondary antibodies (both from Jackson ImmunoResearch Laboratories, purchased from DIANOVA, Hamburg, Germany). Slides were embedded in mounting buffer and examined with a Zeiss Axiophot photomicroscope (Carl Zeiss, Jena, Germany) equipped with a triple immunofluorescence filter system. For immunoelectron microscopy, KG-1 cells were incubated with NM-TF2 supernatant for 1 h, washed, and incubated with colloidal gold-AffiniPure goat anti-mouse IgM

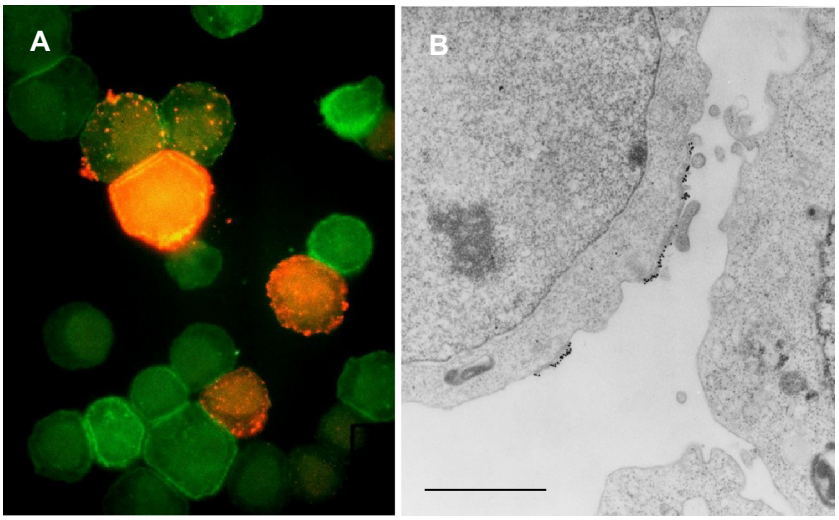

Fig. 1. Thomsen-Friedenreich (TF) antigen clusters at the cellular level shown on KG-1 (human AML cell line) cells as a typical example. A: immunofluorescence picture double-stained for CD45 (LCA, green) and TF (NM-TF2, red). 100×, oil lens. B: immunoelectron microscopic picture, NM-TF2, immunogold labeling, 26,000×, bar 1 μm.

antiserum (EM grade, Jackson), diluted 1:50, for 20 min. After washing cells were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer for 3 days at 4 °C, and postfixed with 1% osmium tetroxide for 1 h. After dehydration with graded ethanol cells were embedded in Poly/Bed 812 (Polysciences Inc., Eppenheim, Germany). Ultrathin sections were counterstained with uranyl acetate and lead citrate, and analysed with a Philips EM 400 T electron microscope at an acceleration voltage of 80 kV.

2.3. ELISA experiments

Non-conjugated glycopeptides and TFα-PAA were coated on MaxiSorp microtiter plates (Nunc, Wiesbaden, Germany, 96 wells) at 5 μg/ml in PBS (50 μl per well) and kept in a moist chamber at room temperature (RT) overnight. Next day the wells were blocked with bovine serum albumin (1% in PBS, 100 μl per well, 2 h). Thereafter, dilutions of purified antibody in PBS/BSA (or, in some cases, supernatant) were added and incubated for 2 h at RT. As second antibody POD-conjugated rabbit anti-mouse immunoglobulin (P0260, DakoCytomation, Hamburg, Germany; diluted 1:2000) was used (90 min, RT). Washing steps were done 3 times each with PBS containing 0.05% Tween 20. Staining was achieved with TMB solution (tebu-bio, Frankfurt, Germany), 100 μl/well. The reaction was stopped with 50 μl of 2.5 N H_2SO_4, and the OD was measured in an ELISA reader at 450 nm versus 620 nm. Blank values were subtracted.

Biotinylated glycopeptides were coated on streptavidin-coated microtiter plates (BioTeZ GmbH, Berlin-Buch, Germany, standard capacity). Further treatment was similar with a few exceptions: glycopeptide concentrations were varied, the second antibody was used at 1:1000, and the reaction time was varied (3–20 min).

Graphs were prepared with GraphPad Prism (San Diego, CA, USA). Values are means ± SD.

2.4. Surface plasmon resonance (SPR) analysis

Binding data were generated by SPR analysis with a BIAcore 2000 instrument (BIAcore, Pharmacia, Upsala, Sweden) and evaluated with the BIAevaluation software 3.1.

2.4.1. Immobilization of ligands

Goat anti-mouse IgM, μ-chain specific, or goat anti-mouse IgG + IgM, H + L antibodies (Jackson) were covalently coupled to the dextran film of the sensorchip CM5 (research grade, BIAcore) by amine coupling (BIAapplication Handbook). For immobilization, 40 μg/ml of the antibodies were dissolved in 10 mM acetate buffer (pH 5.0). The flow rate was set at 10 μl/min. The reaction was stopped with ethanolamine.

