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Surface plasmon resonance and NMR analyses of anti Tn-antigen MLS128 monoclonal antibody binding to two or three consecutive Tn-antigen clusters

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Tn-antigens are tumour-associated carbohydrate antigens that are involved in metastatic processes and are associated with a poor prognosis. MLS128 monoclonal antibody recognizes the structures of two or three consecutive Tn-antigens (Tn2 or Tn3). Since MLS128 treatment inhibits colon and breast cancer cell growth [Morita, N., Yajima, Y., Asanuma, H., Nakada, H., and Fujita-Yamaguchi, Y. (2009) Inhibition of cancer cell growth by anti-Tn monoclonal antibody MLS128. *Biosci. Trends* 3, 32–37.], understanding the interaction between MLS128 and Tn-clusters may allow us to the development of novel cancer therapeutics. Although MLS128 was previously reported to have specificity for Tn3 rather than Tn2, similar levels of Tn2/Tn3 binding were unexpectedly observed at 37°C. Thus, thermodynamic analyses were performed via surface plasmon resonance (SPR) using synthetic Tn2- and Tn3-peptides at 10, 15, 20, 25 and 30°C. SPR results revealed that MLS128's association constants for both antigens were highly temperature dependent. Below 25°C MLS128's association constant for Tn3-peptide was clearly higher than that for Tn2-peptide. At 30°C, however, the association constant for Tn2-peptide was higher than that for Tn3-peptide. This reversal of affinity is due to the sharp increase in K_d for Tn3. These results were confirmed by NMR, which directly measured MLS128-Tn binding in solution. This study suggested that thermodynamic control plays a critical role in the interaction between MLS128/Tn2 and MLS128/Tn3.

Keywords: antibody/isothermal titration calorimetry/surface plasmon resonance/Tn-antigen/thermodynamic.

Abbreviations: Ab, antibody; BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylpropyl)-carbodiimide; ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPR, horseradish peroxidase; ITC, isothermal titration calorimetry; mAb, monoclonal Ab; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; RU, response units; SPR, surface plasmon resonance; Tn antigen, Thomson-Freidenreich (GalNAc-O-Ser/Thr) antigen.

The equilibrium constants between simple mono- or oligosaccharides and binding proteins such as lectins are generally observed in the range of 10^{-3}–10^{-4} M. The affinity constants of such lectins could be improved by subunit multivalency and clustering or the branching structure of oligosaccharides for proteins and ligands, respectively (*1*–*7*). Since oligosaccharides from glycoconjugates are usually exposed on the cell surface *in vivo*, surface plasmon resonance (SPR) provides a relevant model for studying the interaction between carbohydrate moieties immobilized on the solid phase surface and carbohydrate-binding proteins in solution. A good example for this is the selectin family of adhesion molecules; this family is known to mediate the initial attachment of leukocytes to vascular endothelial cells before their firm adhesion to support their subsequent labile rolling interactions (*8*, *9*). Alon *et al.* (*10*) reported that transient tethers had first-order kinetics and other characteristics suggesting a unimolecular interaction between P-selectin and its glycoprotein ligand, PSGL-1. Fast on and off rates, together with the high tensile strength of the selectin bond, as well as dimerization of a selectin and its ligand appear necessary to facilitate rolling of leukocytes at physiological shear stresses (*9*, *10*). SPR provides a useful tool for carrying out affinity and kinetic analyses of P- and L-selectin binding to their physiologically relevant ligands as well as identifying inhibitors of such selectin binding (*11*–*13*).

Tn-antigen, GalNAcα-Ser/Thr, is a tumour-associated carbohydrate antigen that is involved in metastatic processes and is associated with a poor

prognosis, thus representing an excellent target for cancer intervention. MLS128 is a monoclonal antibody that recognizes the structures of two or three consecutive Tn-antigens (Tn2 or Tn3) (*14*, *15*). Osinaga *et al*. (*16*) used SPR to measure the kinetic parameters for anti-Tn-antigen 83D4 mAb (IgM) of their own, MLS128 mAb (IgG_3), and VVLB4 lectin and they reported that MLS128 binds to a synthetic Tn3-peptide with approximately 10 times the affinity that it binds to a synthetic Tn2 peptide. Since the three analytes 83D4, MLS128 and VVLB4, have five, two and four binding sites, respectively, their affinity constants cannot be simply calculated using the same fitting model such as a 1:1 Langmuir model. Thus, previously reported values should be considered as apparent affinities for observed interactions.

The current authors previously found that MLS128 treatment significantly inhibited colon and breast cancer cell growth, providing the first insights into the potential use of this particular type of anti-Tn antigen antibody as a cancer therapy (*17*). Analysis of the mechanisms by which this antibody binds to colon and breast cancer cells is thus an important area of study. To obtain more detailed information on the interaction between MLS128 and Tn2- or Tn3-epitopes, thermodynamic analyses were performed using SPR measurements. Furthermore, NMR and ITC, which measure the binding of MLS128 and its ligands in solution, were performed to compare their results with those of SPR. These studies led to the novel finding that MLS128's affinity for Tn2- and Tn3-epitopes varied at different temperatures, suggesting that subtle differences in binding play a role in the interaction between MLS128 and Tn2- and Tn3-epitopes.

Materials and Methods

Materials

MLS128 monoclonal antibody was prepared as previously described (*14*). Tn3-peptide, Tn2-peptide and a peptide backbone (Tn0-peptide) were synthesized as previously described (*18*). N- and C-terminal free peptides, N-terminal blocked peptides and both terminal blocked peptides were used for SPR and ITC, enzyme-linked immunosorbent assay (ELISA) and NMR, respectively (Table I). The BIAcore 3000 biosensor, CM3-sensor chip, amine coupling kit and Microcal iTC200 Isothermal Titration Calorimetry (iTC200) were from GE Healthcare UK Ltd. Amersham Place (Little Chalfont, England). Tween-20 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (St Louis, MO, USA). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Analysis of MLS128 binding to Tn-peptides using ELISA

Tn2- and Tn3-peptides as well as a control Tn0-peptide were covalently linked to free amino groups on Covalink 96-well plates (Nunc, Roskilde, Denmark) using water-soluble carbodiimide (EDC) as a coupling reagent. Briefly, 25 µl of 100 µM Tn0-, Tn2- and Tn3-peptides were mixed with 25 µl of 5 mM EDC in water and added to wells of Covalink 96-well plates. The coupling reaction was allowed to proceed for 2 h at 37°C. After the plates were blocked with 10 mM Tris–HCl, pH 7.4, containing 150 mM NaCl (TBS) and 3% BSA at 4°C overnight, 50 µl of MLS128 (0.01–10 µg/ml) in TBS containing 0.1% Tween-20 (TBST) were added to the wells and allowed to bind to antigens for 2 h at 37°C or 4°C. After plates were washed with TBST five times, the bound antibodies were incubated with 50 µl of HRP-labelled anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc., PA, USA) for 1 h at 4°C. After wells were washed five times with TBST, bound MLS128 was detected by colour development using a 100 µl ABTS solution (Roche Diagnostics, Basel, Switzerland) and terminated by adding 100 µl of 2% oxalic acid. Absorbance at 405 nm was measured with a plate reader (Bio-Rad, Hercules, CA, USA).

