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The binding of synthetic analogs of the upstream, terminal residue of the O-polysaccharides (O-PS) of *Vibrio cholerae* O:1 serotypes Ogawa and Inaba to two murine monoclonal antibodies (MAbs) specific for the Ogawa lipopolysaccharide (LPS)

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Abstract

The binding of nineteen analogues of the upstream, terminal, monosaccharide residue of each of the O-polysaccharide (O-PS) of *Vibrio cholerae* O:1, serotype Ogawa and Inaba, with two murine monoclonal IgG antibodies both specific for the Ogawa LPS were measured using fluorescence spectroscopy. The use of the deoxy and the deoxyfluoro analogs allowed further refinement of the hydrogen-bonding pattern involved in the binding. Based on the binding characteristics observed for some of the ligands in the Inaba series, the binding of the monosaccharide that represents the upstream, terminal unit of the O-PS of *V. cholerae* O:1 serotype Inaba was redefined. We show for the first time that the upstream, terminal monosaccharide of the Inaba O-PS shows weak binding with these two anti-Ogawa antibodies. The results obtained allow further rationalization of the structural basis for the binding of *V. cholerae* O:1 antigens to their homologous antibodies.

1. Introduction

In the past, we have used synthetic, specifically deoxygenated and fluorinated analogues of saccharides that mimic the structure of antigenic O-polysaccharides (O-PS) to probe hydrogen bonding involved in binding to their murine monoclonal antibodies (MAbs).[1,2] Such studies can reveal involvement of individual hydroxyl groups in the antigen–antibody interaction to mediate hydrogen bonding, and can also identify the source, the antigen or the antibody, that provides the hydrogen atom that is a conduit for this interaction.[3–7]

Using the aforementioned approach, we reported earlier[2] on the binding of a number of antigenic fragments to two murine monoclonal IgG antibodies, namely S-20-4 and A-20-6, both specific for the *Vibrio cholerae* O:1, serotype Ogawa LPS. Important conclusions concerning the size and the shape of the combining site could be made, and for IgG S-20-4, these were later fully confirmed by X-ray analysis[8] of the Fab fragment crystallized with either the terminal mono- or disaccharide determinants of the O-PS of *V. cholerae* O:1, serotype Ogawa. We were also able to show that IgGs S-20-4 and A-20-6 had similar affinities and the same fine specificities for a set of methyl α-glycosides of mono- and oligosaccharides that are the upstream, terminal fragments of the O-PS of *V. cholerae* O:1, serotype Ogawa.[2] A new series of analogues, including specifically monofluorinated derivatives of the terminal determinant of the O-PS in both the Ogawa- and the Inaba series, have recently become available.[9–11] Employing these ligands, we here present an extension of the previous study and report for the first time the finding that the upstream monosaccharide terminus of the serotype Inaba O-PS shows weak binding with these two anti-Ogawa antibodies.

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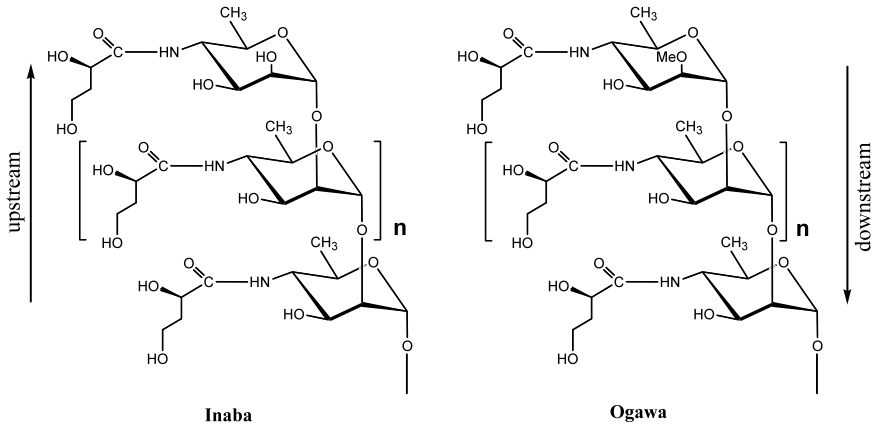


Fig. 1. Comparison of the structures of the O-PSs of *V. cholerae* O1, serotype Inaba and Ogawa.