2.4.2. Binding of the analytes

After docking the chip to the flow cell system, the monoclonal antibody and thereafter different concentrations of the antigen (e.g. aGP) were slowly injected (flow rates were 10 μl/min for the antibody and 30 μl/min for the antigens) into the individual flow cells. Reference experiments were carried out using HBS buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween 20). In addition, control experiments were done with the almost non-binding TF10-PAA (see below) in case of TF30-PAA, or with the irrelevant IgM antibody A89-B/D1 in experiments with aGP. The following parameters were calculated:

k_a: association rate [$M^{-1}\,s^{-1}$].

k_d: dissociation rate [s^{-1}].

K_A: equilibrium binding (affinity) [M^{-1}]; $K_A = 1/K_D = k_a/k_d$.

K_D: equilibrium dissociation [M]; $K_D = 1/K_A = k_d/k_a$.

Values are means ± SEM.

3. Results

3.1. Preliminary data

3.1.1. Immunocytochemistry and immuno-electron microscopy

Immunocytochemistry with NM-TF-2 reveals that the TF-carrying membrane glycoprotein is not homogeneously distributed on tumor cells (as is, for instance, the case with CD45). Instead it is arranged as distinct membrane clusters of different sizes (Fig. 1A). Immuno-electron microscopic pictures confirm this in more detail (Fig. 1B). The diameter of these TF clusters at the cellular level ranges from below 0.1 to 0.5 μm. This type of TF clustering is a common picture among many types of cancer cells, although the carrier molecules of TF vary [14]. An interesting detail is that the TF clusters are based on a characteristic structure of electron dense material immediately below the cell membrane (Fig. 1B).

3.1.2. Binding of anti-TF antibodies to asialoglycophorin (aGP) and to synthetic glycopeptides derived from it

It is no surprise that both antibodies strongly bind to aGP because this was the immunogen employed to their generation. Asialoglycophorin A carries 16 TF moieties at different, single as well as clustered positions [12]. Nine TFs are bound to threonine, and seven to serine. Fig. 2 presents ELISA data obtained with two synthetic glycopeptides representing two different sections of the extracellular domain of the aGP molecule, arbitrarily called glycopeptides A and B

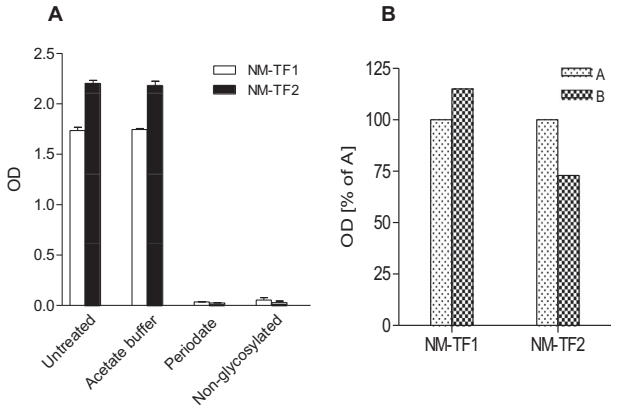

Fig. 2. Basic specificity data of antibodies NM-TF1 and NM-TF2, demonstrated on two synthetic TF-carrying glycopeptides copying two different sequences of native aGP (incidentally called A and B; see Table 2B). A: glycopeptide A; carbohydrate specificity of the epitope shown by its sensitivity toward mild periodate oxidation and by the absence of binding to the non-glycosylated peptide. B: absence of peptide involvement in the epitope shown by antibody binding to two different sequences of aGP (A and B).

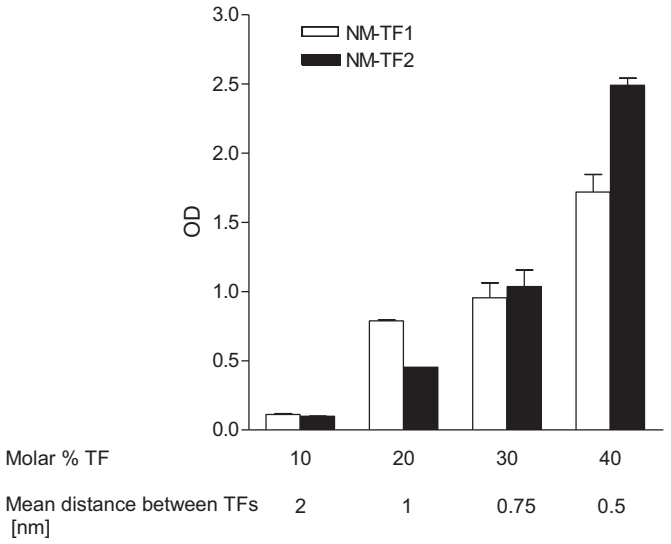

Fig. 3. Binding of antibodies NM-TF1 and NM-TF2 to TF-polyacrylamide (PAA) conjugates of different TF content as indicated (10 to 40 M % TF). The approximate mean distances between TF units are also indicated.