Analysis of temperature-dependent binding of MLS128 using SPR

Tn3-, Tn2- and Tn0-peptides were immobilized to CM3-sensor chips by amine coupling according to the manufacturer's instructions. After activation of the carboxyl residues by 0.1 M NHS–0.4 M EDC, Tn3-, Tn2- or Tn0-peptide in 10 mM HEPES, pH7.4, 150 mM of NaCl were injected into each flow cell to allow coupling reactions. Remaining active carboxyl residues were masked by treatment with 1 M ethanolamine–HCl, pH 8.5, for 14 min at 25°C. The aforementioned procedure was followed with manual injection to better control the amount of ligands immobilized.

Binding analyses were carried out with four concentrations of MLS128 in 10 mM HEPES, pH7.4, containing 150 mM NaCl and 0.005% Tween-20 at a flow rate of 20 µl/min at five different temperatures; 10, 15, 20, 25 and 30°C. During the sets of SPR experiments, flow cells were regenerated by injection of the running buffer containing 3.5 M Guanidine–HCl followed by thorough washing with the running buffer. Tn2-/Tn3-specific binding signals in response units (RU) were obtained by subtracting the RU of Tn0-peptide as a non-specific control from the Tn2/Tn3-binding signals in RU derived from flow cells coated with the T2- and T3-peptides, respectively.

BIAevaluation software version 3.2 was used to fit experimental data by the bivalent model according to the software manual. Non-linear fitting methods were used to simultaneously obtain association and dissociation parameters. The bivalent antibody (A)-antigen (B) binding is described as follows [Equation (1)]:

$$\mathrm{A{+}2B} \underset{kd1}{\overset{ka1}{\rightleftharpoons}} \mathrm{AB{+}B} \underset{kd2}{\overset{ka2}{\rightleftharpoons}} \mathrm{ABB}$$

Reaction 1 Reaction 2

The association and dissociation rate constants (k_a1, k_d1) were determined, and the association constant (K_A1) was calculated using the equation $K_A1 = k_a1/\ k_d1$. Kinetics parameters k_a1, k_d1, k_a2 and k_d2 were calculated as a global fitting for a set of four different concentrations of MLS128. For each set of experiments, 'R_{max}' was calculated by setting 'global' fitting except for Tn3 at 10, 15 or 20°C which was calculated by setting local fitting at four

Table I. List and structures of glycopeptides used in this study.

Tn peptide	Structure	Experiment
Tn0	Acetyl-Gly-Thr-Thr-Thr-βAla-Gly-OH	ELISA
	Gly-Thr-Thr-Thr-βAla-Gly-OH	SPR
Tn2	Acetyl-Gly- Thr- Thr(α-GalNAc)-Thr(α-GalNAc)- βAla-Gly-OH	ELISA
	Gly- Thr- Thr(α-GalNAc)-Thr(α-GalNAc)- βAla-Gly-OH	SPR
	Acetyl-Gly-Thr-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-NH_2	NMR
Tn3	Acetyl-Gly-Thr(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-OH	ELISA
	Gly-Thr(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-OH	SPR
	Acetyl-Gly-Thr(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-βAla-Gly-NH_2	NMR

different concentrations, which tends to vary during continuous SPR measurements. The fitting data were evaluated using the R_{max} values that were calculated from the molecular weight (MW) and RU of the immobilized ligand using the following equation: MLS128 (170 kDa)/Tn2-peptide (955 Da) or Tn3-peptide (1158 Da) × the amount of the immobilized ligands in RU) × (the number of binding sites on the ligand (n, 0.5–1).

Thermodynamic parameters were calculated from the equation, $\Delta G = \Delta H - T\Delta S = -RT\ln K$, where R is the universal gas constant ($1.987\,JK^{-1}mol^{-1}$) and T is the absolute temperature in Kelvins. Transition state analyses were carried out as indicated below (*19*, *20*).

(i) Thermodynamic analysis was performed by substituting K_A and $\Delta G = \Delta H - T\Delta S$ into the van't Hoff's equation $\Delta G = -RT\ln(K_A)$, yielding $\ln(K_A) = \Delta H/(RT) - \Delta S/R$ ($y = a + bx$), which is linear. Plotting $y = \ln(K_A)$ versus $x = 1/T$ gives $a = -\Delta S/R$ and $b = \Delta H/R$.

(ii) Transition state analysis was carried out using the Eyring equation, $k = (\chi T/h)e^{-\Delta H^*/R + \Delta S^*/R}$ where $h = 1.584 \times 10^{-34}$ Js and $\chi = 3.3 \times 10^{-24}$ JK^{-1} are the Plank and Boltzmann constants, respectively, and the asterisk denotes a transition state. A plot of $\ln(kh/\chi T)$ versus $1/T$ should result in a straight line of slope $-\Delta H^*/R$ and intercept $\Delta S^*/R$, $\ln(kh/\chi T) = -\Delta H^*/RT + \Delta S^*/R$.

Isothermal titration microcalorimetry

Isothermal titration microcalorimetry (ITC) was performed at 10°C and 25°C using the ITC 200 Micro Calorimeter (GE Healthcare) (*21*, *22*). MLS128 and Tn2- or Tn3-peptide were prepared in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). To a sample cell containing 200 µl of 48.8 µM MLS128, 2-µl injections of 600 µM Tn2- or Tn3-peptide solution were added every 180 s by a computer-controlled syringe rotating at 1000 rpm while exothermic heat changes were recorded every 2 s. Control experiments were carried out by adding 2 µl injections of 600 µM Tn2- or Tn3-peptide solution to a sample cell containing 200 µl of PBS in the absence of MLS128. The experimental data were fitted to a theoretical titration curve using the program Origin ver. 7.0 (OriginLab, Northampton, MA, USA), with ΔH (enthalpy change in $cal\,mol^{-1}$), K_A (association constant in M^{-1}) and n (number of binding sites/monomer) as adjustable parameters.

