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Further characterization of the saccharide specificity of peanut (*Arachis hypogaea*) agglutinin*,†

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ABSTRACT

2-Dansylamino-2-deoxy-D-galactose (GalNDns) has been shown to bind to peanut (*Arachis hypogaea*) agglutinin (PNA) in a saccharide-specific manner. This binding was accompanied by a five-fold increase in the fluorescence of GalNDns. The interaction was characterized by an association constant of 0.15 mM at 15° and ΔH and ΔS values of $-57.04\ kJ \cdot mol^{-1}$ and $-118.1\ J \cdot mol^{-1} \cdot K^{-1}$, respectively. Binding of a variety of other mono-, di- and oligo-saccharides to PNA, studied by monitoring their ability to dissociate the PNA–GalNDns complex, revealed that PNA interacts with several T-antigen-related structures, such as β-D-Gal*p*-(1→3)-D-GalNAc, β-D-Gal*p*-(1→3)-α-D-Gal*p*NAcOMe, and β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→3)-Ser, as well as the asialo-G_{M1} tetrasaccharide, with comparable affinity, thus showing that this lectin does not discriminate between saccharides in which the penultimate sugar of the β-D-Gal*p*-(1→3)-D-GalNAc unit is the α or β anomer, in contrast to jacalin (*Artocarpus integrifolia* agglutinin), another anti T-lectin which preferentially binds to β-D-Gal*p*-(1→3)-α-D-GalNAc and does not recognize β-D-Gal*p*-(1→3)-β-D-GalNAc or the related asialo-G_{M1} oligosaccharide. These studies also indicated that, in the extended combining region of PNA which accommodates a disaccharide, the primary subsite (subsite A) is highly specific for D-galactose, whereas the secondary subsite (subsite B) is less specific and can accommodate various structures, such as D-galactose, 2-acetamido-2-deoxy-D-galactose, D-glucose, and 2-acetamido-2-deoxy-D-glucose.

INTRODUCTION

The ability of the extract of peanut (*Arachis hypogaea*) to agglutinate sialidase-treated human red blood cells has been known for a very long time[1,2]. The protein responsible for this activity, peanut agglutinin (PNA), has been purified and its macromolecular properties have been characterized[3,4]. The similarity of the immunological behaviour of the agglutinin to the anti-T antibody of the mammalian sera has resulted in its routine use in serological applications to monitor T-poly agglutinability, which results upon exposure of Thomsen–Friedenreich antigen on the red blood cells after treatment with sialidase[5]. On account of its importance as a cell-surface probe for T

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antigen, several studies of its carbohydrate specificity have been carried out, which have provided detailed insights into the nature of its saccharide-binding site[3,6–10]. Notable among these is the thermodynamic analysis[6] of ligand binding, which strongly pointed towards the presence of an extended binding region in the lectin for the T-antigenic disaccharide Gal*p*-(1→3)-GalNAc*. Since the T antigen is an important human carcinoma marker, intensive efforts have been made to identify anti-T probes, such as monoclonal antibodies and lectins. Among these, the ligand-binding specificity of jacalin (*Artocarpus integrifolia* agglutinin), isolated from the seeds of jackfruit, has been studied in considerable detail in our laboratory[11–14]. We report herein interesting features of ligand binding to PNA and compare the subtle but important differences in carbohydrate specificity between PNA and jacalin in order to reveal the advantages of each lectin as anti-T probes, thus preventing the wrong assignment of an unknown cell-surface antigen as T antigen. For this purpose, it was necessary to quantify the degree of association of several oligosaccharides with PNA and we report the binding of 2-(*N*-dansylamino)-2-deoxy-D-galactose to PNA and its use in substitution titrations as an indicator ligand.

EXPERIMENTAL

Materials. — Peanut agglutinin was purified by affinity chromatography on a matrix of lactose-coupled, epoxy-activated Sepharose[15]. 2-(*N*-dansylamino)-2-deoxy-D-galactose (GalNDns) was synthesized as described earlier[16,17]. D-Galactose, methyl α- and β-D-galactopyranoside (α- and β-GalOMe), 2-acetamido-2-deoxy-D-galactose, melibiose, β-Gal*p*-(1→3)-GlcNAc, and β-Gal*p*-(1→3)-Ara were products of Sigma Chemical Co., St. Louis, Missouri, U.S.A. α-Gal-(1→3)-α-GalOMe, β-Gal*p*-(1→3)-β-Gal*p*OMe, and β-Gal*p*NAc-(1→3)α-Gal*p*OMe were obtained from Carbohydrate International, Arlov, Sweden. β-Gal*p*-(1→3)-β-Glc*p*NAcOMe, β-Gal*p*-(1→3)-GalNAc, β-Gal*p*-(1→3)-α-Gal*p*NAcOMe, β-Gal*p*-(1→3)-α-Gal*p*NAc-(1→3)-Ser, asialo-G_{M1} tetrasaccharide, and lacto-*N*-tetraose were from Biocarb Chemicals, Lund, Sweden. Methyl 2-acetamido-2-deoxy-α-D-galactopyranoside was prepared by the method of Preston and Preston[18].