(Table 2B). Fig. 2A demonstrates that binding is carbohydrate-dependent, since both the glycopeptide A after treatment with periodate under carbohydrate-specific conditions according to [15], which destroys the terminal Gal, as well as the nonglycosylated peptide are completely negative in ELISA. Fig. 2B shows that both glycopeptides are recognized by the anti-TF antibodies, demonstrating that their epitopes do not involve parts of the peptide sequence. NM-TF2 apparently slightly prefers glycopeptide A. It should be noted that both glycopeptides contain the same number and types of TF (2 × Thr-TF, 2 × Ser-TF) but positioned at different distances: whereas glycopeptide A carries a cluster of two TFs in immediate neighborhood, glycopeptide B carries only separate TFs (see Table 2B). From these data no preference with respect to TF distribution patterns can be concluded.

3.1.3. Binding constants of both antibodies

Binding data (functional affinities) toward different TF carrying antigens were determined by surface plasmon resonance experiments (Table 3). It can be seen that both antibodies revealed almost identical affinities to aGP (K_A ~$6.5 \times 10^8\ M^{-1}$). Binding to aGP is stronger than that to artificial TF carrying molecules (either TF30-PAA [containing 30 M % TF] or synthetic AFGP). On the other hand, binding is lower compared to another IgM antibody recognizing a mixed peptide-TF epitope of aGP, A63-C/A9.

3.2. Binding to different TF carrier models

In order to examine whether the antibodies recognize molecular clusters, and if so, how they look like, more sophisticated models had to be designed. We decided to employ three different sets of synthetic glycoconjugates carrying TF in defined distribution patterns.

3.2.1. Model 1: binding to TF-PAA conjugates

The first model consisted of TF-PAA conjugates [16] with variable molar proportions of TF. An increase of the molar percent of TF leads to shorter mean distances between neighboring TFs as given in Fig. 3 (X-axis, lower line). The (non-biotinylated) PAA conjugates were coated at equal concentrations (5 µg/ml), which leads to saturation of coating. The results are shown in Fig. 3. As expected, OD values increased with the molar per cent of TF. This was true for both antibodies. However, two details were unexpected: First, TF-PAA carrying 10 M % TF was only marginally recognized, and second, the increase in OD was not proportionate to the increase in the TF content (especially in case of NM-TF2). We concluded that the vicinity of TF moieties appeared to be the crucial factor for antibody binding. Distances of > 1 nm between neighboring TFs were obviously too wide for significant binding. This was surprising, since IgM molecules should easily be able to span distances of up to 40 nm.

3.2.2. Model 2: binding to mono- and multivalent AFGP glycopeptides

Antifreeze glycoprotein (AFGP) [17–20] with its simple structure carrying multiple TFs at distances of about 1 nm appeared to be a promising model for our purpose. First we examined in ELISA whether the antibodies bound to native AFGP prepared from *Pleuragramma antarcticum* [18], which was the case (data not shown). We then employed three sets of synthetic AFGP glycopeptides of different length

Table 3
Binding constants of NM-TF1 and NM-TF2.

Test antibody	NM-TF1	NM-TF2	NM-TF2	NM-TF2	A63-C/A9
Antigen	aGP	aGP	$syAFGP_6$	TF30-PAA	aGP
Control	A89-B/D1	A89-B/D1	A89-BD1	TF10-PAA	A89-B/D1
K_A [M^{-1}]	6.57×10^8	6.49×10^8	2.26×10^8	4.02×10^6	4.94×10^9
K_D [M]	1.52×10^{-9}	1.54×10^{-9}	4.42×10^{-9}	2.49×10^{-7}	2.02×10^{-10}
k_a [$M^{-1}\ s^{-1}$]	9.97×10^4	1.53×10^5	1.84×10^4	3.19×10^3	2.05×10^5
k_d [s^{-1}]	1.52×10^{-5}	2.36×10^{-4}	7.96×10^{-5}	7.93×10^{-4}	4.14×10^{-5}

Association and dissociation constants for each antigen were calculated from the evaluation of four overlaid BIAcore curves, respectively. Antibody, antigen, and the non-binding reference antibody (or the non-binding reference antigen) are shown. TF30-PAA contains 30% TF; TF10-PAA 10% TF. Concentrations of aGP were 16, 32, 64, 96 nM, and 50, 100, 150, and 200 nM for $syAFGP_6$, and 200, 400, 600, and 800 nM for the TF30-PAA conjugate. Binding constants of A63-C/A9 were taken from [10].