NMR experiments

^{1}H-NMR spectra were recorded with a 600 MHz spectrometer (DRX-600, BrukerBiospin) equipped with a triple resonance inverse (TXI) probe. MLS128 (50 µM, 500 µl) in 10 mM sodium phosphate buffer (pH 6.0) containing 150 mM NaCl was prepared for NMR experiments, and 10% D_2O was added to provide a lock signal. Tn2 or Tn3-peptide was added to the corresponding NMR samples to reach a molar ratio of 1:2. In ^{1}H-NMR experiment, water suppression was achieved using the WATERGATE pulse sequence with a 3–9–19 pulse train, and probe temperature was set to 5, 20 and 35°C. Either 32 scans (Tn-peptides only) or 512 scans (Tn-peptides with mAb) were required to obtain a good signal-to-noise ratio (S/N). Chemical shifts indicated in parts per million (ppm) were calibrated based on the outer standards of the chemical shift of 4,4-dimethyl-4-silapentane-1-sulphonic acid at 0 ppm. NMR data were processed with XWIN-NMR (ver.3.5) and the spectra were displayed using XWIN-PLOT (ver.3.5).

Results

Analysis of MLS128 binding to Tn2-peptide or Tn3-peptide using ELISA

MLS128's specificity for Tn3 was originally suggested by ELISA experiments at 4°C utilizing glycophorin A peptides (*15*). Later, MLS128's affinity for binding to Tn3-peptide was confirmed to be an order higher than that for binding to Tn2-peptide according to SPR at 25°C (*16*). ELISA at 4°C indicated that MLS128 binding to Tn3-peptide was much greater than that to Tn2-peptide (Fig. 1A), which is consistent with results

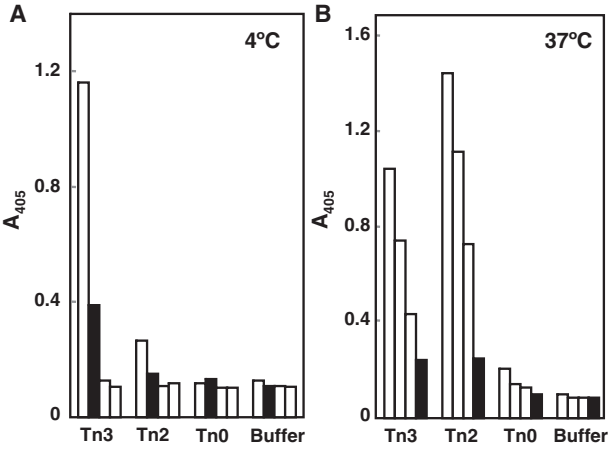

Fig. 1 Binding of MLS128 to Tn3- and Tn2-peptides or control Tn0-peptide as determined by ELISA at 4°C (A) or 37°C (B). Tn0-, Tn2- and Tn3-peptides were immobilized by amine coupling using Covalink plates and NHS reagent via the free carboxyl-terminal of the peptide back bone. (A) The binding assays were carried out at 4°C using MLS128 at a concentration of 10 µg/ml, followed by serial dilutions from left to right: 1, 0.1 and 0.01 µg/ml. (B) The binding assays were carried out at 37°C using MLS128 at a concentration of 100 µg/ml, followed by serial dilutions from left to right: 20, 4 and 0.8 µg/ml. Of four concentrations examined, comparable concentrations between the two experiments performed at 4°C and 37°C are highlighted in closed bars.

of SPR (*16*). At 37°C, however, MLS128's binding to Tn2-peptide was surprisingly found to be comparable or even greater than its binding to Tn3-peptide, as shown in Fig. 1B. Since Tn2-binding activity was measured using a set of higher concentrations of MLS128 than those for Tn3-binding assays in the experiments shown in Fig. 1, of four concentrations examined comparable concentrations between the two experiments performed at 4°C and 37°C are highlighted in closed bars. The results suggested that MLS128's affinity for Tn2 and Tn3 changed in a temperature-dependent manner. This observation led us to further investigation of the kinetics of MLS128 binding to Tn3- and Tn2-peptides at various temperatures using SPR, ITC and NMR.

SPR analysis of temperature-dependent binding of MLS128 to Tn2- or Tn3-peptide

Figure 2 shows the results of SPR analyses at three different temperatures using the same Tn2- and Tn3-peptide immobilized-sensor chips. Results clearly demonstrate that although MLS128 binding to Tn3-and Tn2-peptide at 10°C and 15°C has similar kinetics in both the association and dissociation phases, association and dissociation kinetics at 25°C differed significantly from those at 10°C or 15°C. Notably, the appearance amount of maximum RU value for MLS128 binding to Tn2-peptide at 25°C was much lower than that for binding to Tn3-peptide. In addition, the dissociation rate for the MLS128/Tn2-peptide complex was slower than the dissociation rate for the MLS128/Tn3-peptide complex, which was markedly fasted. At 625 s after addition of the running buffer, therefore, the amount of MLS128 remaining on the Tn2-peptide immobilized-sensor

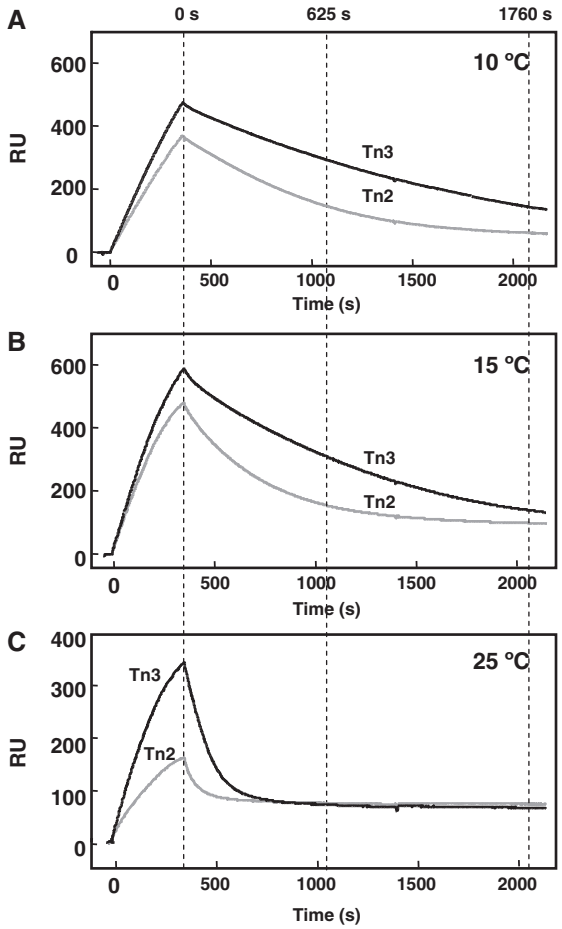

Fig. 2 Temperature dependence of the MLS128/Tn2-peptide and MLS128/Tn3-peptide interactions as determined by SPR analysis. MLS128 (5.9 nM) was associated with Tn2-peptide (grey line) or Tn3-peptide (black line) for 6 min (360 s) at a flow rate of 20 µl/min and then dissociation phases were observed for 29.5 min (1760 s). SPR analyses were performed at 10°C (**A**), 15°C (**B**) and 25°C (**C**) on the same chips that were immobilized with Tn0 (peptide backbone), Tn2- and Tn3-peptides (120 RU, 190 RU and 270 RU, respectively). Specific RU values were shown after subtraction of RU responses of Tn0-peptide from the RU responses of T2-peptide or Tn3-peptide. The broken line at 625 s indicates the time at which dissociation rates of Tn2- and Tn3-peptides crossed at 25°C.