Fluorescence measurements. — Fluorescence spectra were recorded with a Shimadzu Fluorescence Spectrophotometer. Titrations of fluorescence for the binding of GalNDns to PNA was performed on a Union Giken FS 501A fluorescence polarizer, essentially as described earlier for the binding of GalNDns to other lectins[11,17]. The fluorescence intensity of GalNDns increased five-fold at infinite concentration of the lectin (Fig. 1). The association constant (K_a) was determined graphically from plots of log $[P]_f$ *vs.* log $[(F_c - F_0)/(F_\infty - F_c)]$ (Fig. 2) according to *Eq. 1*[19],

$$\log [(F_c - F_0)/(F_\infty - F_c)] = \log K_a + \log[P]_f \qquad (1)$$

* All sugars mentioned in this paper have the D configuration.

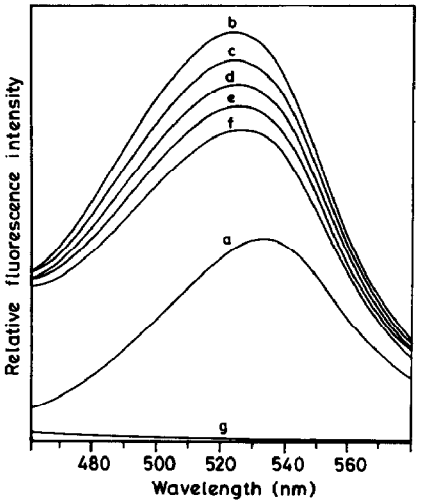

Fig. 1. Binding of GalNDns to PNA and reversal by α-Gal*p*OMe: The fluorescence intensity (curve a) of GalNDns (3.78μM, 2.0 mL) increased significantly (curve b) upon addition of PNA [175 μL, 644μM stock (protomer)] resulting in 34% saturation. The fluorescence intensity decreased (curves c–f) after the addition of successive aliquots (40 μL each) of α-Gal*p*OMe (20mM). Curve g is a buffer control.

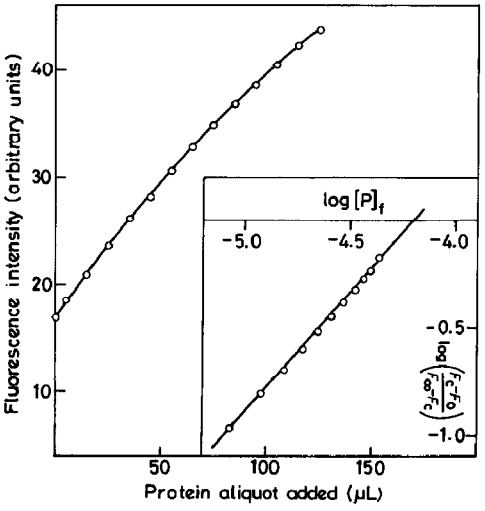

Fig. 2. Titration of GalNDns with PNA at 15°: The fluorescence intensity is plotted as a function of added protein concentration. A 3.78μM solution of GalNDns (1.8 mL) in 20mM phosphate buffer containing 0.15M NaCl was titrated with 644μM PNA (protomer). Inset gives a graphical representation for the determination of the association constant (K_a = 0.15mM). See Experimental section for details.

where F_0, F_c, and F_∞ are the fluorescence intensities of GalNDns in the absence, in the presence, and at infinite concentration of PNA, and $[P]_f$ is the free protein concentration. Binding of nonfluorescent, inhibitory sugars was studied by monitoring their ability to displace GalNDns from its complex with PNA in substitution titrations. Association constants for the indicator ligand and the competing ligand were determined from plots of $[L]_f$ *vs.* $\{[P]_t/[PD]-1\}\cdot[D]_f$ (Fig. 3) according to *E*q. 2[20,21],

$$\{[P]_t/[PD]-1\}\cdot[D]_f = K_L/K_D\cdot[L]_f + 1/K_D \quad (2)$$

The enthalpy of interaction was obtained from Van 't Hoff plots of the temperature dependent association constants. Free-energy of interaction was obtained from *Eq. 3*

$$\Delta G = -RT\ln K_a \quad (3)$$

and the entropy changes were obtained from *Eq. 4*.