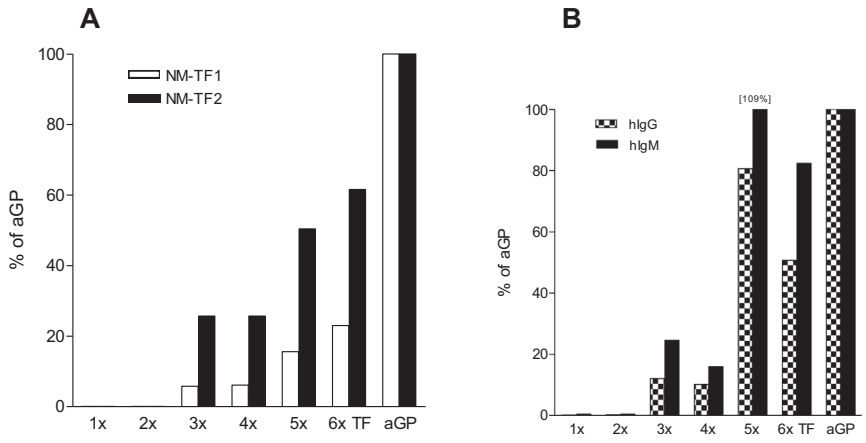

Fig. 4. A: Binding of antibodies NM-TF1 and NM-TF2 to synthetic anti-freeze glycoprotein (AFGP) sequences (set I) of different length (carrying 1–6 TF units), calculated as percent OD compared to aGP. B: similar experiment as in A, but performed with humanized antibodies of IgM or IgG isotype (hIgM, hIgG), respectively, both derived from NM-TF2. Absolute OD values in case of hIgG were significantly lower compared to hIgM.

(Table 2A). Set I consisted of non-biotinylated glycopeptides ranging from 1 to 6 units of (-AT[*TF*]A-)$_n$, (n = 1–6) [20]. They were coated at 5 μg/ml (saturated coating conditions). The results are shown in Fig. 4A. OD values to AFGP were generally lower compared to aGP. However, a striking increase of OD values was observed from 1 × TF to 6 × TF, which looks similar to the graph obtained with TF-PAA conjugates (Fig. 3). In case of AFGP short glycopeptides containing 1 or 2 TF saccharides were completely negative (Fig. 4A). This lack of binding poses again the same problem to the interpretation of the results. IgM antibodies are able to multiple binding, which should apply here. In any case, IgM should perform differently from IgG in this respect. To our surprise, this was not the case. This is shown in Fig. 4B, in which humanized IgG and IgM antibodies derived from NM-TF2 were examined under the same conditions as in Fig. 4A, except that (in this case only) supernatants were used, and that anti-human Ig secondary antibodies were employed. Both isotypes revealed a similar binding pattern toward the AFGP glycopeptides of different length, and both did not bind to short (1 or 2 TF containing) conjugates. The only obvious difference was that in absolute terms the OD values measured with hIgG were only around 25% of the OD values obtained with hIgM (data not shown). The general lack of binding to 1 or 2 TF-carrying glycopeptides suggests that a special type of clustering is required for effective binding.

Therefore we started further experiments with two new sets of AFGP glycopeptides (II and III) which were C-terminally biotinylated (Table 2A), thus allowing for better defined coating conditions. Set III was prepared 'artificially' with Ser instead of Thr as carrier for TF. The assay conditions were as follows. Coating was done at saturating or non-saturating conditions (0.2 μg/ml or 0.05 μg/ml, respectively), and a low antibody concentration was chosen (0.65 μg/ml). The results are shown in Fig. 5. Two aspects of the outcome were again surprising. First, it was unexpected that both antibodies bound much stronger to Ser-TF than to Thr-TF. Second, the previously described increase in OD from 1 × TF to 5× (or 6×) TF (Fig. 4) was only confirmed at saturating coating conditions. At non-saturating coating conditions none of the glycopeptides resulted in significant binding. This is most clearly seen on the lower panel of Fig. 5 (C, D) due to the generally higher OD values obtained with Ser-TF containing glycopeptides. At saturating coating conditions, the increase in OD from 1 × TF to 5 × TF was more than proportionate to the number of TFs, especially in case of NM-TF2 (Fig. 5D). With NM-TF1, the binding to AFGP type glycopeptides was generally weak compared to its binding to aGP (see Fig. 4A) and also lower compared to NM-TF2 (Fig. 5A,C versus B,D).

The AFGP data confirm the absence of binding to single TFs. The necessity of dense coating for significant antibody binding is interpreted as follows. First, the presence of > 2 TFs per molecule is apparently in itself not sufficient for binding. This may be due to reasons of spatial arrangement. Second, even under saturating coating conditions, a certain minimal length of the TF presenting molecule is required for antibody binding, probably due to higher flexibility. These experiments again point toward the necessity of dense clusters of TF for antibody binding, but do not sufficiently define these clusters.