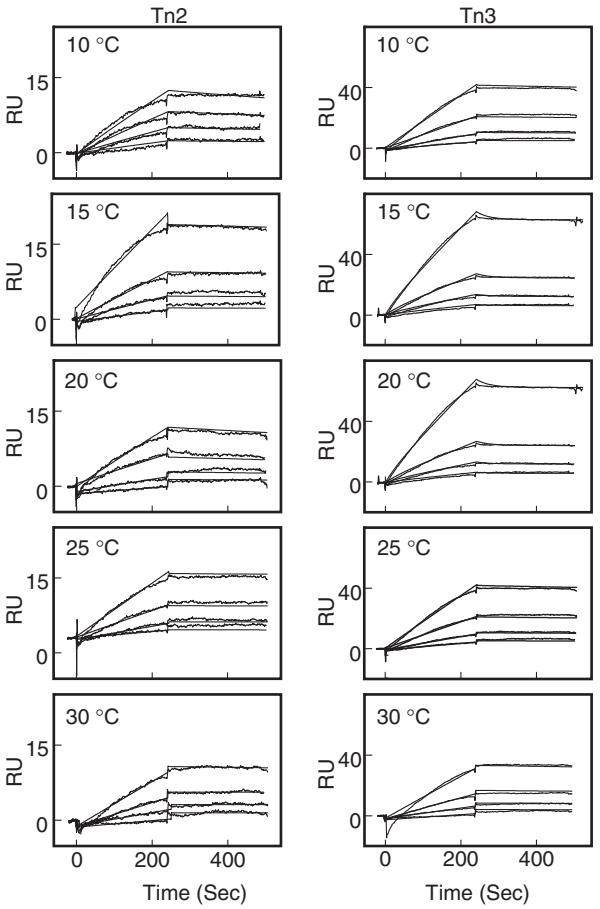

Fig. 3 SPR analyses of the MLS128/Tn2-peptide and MLS128/Tn3-peptide interactions at five different temperatures. Kinetic measurements were performed using sensor chips coated with the low density of Tn0-, Tn2- or Tn3-ligand (30 RU, 22 RU or 44 RU, respectively). Binding analyses were carried out at four concentrations of MLS128 (5.9, 2.9, 1.5 and 0.74 nM). MLS128 was associated with Tn2-peptide (left panel) or Tn3-peptide (right panel) for 240 s and then dissociation phases were observed for 240 s at 10, 15, 20, 25 and 30°C, from which kinetic parameters of the interaction between MLS128 and Tn2- or Tn3-peptide were determined (Table II).

chip exceeded the MLS128 bound to the Tn3-peptide immobilized-sensor chip (Fig. 2C). Since Tn-peptide/MLS128 complexes were thoroughly washed to remove non-specific binding in ELISA experiments, the RU values after thorough washing of the sensor chips are presumably equivalent to the binding activity according to ELISA. As shown in Fig. 2, the ratio of MLS128 binding to Tn3:Tn2 1760 s after washing was calculated to be 2.4:1 at 10°C, 1.5:1 at 15°C and 0.9:1 at 25°C. These results were essentially consistent with the results of ELISA (Fig. 1) in that MLS128's affinity for Tn2 and Tn3 is temperature dependent.

The initial SPR analyses as described above were done using sensor chips coated with high-density ligands, which may have caused heterogeneity in analytes or ligands or rebinding of analytes (*23*). Kinetic measurements were therefore performed using sensor chips coated with the lowest density of ligands; this should still provide a good response without disturbance by secondary factors such as mass transfer or steric hindrance. SPR was carried out under such conditions at five different temperatures to further investigate the kinetics of MLS128 binding to Tn2- or Tn3-peptide-coated sensor chips. The results of SPR performed at 10, 15, 20, 25 and 30°C are shown in Fig. 3, from which kinetic parameters of the interaction between MLS128 and Tn2- or Tn3-peptide were determined (Table II).

First, the results revealed that MLS128's association constant (K_A) for both antigens decreased as the assay temperature increased. Second, <25°C MLS128's association constant for Tn3-peptide was clearly higher than that for Tn2-peptide. At ~25°C, the association constant for Tn3-peptide was nearly the same as that for Tn2-peptide. At 30°C, however, the association constant for Tn2-peptide was higher than that for Tn3-peptide. These results clearly demonstrated that MLS128 has higher affinity for Tn2-peptide than

Table II. Kinetics parameters of the interaction between MLS128 and Tn2-peptide or Tn3-peptide.

	Tn2				Tn3			
Temperature °C	k_a1 $M^{-1}s^{-1}\times10^{-3}$	k_d1 $s^{-1}\times10^{3}$	K_A1 $M^{-1}\times10^{-4}$	R_{max}[a] $RU\times10^{-3}$	k_a1 $M^{-1}s^{-1}\times10^{-3}$	k_d1 $s^{-1}\times10^{3}$	K_A1 $M^{-1}\times10^{-4}$	R_{max}[a] $RU\times10^{-3}$
10	2.19 ± 0.13	28.8 ± 5.1	7.60	3.92	4.73 ± 0.01	7.89 ± 0.06	59.9	5.87
15	1.82 ± 0.05	32.1 ± 9.1	5.67	3.92	4.73 ± 0.24	11.2 ± 0.01	42.2	6.35
20	1.13 ± 0.02	17.8 ± 8.3	5.22	3.92	2.06 ± 0.12	11.1 ± 1.23	18.6	5.60
25	1.33 ± 0.02	24.5 ± 0.1	5.43	3.92	2.18 ± 0.07	48.2 ± 40.0	4.52	7.04
30	0.68 ± 0.11	17.1 ± 5.6	3.67	3.92	1.73 ± 0.03	99.3 ± 21.8	1.74	7.04

[a] R_{max} was calculated by setting global fitting except for Tn3 at 10, 15 or 20°C which was calculated by setting local fitting at four different concentrations (Fig. 3). R_{max} shown for Tn3 at 10, 15, or 20°C is the average of four values.