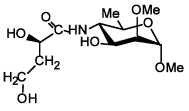
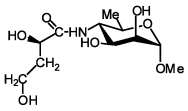
2. Results and discussion

The O-PS of *V. cholerae* O:1 is a homopolymer made up of (1→2)-linked 4-amino-4,6-dideoxy-α-D-mannopyranosyl (perosaminyl) residues whose amino group is acylated with 3-deoxy-L-*glycero*-tetronic acid. The two polysaccharides apparently differ in that in the serotype Ogawa there is a 2-*O*-methyl group in the terminal upstream residue, whereas in the Inaba serotype C-2 position holds a hydroxyl group[12–14] (Fig. 1).

Table 1 shows the binding of the monosaccharides in the form of their methyl α-glycosides (**1** and **2**) that represent, respectively, the Ogawa and Inaba upstream termini. In continuation of our previous studies,[2] we here report on the binding of another series of synthetic analogues of the upstream, monosaccharide termini of the O-polysaccharides of *V. cholerae* O:1, serotype Ogawa and Inaba to the same two monoclonal murine IgG antibodies.

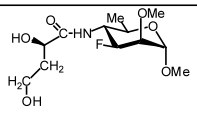
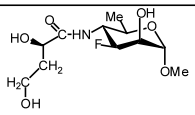
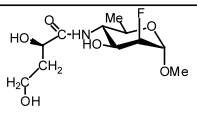
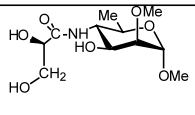
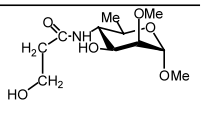
Table 2 lists the binding constants of the nineteen analogues of the methyl glycosides of the terminal Ogawa and Inaba-monosaccharides with these MAbs that are specific for the Ogawa LPS, namely the IgGs S-20-4 and A-20-6. We reported earlier that we had not observed binding of the terminal Inaba monosaccharide to those MAbs. However, when the work reported here was in progress, the binding patterns of some of the new ligands measured strongly indicated that the Inaba monosaccharide should bind weakly to these monoclonal IgGs. To wit: In Table 2, note that the 3-deoxy-3-fluoro Ogawa analogue (**3**) binds to the two monoclonal IgGs with a K_a that is similar ($\sim 0.5\times$) to that of the Ogawa terminal saccharide itself (**1**, Table 1). Our previous[2] solution and crystallographic studies[8] showed that the 3-OH group in **1** is involved in hydrogen bonding. We have also shown in the past[3–7] that fluorine substitution allows hydrogen-bond reception due to fluorine's electronegativity. Thus, the observed binding of the 3-fluoro derivative **3** is not unexpected. That the corresponding 3-fluorinated monosaccharide in the Inaba series (**4**) binds to these anti-Ogawa antibodies, albeit with a binding constant that is $\sim 1.7\times 10^2$ times less than that of **3**, suggests that the Inaba monosaccharide **2** itself should bind to these antibodies with a K_a similarly reduced when compared to **1**, i.e., perhaps around 10^3. This prompted us to measure the K_a of **2** with the IgGs S-20-4 and A-20-6 using a substantially higher concentration of ligand than was the case in our previous study.[2] (This, because if an antibody is screened for possible binding to a ligand, and the ligand solution is used at a low concentration, set for an expected affinity of 10^5–10^6, while in fact the

Table 1
The binding constants (K_a, M^{-1}) and free-energy association ($-\Delta G°$, kJ/mol) for vibriocidal immunoglobulins G specific for the LPS of *V. cholerae* O:1, serotype Ogawa with the synthetic terminal monosaccharide determinants of the O-PS of *V. cholerae* O:1, serotype Inaba and Ogawa