3.2.3. Model 3: binding to short glycopeptides modeling aGP clusters

The data obtained so far suggest that the antibodies NM-TF1 and NM-TF2 reveal cluster binding patterns not explained by typical multiple IgM binding. In order to get more insight into the type of clusters recognized, we designed a novel panel of poly-Gly chains carrying either a single Thr-TF or clusters of Thr-TF/Ser-TF (Table 2C). The poly-Gly chains permit maximum flexibility to the glycopeptides. An additional glycopeptide was synthesized similar to 2 × TF except that both TFs were separated by 9 × Gly (corresponding to an approximate distance of 3 nm). This construct was called 2 × 1 TF. All glycopeptides were C-terminally biotinylated, and coated at equimolar concentrations (either 50 nM, leading to saturated coating, or 10 nM, leading to non-saturated coating).

The results are presented in Figs. 6 and 7. In Fig. 6 once again the glycopeptide carrying a single TF was only marginally recognized. Increasing numbers of clustered TFs led to an increase in binding. NM-TF1 and NM-TF2 differed slightly in this respect. An intriguing result was the difference in antibody binding between the two glycopeptides carrying 2 TFs each. NM-TF2 at saturating coating conditions recognized two clustered TFs to some extent (Fig. 6A, "2 × TF"), but not the same two TFs at a distance of 3 nm (Fig. 6A, "2 × 1 TF"). This suggests again that a close vicinity of at least two TFs is obviously necessary for antibody binding. However, recognizing of 2 × TF apparently depends on additional factors such as the density of coating. Fig. 6B shows that at low coating conditions NM-TF2 almost exclusively binds to the quadruple TF cluster. Fig. 7 finally confirms the conclusions previously drawn by demonstrating that the (humanized) IgG derivative from NM-TF2 reveals a similar binding pattern compared to its IgM counterpart. The only deviation seen was a difference in binding to the 2 × TF glycopeptide. The total OD values of the hIgG variant compared to hIgM obtained under identical ELISA conditions, however, were clearly lower (around 1/20 compared to hIgM, data not shown). This points toward an avidity enhancing effect of multimeric (IgM-typical) binding.

4. Discussion

This study was designed to examine and possibly identify the type of clusters actually recognized by the anti-TF IgM antibodies NM-TF1 and NM-TF2. Originally, it was anticipated that the structure best bound by the antibodies would be, in principle, a collection of TFs at distances suited for multivalent IgM binding, thereby leading to measurable functional affinities. However, the results of the study were at first glance not in agreement with this expectation.