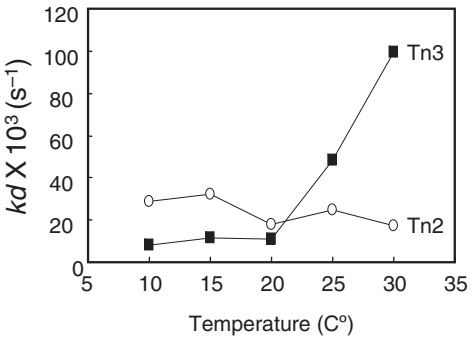

Fig. 4 Comparison of MLS128's k_d1 values for Tn2- and Tn3-peptide according to SPR analyses at different temperatures. The striking difference in interaction between MLS128 and Tn2- or Tn3-peptide is presented graphically. The kinetic parameters for experiments overall are listed in Table II.

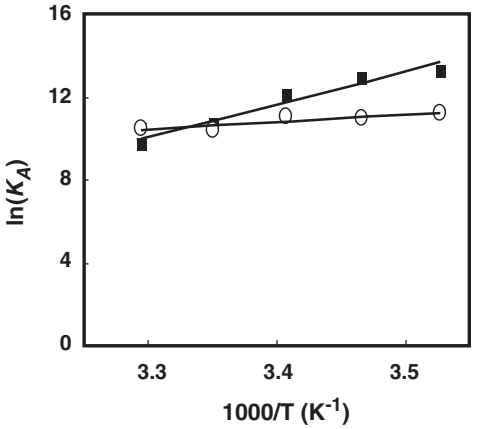

Fig. 5 van't Hoff plots for the MLS128/Tn2-peptide and MLS128/Tn3-peptide interactions, determined from SPR analysis as summarized in Table II. Open squares and closed squares indicate data points for Tn2- and Tn3-peptide, respectively. Thermodynamic analysis was performed by substituting K_A and $\Delta G = \Delta H - T\Delta S$ into the van't Hoff's equation $\Delta G = -RT\ln(K_A)$, yielding $\ln(K_A) = \Delta H/(RT) - \Delta S/R$ ($y = a + bx$). Plotting $y = \ln(K_A)$ versus $x = 1/T$ gives $a = -\Delta S/R$ and $b = \Delta H/R$.

Tn3-peptide at 30°C. This reversal of affinity is due to the sharp increase in *kd*, as depicted in Fig. 4, and confirms original suppositions (Fig. 2). These results are essentially consistent with those of ELISA (Fig. 1), which found a clear temperature-dependent difference in the binding activity of MLS128 between 4°C and 37°C.

The equilibrium constants derived from SPR analyses at varying temperatures (Table II) were used to determine van't Hoff enthalpies by plotting $\ln(K_A)$ versus 1/T (Fig. 5). The van't Hoff plots for both Tn2- and Tn3-peptides were linear, which is consistent with an invariant binding mechanism across the temperature range studied. The linear fitting lines for Tn2- and Tn-3 peptides crossed at 26°C, indicating that MLS128's association constant for Tn2-peptide is higher than its constant for Tn3-peptide at temperatures >26°C.

Applying the transition state theory to the SPR rate constants yielded the Eyring plots shown in Fig. 6. The plots are linear, which is consistent with ΔH^* and ΔS^* for both reactions having no temperature dependence across the range studied (10–30°C). Thermodynamic parameters were calculated from Eyring plots using kinetic rate constants for Tn2- and Tn3-ligands determined using SPR at 25°C (Table III). Free energy ΔG at 25°C was nearly the same for Tn2-peptide and Tn3-peptide binding to MLS128 although the entropic and enthalpic values differed significantly. Tn3 binding to MLS128 is enthalpically driven but entropically unfavourable. In contrast, the binding of Tn2 to MLS128 does not seem to involve entropic changes.

The coordinates tracing the lowest energy continuous pathway between free and bound mAb on the potential energy surface for Tn2- and Tn3-peptides are illustrated in Fig. 7. Although the free energy path for both ligands was similar, the enthalpic and entropic terms for the bound state differed, suggesting that distinct thermodynamic parameters drive complex formations for Tn2- and Tn3-peptides with MLS128.

Isothermal titration calorimetric analysis of MLS128 interaction with Tn2- and Tn3-peptides

Interaction of MLS128 with Tn2- and Tn3-peptides was assayed by ITC to determine solution-based equilibrium constants at 10°C and 25°C (Fig. 8). Although the titration curves did not reach the maximum binding due to the limitation of mAb concentrations used in these experiments, based on ITC binding isotherms for the two ligands at 10°C (Fig. 8A) and 25°C (Fig. 8B) the K_A values were calculated to be $2.8\times10^4\,M^{-1}$ (Tn2) and $2.9\times10^4\,M^{-1}$ (Tn3) at 10°C,

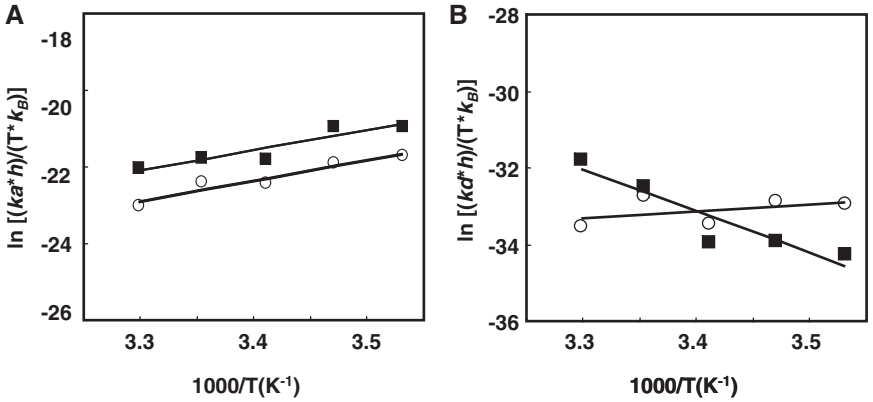

Fig. 6 Eyring plots for MLS128/Tn2-peptide and MLS128/Tn3-peptide interactions determined by SPR analyses. Open squares and closed squares indicate data points for Tn2- and Tn3-peptide, respectively. Plots show ln[(ka*h)/(T*k_B)] of the interaction between MLS128 and Tn2 (open circle) or Tn3 (black square) derived from the parameters listed in Table II; (**A**) is calculated from the association rate constants and (**B**) is calculated from the dissociation rate constants.