Ligand		Mono Ogawa	Mono Inaba
Structure			
		1	**2**
S-20-4	K_a	2.86×10^5	3.41×10^2
	ΔG	31.1 kJ	14.5 kJ
	r^2	0.94	0.85
	ΔF%	9.37%	47%
A-20-6	K_a	3.16×10^5	4.77×10^2
	ΔG	31.37 kJ	15.3 kJ
	r^2	0.96	0.71
	ΔF%	9.61	12.6

K_a is much lower, it is likely that the test addition of the far-too-dilute antigen solution will not result in an observable change in fluorescence, thus incorrectly indicating a lack of binding.) It can be seen in Table 1 that using a more concentrated ligand solution, the Inaba monosaccharide **2**, indeed, showed weak binding with the two IgGs, their K_as being some 10^3 times less than with the Ogawa monosaccharide **1**. This observation can be perhaps explained as follows: It was found in our work on the X-ray diffraction of IgG S-20-4 bound to the terminal Ogawa saccharides[8] that the 2-*O*-methyl group of the Ogawa disaccharide occupies a hydrophobic pocket formed by the Trp 95 residue and the Tyr 32 residue, both of the light chain. That methyl group is not present in the upstream, terminal Inaba monosaccharide, and the resulting 2-OH group is thus no longer nonpolar. In this saccharide, the now absent electron-donating effect of the methyl group may make the oxygen of the neighboring 3-OH group somewhat less negative. This could reduce its capacity to partake in the bifurcated hydrogen bond found previously.[8] As to the other binding patterns that could still come into play in the Inaba terminus: If the tetronic acid residue could still be able to bind as in the Ogawa terminus, the

Table 2
The binding constants (K_a, M^{-1}) and free-energy association ($-\Delta G°$, kJ/mol) for vibriocidal immunoglobulins G specific for LPS of *V. cholerae* O:1, serotype Ogawa with synthetic analogs of the terminal monosaccharide determinants of the O-PS of *V. cholerae* O:1, serotype Inaba and Ogawa

						
		3	**4**	**5**	**6**	**7**
S-20-4	K_a	1.15×10^5	6.84×10^2	1.06×10^4	1.40×10^5	6.25×10^3
	ΔG	28.87 kJ	16.17 kJ	23.0 kJ	29.36 kJ	21.6 kJ
	r^2	1.00	0.95	0.96	0.94	1.00
	$\Delta F\%$	-10.3	-12.85	-18.35	-17.00	-14.6
A-20-6	K_a	5.75×10^5	6.84×10^2	1.01×10^4	4.04×10^5	6.04×10^3
	ΔG	32.86 kJ	16.17 kJ	22.84 kJ	31.98 kJ	21.57 kJ
	r^2	0.90	0.75	0.91	0.92	0.90
	$\Delta F\%$	-6.02	-11.8	-22.42	-11.55	-7.56

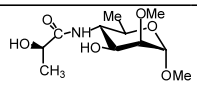
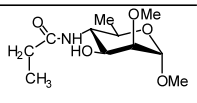
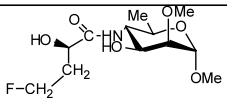
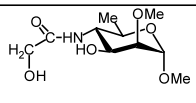
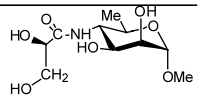
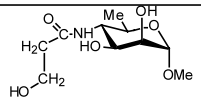
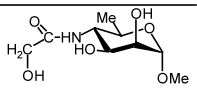
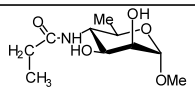
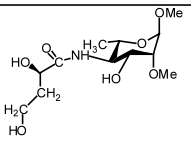
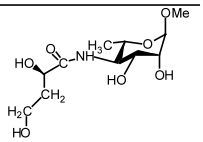
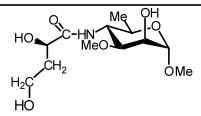
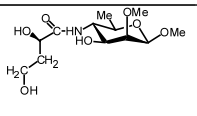
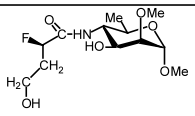
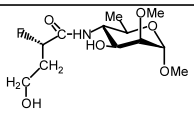
Structure						
		8	**9**	**10**	**11**	**12**
S-20-4	K_a	2.09×10^5	2.73×10^3	4.67×10^5	7.55×10^3	3.32×10^3
	ΔG	30.35 kJ	19.60 kJ	32.3 kJ	22.12 kJ	20.09 kJ
	r^2	0.86	0.91	0.97	0.94	0.89
	$\Delta F\%$	-9.15	-9.76	-11.01	-16.49	-6.73
A-20-6	K_a	2.90×10^5	1.12×10^3	5.23×10^5	1.30×10^4	4.50×10^3
	ΔG	31.16 kJ	17.40 kJ	32.62 kJ	23.47 kJ	20.84 kJ
	r^2	0.95	0.97	0.96	0.96	0.95
	$\Delta F\%$	-8.33	-7.37	-10.66	-10.58	-5.39