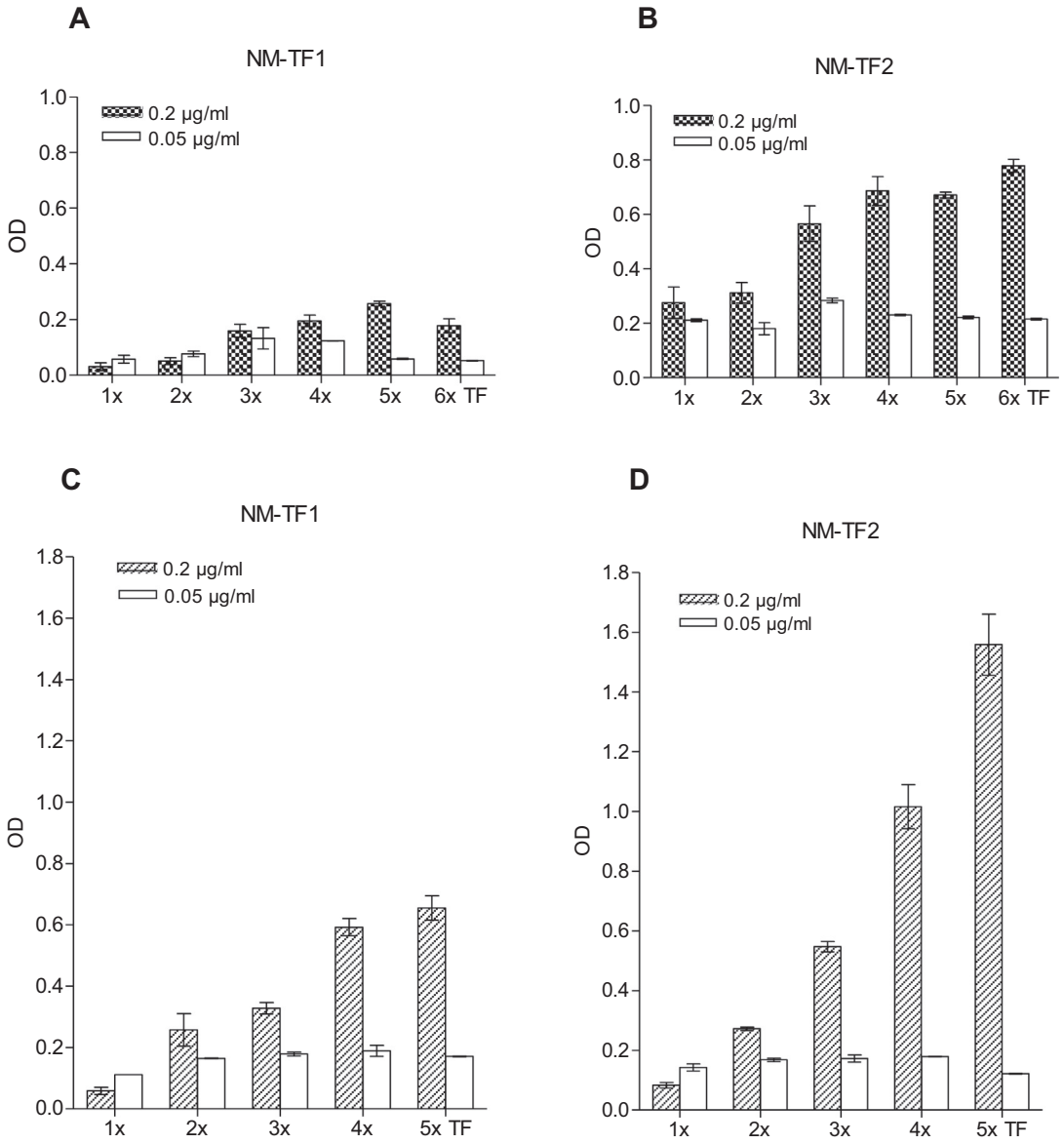

Fig. 5. Binding of antibodies NM-TF1 and NM-TF2 to biotinylated synthetic anti-freeze glycoprotein (AFGP) sequences of different length (carrying 1–6 or 1–5 TF units, respectively), performed under defined coating conditions: saturated (0.2 µg/ml) or non-saturated (0.05 µg/ml). A, B (set II): Thr-bound TF (corresponding to native AFGP). C, D (set III): Ser-bound TF.

Before discussing the results in detail, we should define what is meant by the term "cluster". As shown in Fig. 1, TF occurs in distinct clusters at the cellular membrane of many human tumor cells. This is often observed with other carbohydrate tumor markers, too. These clusters occur at a magnitude far above the molecular level, to which our ELISA data belong.

At the molecular level we may distinguish between clusters on the side of the combining site (paratope) of the antibody, and clusters on the side of the antigen (epitope). The former are essentially depending on the antibody class and on structural details of the antibody itself, whereas the latter can be quite diverse. Multiple epitopes can be localized on a single molecule, which is often the case with glycans carried on large proteins (*molecular* or *structural clusters*). Alternatively, molecules carrying single epitopes can be expressed in close vicinity on a surface (*density clusters*). ELISA data are not able to answer structural questions. However, they can exclude certain possibilities, as is the case in the present experiments. To this end it should be helpful to consider relevant molecular dimensions, which are summarized in Table 4.

In the experiments described here we have employed three different sets of synthetic glycoconjugates modeling both *structural* and *density* clusters of TF. From the obtained data we draw the following conclusions for the binding behavior of the antibodies NM-TF1 and NM-TF2.

- Single TF units were not detected (or at very low affinities not detectable in ELISA experiments). This behavior would not be surprising in case of IgG anti-carbohydrate antibodies, but it was not expected in case of IgM antibodies.
- The most critical factor for the strength of binding (in terms of maximum OD) was found to be a very close *structural* (*molecular*) vicinity of at least two TF units, whereby a distance of ≤ 1 nm was required. Best binding was obtained with 4 TFs in immediate neighborhood.
- We explain this density dependency as follows. Considering the dimensions of immunoglobulin molecules (Table 4), the vicinity of ≤ 1 nm between TFs necessary for binding excludes the involvement of two separate Fab arms, either by two monomers or by the two arms of one monomer. On the other hand, two or more TFs at a distance of ≤ 1 nm should well fit into a single binding pocket. We