Table III. Thermodynamic parameters for the binding of Tn2-peptide or Tn3-peptide to MLS128 with SPR (25°C).

Ligand	Tn2-peptide	Tn3-peptide
ΔG (kcal mol^{-1})	−6.3	−6.5
ΔH (kcal mol^{-1})	−7.0	−31.1
$-T\Delta S$ (kcal mol^{-1})	0.7	25.3

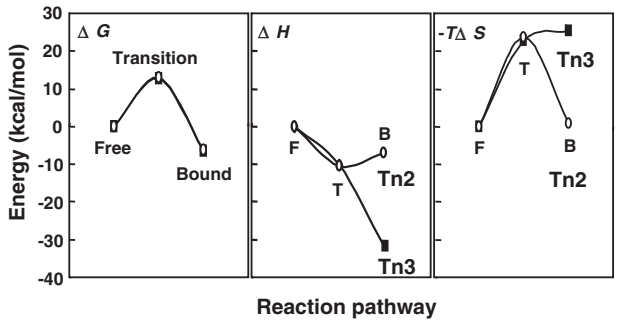

Fig. 7 Free-energy profiles for interaction between MLS128 and Tn2- or Tn3-peptide. Plots show ΔG, ΔH and $-T\Delta S$ for MLS128 interaction with Tn2-peptide (open squares) and Tn3-peptide (closed squares); values are results of van't Hoff (for Bound) and Eyring analyses (for Transition).

and $2.2 \times 10^4\,M^{-1}$ (Tn2) and $2.2 \times 10^4\,M^{-1}$ (Tn3) at 25°C. The results indicated that affinity for Tn2-pepitde and Tn3-peptide decreased by 1.3-fold when the temperature increased from 10°C to 25°C. The results are consistent to some extent with those of SPR since changes in affinity for binding to Tn2- and Tn3-peptide were 1.4- and 13-fold, respectively although an expected level of difference from SPR experiments was not achieved due to the experimental limitation as described above.

^{1}H-NMR experiments

^{1}H-NMR experiments were performed in order to analyse the binding of Tn2- and Tn3-peptides to MLS128 in solution (Fig. 9A and B, respectively). ^{1}H-NMR spectra of Tn2- and Tn3-peptides were recorded at 5°C with MLS128 at a molar ratio of 1:1 based on the number of binding sites. MLS128 produced very broad ^{1}H-NMR signals in the background due to its high MW (150 kDa), and the signal pattern did not change significantly upon titration of ligands under experimental conditions (data not shown). In contrast, both Tn2 and Tn3 peptides exhibited relatively sharp ^{1}H-NMR signals in the presence of MLS128, which suggests a slow exchange (Figs. 9 Aa/Ba versus Ab/Bb). Based on the association constants (K_a) determined by SPR and ITC analysis, nearly half of the ligands are free in solution under slow exchange conditions. The free peptide fraction produces sharp signals in both spectra. Slight line broadening was observed with Cγ-methyl groups of Thr (1.4–1.2 ppm) even under slow exchange conditions. These observations indicate that both Tn2 and Tn3 peptides bind to MLS128 in a slow exchange and that Thr residues consecutively located at the centre of the peptide are involved in that binding.

Furthermore, the binding event was analysed by focusing on temperature dependency. To that end, ^{1}H-NMR spectra were additionally recorded at 20°C and 35°C, respectively (Figs. 9Ac/B9c and A9d/B9d). The increase in the temperature considerably augmented signal broadening. The sharpness of the ligand signals at 5°C (Fig. 9Ab and Bb) diminished in experiments at 20°C (Fig. 9Ac and Bc), and considerably broader signals were obtained at 35°C (Fig. 9Ad and Bd). In terms of the quantification of the exchanging properties based on the line-shape, a singlet signal originating from the acetamide group of GalNAc3 at 2.0 ppm was used because GalNAc3 is located in the centre of the Tn2/Tn3 peptides. The acetamide signal of Tn2 peptide basically indicate a sharp singlet (linewidth; 3.1 Hz; without MLS128, and 5.1 Hz with MLS128) at 5°C. Same values were obtained in the case of Tn3 peptide (3.1 Hz; without MLS128 and 5.1 Hz with MLS128 at 5°C). On the other hands, Tn3 peptide behaved more sensitive to the temperature increase than Tn2 peptide. Notably, the acetamide

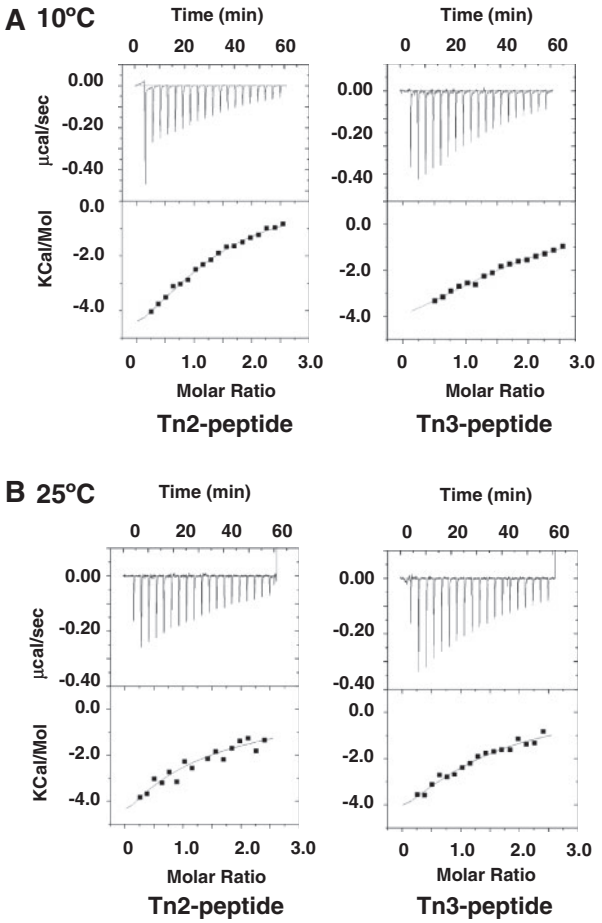

Fig. 8 ITC profiles of MLS128 and Tn2-and Tn3-peptides at 10°C and 25°C. MLS128 (48.8 µM) was titrated with 600 µM Tn2-peptide (left panel) or Tn3-peptide (right panel) at 10°C (**A**) and at 25°C (**B**). The upper panels show calorimetric data obtained from each titration point. The lower panels show the integrated curves of the area of each titration peak with values converted to kcal/mol.

methyl-signal of Tn2 show line-width of 5.7 Hz at 25°C and 11.0 Hz at 35°C, whereas Tn3 peptide provided the line-width of 6.2 Hz at 25°C and 14.3 Hz at 35°C (Fig. 9A spectra b and c versus 9B spectra b and c). These temperature-dependent dynamics took place in NMR on a scale of nanoseconds or microseconds. Temperature-dependent signal broadening suggests that the ligand–protein exchange rate increased markedly to an intermediate- to fast-exchange rate at higher temperatures. In summary, slow exchange at 5°C in ^{1}H-NMR changed to intermediate to fast exchange at 35°C.