Table 2 (*Continued*)

	Structure					
		13	**14**	**15**	**16**	**17**
S-20-4	K_a	6.34×10^2	1.54×10^3	7.79×10^3	1.80×10^3	1.20×10^5
	ΔG	15.98 kJ	18.18 kJ	22.20 kJ	18.57 kJ	28.98 kJ
	r^2	0.96	0.90	0.84	0.95	0.96
	$\Delta F\%$	-13.19	-8.57	-8.07	-13.62	-9.85
A-20-6	K_a	6.32×10^2	1.02×10^3	1.24×10^4	9.56×10^2	1.01×10^5
	ΔG	15.98 kJ	17.16 kJ	23.35 kJ	17.00 kJ	28.55 kJ
	r^2	0.94	0.98	0.95	0.99	0.98
	$\Delta F\%$	-13.01	-8.08	-10.11	-12.76	-11.99

	Structure				
		18	**19**	**20**	**21**
S-20-4	K_a	3.89×10^3	6.94×10^3	2.60×10^3	1.16×10^3
	ΔG	19.6 kJ	21.9 kJ	19.5 kJ	17.48 kJ
	r^2	0.83	0.94	0.89	0.91
	$\Delta F\%$	-8.86	-6.32	-7.90	-11.2
A-20-6	K_a	7.42×10^3	1.75×10^4	1.48×10^3	1.28×10^3
	ΔG	22.08 kJ	24.20 kJ	18.08 kJ	17.73 kJ
	r^2	0.79	0.97	0.97	0.92
	$\Delta F\%$	-8.21	-5.68	-4.86	-9.77

other bifurcated hydrogen bond,[8] from the -NH- of the aspartic acid 33 (H-chain) and the >C=O group of the histidine 95 (H-chain) to, and from, the 2′-OH of the tetronic acid side chain, may still be possible. Although scientifically interesting, it is not clear if so moderate a binding could significantly affect a cross-reaction of the Inaba strain to anti-Ogawa antibodies. Similarly, the 2-fluoro derivative **5** has a K_a some 25 times less than that of **1**. Again, the electronegative fluorine may not have much, if any, interaction with the hydrophobic pocket mentioned above. It could, again, affect the 3-OH group in the way it partakes in the bifurcated hydrogen bond.

The effect of shortening the amido side chain by removal of the 3′-methylene group in the Ogawa monosaccharide, i.e., the transition of **1** to the corresponding *N*-dihydroxypropyl derivative **6**, is small (Table 2), and these two ligands show similar K_as with the two IgGs. Removal of the 2′-OH group from the shortened side chain, i.e., **6** → **7**, reduces the K_a another 22 times. The finding that the 4-(*N*-2-hydroxypropyl) derivative **8** binds almost as well as does **1**, shows the importance of the 2′-OH group in the binding. It also shows that the terminal hydroxymethyl group of the tetronic acid contributes only negligibly to the binding of **1**. That is further indicated by the only small increase

in the binding of the 4′-fluoro derivative **10**, when compared to **1**. This confirms our previous finding that the weak interaction of the 4′-OH is not critical for binding.[8] That the 3′-OH still very slightly enhances binding is shown by the fact that the 3-hydroxypropyl **7** binds marginally better than the propyl derivative **9**. The K_a of the *N*-hydroxyacetyl analog of **1**, (**11**), is nearly 40 times less than that of **1**, showing that the 2′-OH in **1** is important only in association with a neighboring methylene group. Crystallographic analysis of the antibody complexes of Fab S-20-4 with the methyl α-glycosides of the Ogawa mono- and disaccharide[8] has shown four bifurcated hydrogen bonds. These are involved between the 2′-OH of the tetronic acid residue and the 3-OH of the perosamine residue with the antibody's amino acid residues Asp-33H and His-95H. Disruption of that network apparently leads to a noticeable reduction in binding.