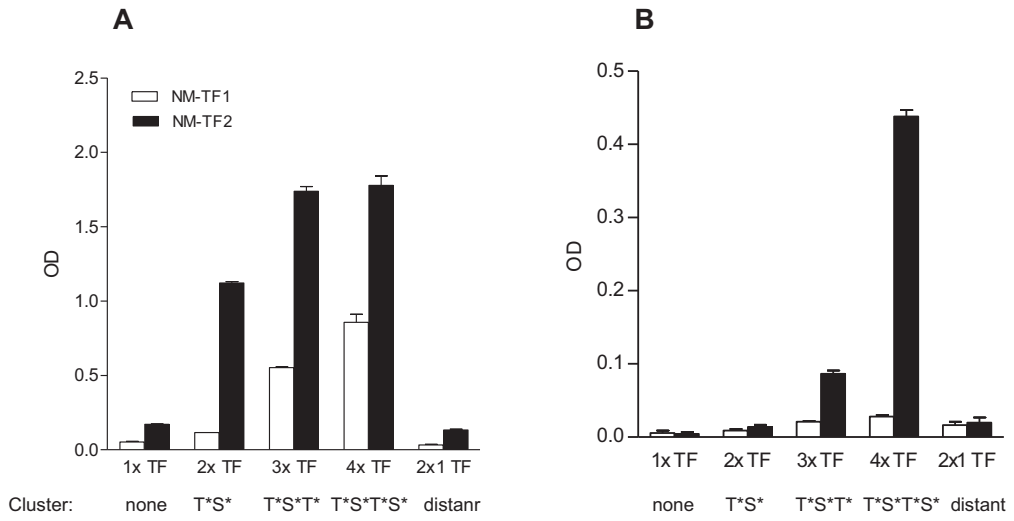

Fig. 6. Binding of antibodies NM-TF1 and NM-TF2 to biotinylated synthetic glycopeptides modeling molecular clusters of aGP (see Table 2C). A: saturating coating conditions (50 nM); B: non-saturated conditions (10 nM).

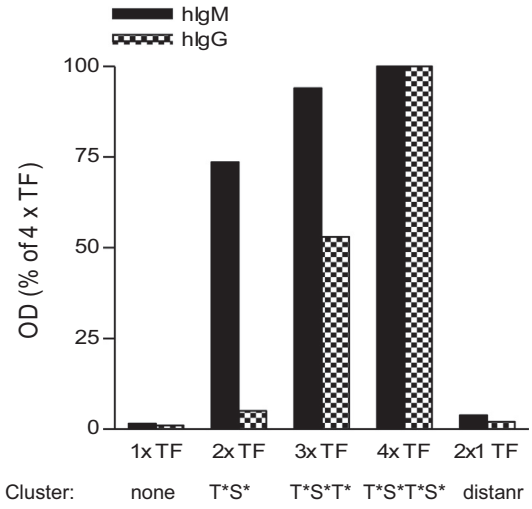

Fig. 7. Binding of humanized antibodies of IgM and IgG isotypes, respectively, both derived from NM-TF2, to biotinylated synthetic glycopeptides modeling molecular clusters of aGP (see Table 2C). Saturating coating conditions. In case of hIgM 1 µg/ml, coating with 50 nM; in case of hIgG 2 µg/ml, coating with 100 nM. Values are calculated as per cent OD compared to the highest OD value (4× TF). In direct comparison under equal ELISA conditions (1 µg/ml antibody, 50 nM coating), OD values of hIgG were significantly lower (only about 5%) compared to hIgM.

therefore suggest that a "multiple epitope" consisting of up to four TF disaccharides in close vicinity provides the best fit for the combining site of both antibodies, NM-TF1 and NM-TF2. The exact number of TFs belonging to the multiple epitope described here is still open. On the one hand one may argue that a single pair of TFs would be sufficient to explain the data. However, we personally tend to the view that it is in fact a quadruple epitope because the binding of two TFs alone is inconsistent, and clearly depends on dense coating. It may also be argued that a quadruple epitope is present on aGP (aa 10–13) [12], and it might be that this sequence, which, by the way, is T*S*T*S*, had been the primary immunogenic structure in generating both antibodies. We did not try experimentally clusters of > 4 TFs. They are probably too big for a single binding pocket.

– Another interesting aspect of this study is the role of the amino acid carrying TF. It is known that either threonine or serine carry *O*-glycans. We found that both antibodies preferred Ser-TF over Thr-TF. This does not imply a mixed peptide-carbohydrate epitope since, for instance, PAA-conjugates of TF (void of amino acids) are also bound (model 1). We suggest that a greater flexibility and/or a better spatial orientation of the Ser-bound TF moiety are responsible for this effect. These properties have already been described for glycopeptides carrying Tn [28] and should be applicable in our case. The Thr-TF containing AFGP is a stiff molecule with the TF moieties directed to one side [18,20], whereas the Ser-TF analogue behaves differently and shows no thermal hysteresis [20].
– Our data also suggest that multiple epitopes can be mimicked by separate TF-carrying molecules in close vicinity (*density clusters*). Such cases apparently exist in model 2 under saturating coating conditions (see Figs. 4 and 5), in model 3 in case of the glycopeptide 2 × TF under saturating coating conditions (Fig. 6 A), and also in case of the preliminary experiments shown in Fig. 2B. Our data also suggest that density clusters require a minimal length and flexibility of the molecule in order to allow TFs to join into one single binding pocket. However, it is interesting to note (and not fully understood) that single TF carrying glycopeptides also in case of model 3 are obviously excluded. In case of experiments using streptavidin-coated microtiter plates, a close vicinity of (biotinylated) glycopeptides is already provided by the four biotin binding sites of each streptavidin molecule within an area with a diameter of about 4 nm (Table 4), provided that all or at least two or three of the four sites are occupied. This explains why OD values also in these experiments depend on the coating conditions (saturated versus non-saturated). In case of the microtiter plates used in our experiments, the possibility of joint binding of TFs from glycopeptides anchored on *different* streptavidin molecules is almost zero because of the actual distances between them (Table 4). (The calculated distances – based on the given binding capacity of the microtiter plate - vary depending on the number of binding sites of streptavidin actually occupied; if all four are captured, the calculated mean distance is 15.6 nm).
– The suggestion of multiple TF epitopes is strongly supported by the fact that humanized IgG derivatives of NM-TF2 showed the same dependency as their IgM counterparts on either quadruple TF clusters (Fig. 7) or on longer AFGP glycopeptides (Fig. 4B). At the same time a considerable increase in avidity is evident in case of IgM.
– The existence of antibodies recognizing *multiple epitopes* (which means the existence of corresponding paratopes) has already been described in some other cases of anti-carbohydrate antibodies