Discussion

Ever since finding that MLS128 treatment significantly inhibited colon and breast cancer cell growth (*17*), the current authors and colleagues have diligently sought to understand the mechanisms by which MLS128 inhibits cancer cell growth. MLS128's inhibition of cancer cell growth is primarily by binding to a receptor on plasma membranes. Thus, understanding the interaction between MLS128 and its ligand is an important step in developing cancer therapeutics. MLS128 was previously reported to have specificity for Tn3 rather than Tn2 (*16*), so the current study intensively analysed the interaction between MLS128 and Tn2 or Tn3-antigen using SPR, NMR and ITC. MLS128's dissociation constants for Tn3-epitope were found to drastically increase as the assay temperature rose over 25°C, whereas those for Tn2-epitope remained nearly the same, resulting in an intriguing switch from Tn3-specificity to Tn2/Tn3-bispecificity at temperatures >25°C.

Breast-cancer-related 83D4 and colon-cancer-related MLS128 are two of several anti-Tn antigen mAbs reported (*14*, *24*, *25*), and comparison of their affinity for Tn-antigen clusters (*16*) has led to an interesting question of how Tn2- or Tn3-specificity is determined. Both 83D4 and MLS128 bind Tn2- and Tn3-residues but not to single Tn-antigen (Tn1), as Osinaga *et al.* (*16*) clearly indicated in Table 1 of their paper. Of particular interest was the fact that while 83D4 has affinity for both Tn2- and Tn3-peptides on the order of 10^{-8} M, MLS128 has affinity for Tn3-peptide on the order of 10^{-8} M but affinity for Tn2-peptide an order lower. Tn-antigen-specific tetrameric *Vicia villosa* isolectin B4 (VVLB4) binds to Tn1 epitope with affinity on the order of 10^{-7} M but does not bind to Tn2- or Tn3-epitopes. The crystal structure of VVLB4 was elucidated (*26*), and this work indicated the role of a single protein residue, Tyr127, as a structural determinant for Tn-binding specificity. To the extent known, however, precise interaction between anti-Tn-antigen mAbs and Tn2- or Tn3-antigen has yet to be reported.

A study of the thermodynamics of MLS128 binding to Tn2- or Tn3-peptide was conducted since analysis of MLS128 binding to Tn2- or Tn3-peptide at 37°C using ELISA provided results that did not appear to agree with previous SPR results. Previous results had found a single-order difference in affinity for binding of MLS128 to Tn2-peptide than for binding to Tn3-peptide at 25°C (*16*). Intensive SPR analyses at five different temperatures revealed unique characteristics of temperature-dependent MLS128 binding to Tn2/Tn3 ligands and indicated that 25°C is the critical temperature at which MLS128 has the same affinity for Tn3 and Tn2. Although IgG or IgM has two or five binding sites for ligands, the 1:1 (Langmuir) binding model is most commonly used among researchers. The kinetic parameters of MLS128 mAb (IgG_3), 83D4 mAb (IgM) and VVLB4-lectin determined by Osinaga *et al.* using the 1:1 binding model represent apparent (sum) kinetics. In fact, the current authors previously reported that MLS128 has 2.1×10^{-8} M affinity for Tn2-peptide using the single-site Langmuir binding model (*18*). In the current study, however, sensorgrams were fitted to the bivalent analyte model to reveal true kinetics using one binding site. The bivalent analyte model is schematically represented in Equation (1) shown in the 'Materials and Methods' section, in which one MLS128 (A) binds with two Tn2–antigen (B) or Tn3-antigen (B) immobilized on the sensor chip. In this model, MLS128 binds with one Tn2-peptide or Tn3-peptide unit (reaction 1) and