In the corresponding Inaba series, the reduction in binding in going from the *N*-2,3-dihydroxypropyl derivative **12** to the *N*-3-hydroxypropyl derivative **13** is not as pronounced, i.e., in terms of K_a only by a factor of 5. The observation that the 2′,3′-dihydroxypropyl **12**, hydroxyacetyl **14** and propyl **15** analogs of **2** all bind better than **2** itself is rather puzzling. Another finding for which we cannot offer a plausible explanation at this time is that the monosaccharide analogues having the perosamine in the L-form rather than the D-form, do show considerable binding. As shown in Table 2, both L-manno analogues bind to the two monoclonal IgGs, the Ogawa analog **16** some 1.6×10^2 times less than does **1**, while the Inaba analog **17** actually binds some 3×10^2 times better than **2** itself. It was shown by others[15,16] that *V. cholerae* 076 and 0144 have homopolymers of *N*-{(*S*)-(+)}- and *N*-{(*R*)-(−)-2-hydroxypropionyl}-α-L-perosamine, respectively, as their O-polysaccharides, and they showed serological cross-reaction. However, neither the LPS of 076 nor of 0144 cross-reacted with *V. cholerae* O:1 serotype Ogawa or Inaba, or some other strains whose O-PS contained D-perosamine residues.[15,16] Thus, the cited works showed that the absolute configuration of the perosamine in the O-PS is important for serological specificity. The monosaccharides **1** and **2** here tested have the same (L,S) configuration of the tetronamide moiety as that present in the O-PS of the serotypes Ogawa and Inaba.

Again, in the Inaba series, the 3-*O*-methyl analogue of **2**, **18**, binds with a K_a ca. 10 times higher than **2** itself. This could be due to the fact that a major hydrogen bond to the O-3 of **18** is still possible: No hydrogen donation from the 3-OMe of **18** *to* the His-95H is possible, unlike the case for the 3-OH in **1** and **2**, but the hydrogen bond to O-3 in **18** *from* the Asp-33H could be stronger due to the electron-donating capacity of the methyl group on that oxygen at position 3.

In view of the α-interglycosidic linkage in the O-PS, the decreased binding of the β anomer **19** to the two antibodies must reflect the unfavorable stereochemistry of the glycosidic linkage.

We have previously found that when in **1** the L-tetronic acid is changed to a D-tetronic acid, the binding constant is markedly reduced.[2] The similar reduction in binding of both the 2′-fluoro derivatives (L-*glycero*, **20** and D-*glycero*, **21**) for the two MAbs indicates that the capability of the 2′-OH in the tetronic acid residue to not only accept, but also to donate a hydrogen bond to the aspartic acid 33 H of the antibody is important.

3. Experimental

Pure monoclonal immunoglobulins were obtained from the ascites fluids containing IgGs A-20-6 and S-20-4 as previously described.[8,17] The previously reported, amorphous methyl 4,6-dideoxy-4-(2,3-dideoxy-2-fluoro-L-*glycero*-tetronamido)-2-*O*-methyl-α-D-mannopyranoside (**15**) solidified on standing. Recrystallization from EtOAc–ether gave material melting at 92.5–93.5 °C. Anal. Calcd for $C_{12}H_{22}FNO_6$: C, 48.81; H, 7.51; N, 4.74. Found: C, 48.86; H, 7.64; N, 4.73. The preparation and full characterization of the other monosaccharides has been reported.[9,11,18]

Affinity constants were determined as described previously.[19,20] In Section 2, atoms or groups of atoms located on the *N*-acyl side chain are denoted with a prime (′).

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