Table 4
Data table of dimensions of ELISA vessels and relevant molecules.

Materials used	Approximate size or capacity	Source
IgM dimensions:		
Overall diameter of the pentamer		
Extended conformation	30–45 nm	[21–23][a]
Turtle conformation	25–40 nm	[22,23][a]
Mushroom or bell shape conformation	10–16 nm	[21,22]
Core		
Extended conformation	10–20 nm	[21–23]
Distance between two adjacent monomers		
Extended conformation	10–20 nm	[23]
Bell shape conformation	15 nm	[22]
Range of potential distances of binding pockets in the bell		
Shape conformation	10–40 nm	[22,23][a]
Common features of IgG and IgM:		
Single Fab arm		
Length	5.5–11 nm	[21,22][a]
Width	2.3–5.6 nm	[21]
Area of the binding pocket (CDR area)	28 nm^2	[24]
Distance between the two binding pockets of [F(ab)$_2$]	5 nm	[22][a]
Microtiter well dimensions (96 well type):		
Surface area (250 µl filled)	293 mm^2	[c]
Surface area (50 µl filled)	82 mm^2	[c]
Biotin binding capacity per well (250 µl filled)	2 pmoles	[b]
Biotin binding capacity per well (50 µl filled)	0.56 pmoles	[b]
Mean distance of streptavidin positions on the well surface	7.8–15.6 nm	[d]
Streptavidin diameter	4 nm	[25]
PAA-TF conjugates		
Diameter of the (almost spherical) molecules	15 nm	[e]
Distance between TF moieties in cis-disposition		[f]
10 M % TF	2 nm	
20 M % TF	1 nm	
40 M % TF	0.5 nm	
AFGP glycopeptide dimensions:		
Length of a disaccharide (e.g. TF)	≈2 nm	[g]
Width of saccharides	0.84 nm	[g]
Distance between two TF positions	0.936 nm	[18]
Size of biotin	3 nm	[25]
Peptide general data		
Distance between two peptide bonds	0.3 nm	[26]

[a] Own estimation using data from the indicated references.
[b] Data provided by BioTeZ GmbH, Berlin-Buch.
[c] Own calculation.
[d] Calculated with a formula provided by Dr. W. Kössler, Humboldt University, Berlin (see text).
[e] Data from the homepage of Lectinity Holding.
[f] N.V. Bovin, personal communication.
[g] Approximation from data in [27], Table 3-1.

[29–31].

– Functional (avidity) association and dissociation constants (Table 3) were measured under conditions providing multiple TF epitopes as antigens, either native aGP or synthetic glycoconjugates. K_A values toward aGP (carrying the quadruple TF cluster aa10–13) are comparably high for anti-carbohydrate antibodies. This may be because obviously all four TFs are involved in interactions with the combining site.

5. Conclusions

Since both antibodies have been employed successfully and with high specificity in immunostaining of human tumors, we conclude that multiple TF epitopes (*structure*- or *density*-related clusters) must be present on cancer cells. This is indirectly confirmed by (or at least in agreement with) tumor vaccination studies targeting TF [32,33], which were successful only by employing TF cluster vaccines, either natural aGP [32] or synthetic TF trimers [33]. In case of the closely related tumor marker Tn it was found that antibodies recognizing clusters encompassing both Ser-Tn and Thr-Tn revealed the best tumor specificity [31]. With respect to the antibodies NM-TF1 and NM-TF2, their restriction on dense clusters of Ser-TF and/or Thr-TF may contribute to their relatively high avidity and explicit tumor specificity. Molecular and cellular clusters as visible in Fig. 1 may even be two sides of the same phenomenon, and may be symptomatic for tumor cells.

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