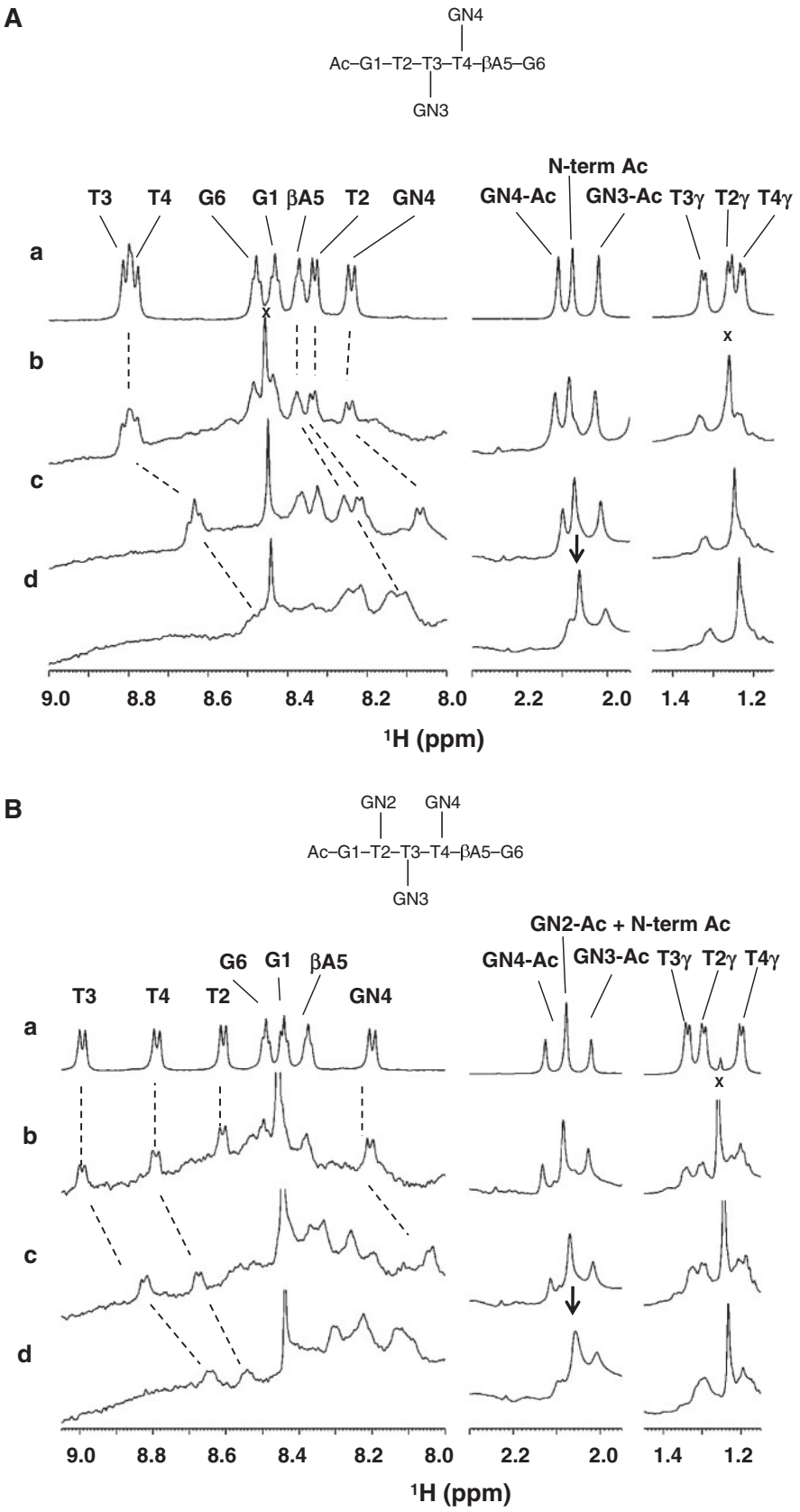

Fig. 9 ^{1}H-NMR spectra of Tn2 or Tn3-peptide in the absence or presence of MLS128. (A) ^{1}H-NMR spectra of Tn2 with MLS128. Amide region (9.0–8.0 ppm) and aliphatic regions (2.4–1.9 ppm and 1.5–1.1 ppm) were apparent. The ^{1}H-NMR spectrum of Tn2 alone at 5°C is shown as reference (a). ^{1}H-NMR spectra of Tn2 in the presence of MLS128 mAb were recorded at 5°C (b), 20°C (c) and 35°C (d). GN; *N*-acetyl-α-D-galactosamine. (B) ^{1}H-NMR spectra of Tn3 with MLS128. Amide region (9.1–8.0 ppm) and aliphatic regions (2.4–1.9 ppm and 1.5–1.1 ppm) were apparent. The ^{1}H-NMR spectrum of Tn3 alone at 5°C is shown as reference (a). ^{1}H-NMR spectra of Tn3 in the presence of MLS128 mAb were recorded at 5°C (b), 20°C (c) and 35°C (d). GN; *N*-acetyl-α-D-galactosamine.

then another antigen (reaction 2). The kinetic parameters for each reaction can be calculated using BIAevaluation software, in which the association constant of reaction 1 (K_A1) corresponds to the binding affinity of MLS128. Affinity constants calculated using the bivalent analyte model, which turned out to be two-orders lower than those reported by Osinaga *et al*. (*16*), should reflect real binding between MLS128 and Tn2- or Tn3-peptide. The K_A1 calculated from SPR data was in a range comparable to K_A values estimated using ITC, substantiating the validity of the current SPR results although the ITC experiments shown in Fig. 8 were still preliminary due to the limitation of mAb concentrations.

The thermodynamics of binding of Tn3- and Tn2-peptides studied using SPR suggested that Tn3 binding to MLS128 is enthalpically driven but entropically unfavourable, whereas the binding of Tn2 to MLS128 does not seem to involve entropic changes. MLS128 bound with Tn3 appears to be more stable than that with Tn2 presumably due to a larger area of the contact surface for the interaction with Tn3 than Tn2. This interaction seems to require hydrogen bonding interactions, which is consistent to the observation that sensorgrams for MLS128 binding to both Tn3- and Tn2-peptides were nearly identical at 10°C when recorded in the presence of 400 mM NaCl (data not shown). These results indicate that induced fitting of the complex may occur upon MLS128 binding to Tn3. In contrast, the fact that no entropy change observed with MLS128 binding to Tn2 suggests that the binding of Tn2 to MLS128 utilizes 'lock and key' recognition without involving conformational changes. Unlike thermodynamic studies on lectins as previously published (*4*, *5*, *27*), detailed thermodynamic characterization of carbohydrate antibody complexes have scarcely been reported except mAb for a *Salmonella* O-antigen (*28*). To obtain insights into the mechanism by which MLS128 recognizes Tn3 or Tn2, further investigation using NMR is in progress. Initial results are described in the current manuscript.

NMR analyses revealed that sharp acetamide methyl signals at 2.0–2.1 ppm behaved as sensitive probes to analyse the binding between Tn2- and Tn3- peptide and MLS128. The methyl signal originating from the N-terminal acetamide methyl group provided a characteristic sharp singlet even at 35°C, which is apparent in contrast to the broad signals of other acetamides at GalNAc (indicated as arrows). The differences in line width indicate that the N-terminal acetamide group retains flexibility and is separate from the binding interface of MLS128. Furthermore, the N-terminal acetyl group originating from Tn3 provides a broader signal, while the corresponding signal from Tn2 suffers only limited signal broadening. The result might be due to differences in binding faces. MLS128 may have a wider face to bind with Tn3 that encompasses binding to the N-termini. In contrast, mAb-Tn2 interaction may involve use of a limited binding face.

Comparison of the spectra at the transition temperature (20°C) from slow exchange to intermediate/fast exchange (Fig. 9A and B; spectra c) indicated that Tn3 produced broader signals than Tn2 because of its faster exchange. The singlet signals originating from the acetamide group of GalNAc3 and GalNAc4 at 2.0 and 2.1 ppm in particular are characteristic. These results suggest that the exchange rate for Tn3-peptide is higher than that for Tn2-peptide under the conditions in this study, which is consistent with the SPR data (Table II).

In summary, SPR and NMR analyses revealed the unique temperature-dependent nature of MLS128 and Tn3/Tn2 interactions. The long-term goal of the current research is to understand what causes differences between MLS128's interaction with Tn2-antigen and its interaction with Tn3-antigen at the molecular level. Initial NMR characterization of the interaction between anti-Tn-antigen mAb and Tn2- or Tn3-antigen is shown in Fig. 9. To further investigate ligand-MLS128 interactions, additional studies have (i) cloned genes encoding MLS128 variable domains and measured expression of the gene in single chain antibody (scFv) and its Fc-conjugated forms (scFv-Fc) (*29*) and (ii) measured expression and purified MLS128 scFv protein with full binding activity as indicated by NMR and ITC analyses. Two manuscripts have been submitted describing these studies.

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Conflict of interest

None declared.

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