$$\Delta S = (\Delta H - \Delta G)/T \quad (4)$$

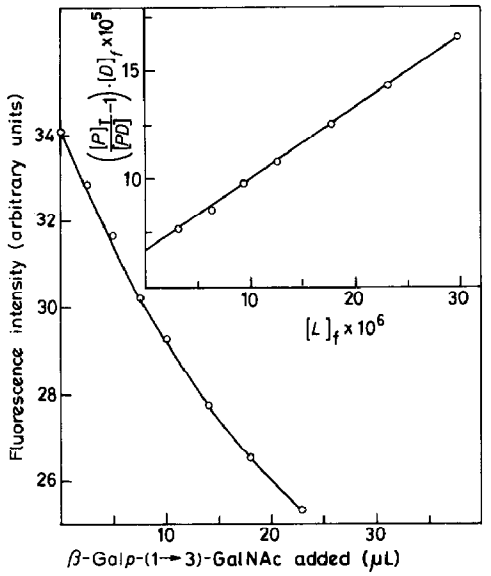

Fig. 3. Competitive binding of β-Gal*p*-(1→3)-GalNAc to PNA in the presence of GalNDns: To a 3.78μM solution of GalNDns (1.8 mL) was added 644μM PNA (in protomer, 75 μL) at 15°. The mixture was then titrated with β-Gal*p*-(1→3)-GalNAc (2.61mM). The fluorescence intensity was plotted as a function of β-Gal*p*-(1→3)-GalNAc added. Inset gives a graphical representation for the determination of association constants for the indicator ligand (GalNDns; K_a 0.149mM) and the competing ligand [β-Gal*p*-(1→3)-GalNAc, K_a 0.49mM].

RESULTS

The association constants for the binding of several saccharides to PNA have been determined in this study using the method of fluorescence substitution titration with GalNDns as the indicator ligand. The fluorescence intensity of GalNDns was enhanced five-fold when all the sugar molecules were bound by the lectin (Fig. 1). Addition of inhibitory saccharides totally reversed this effect. At 15°, GalNDns was

found to have an association constant of 0.15mM. Thermodynamic parameters obtained from Van 't Hoff plots were, $\Delta H = -57.04$ kJ·mol^{-1} and $\Delta S = -118.1$ J·mol^{-1}·K^{-1}. Since a new method was adopted to quantify the association of saccharides, it was considered necessary to determine the binding constants for a few saccharides, such as Gal, αGalOMe, and βGalOMe, for which values were known from earlier studies. The values obtained for these sugars (Table I) were in the range of those obtained by other investigators[9,22]. Apart from the association constants determined for the interaction of various saccharides with PNA, Table I also lists the corresponding K_a values obtained for their association with jacalin, taken from earlier studies[11,14], in order to compare their saccharide-binding behaviour. αGalNAcOMe with an association constant of 68mM was found to be a poor ligand, which is not unexpected as the lectin binds very poorly to GalNAc. Two disaccharide derivatives of T-antigen, β-Gal*p*-(1→3)-α-Gal*p*NAcOMe and β-Gal*p*-(1→3)-α-Gal*p*NAc-(1→3)-Ser, bound with affinities very similar to that for β-Gal*p*-(1→3)-GalNAc. The association constant for the

TABLE I

Association constants and relative affinities for the binding of saccharides to peanut agglutinin and jacalin at 15°

Sugar	*PNA*		*Jacalin*[a]	
	$10^{-3} \times K_a$ (M^{-1})	*Relative affinity*[b]	$10^{-3} \times K_a$ (M^{-1})	*Relative affinity*[b]
GalNDns	15.04	21.80	24.00	17.90
Galactose	0.69	1.00	1.34	1.00
α-Gal*p*OMe	2.90	4.20	63.00	47.00
β-Gal*p*OMe	1.18	1.71	0.29	0.21
GalNAc	0.15	0.22	4.50	3.36
α-Gal*p*NAcOMe	0.68	0.99	122.00	91.00
Melibiose (α-Gal*p*-(1→6)-Glc)	0.81	1.17	6.76	5.05
β-Gal*p*-(1→3)-GalNAc	49.00	71.01	250.00	186.57
β-Gal*p*-(1→3)-α-Gal*p*NAcOMe	60.10	87.10	1520.00	1134.33
β-Gal*p*-(1→3)-α-Gal*p*NAc-(1→3)-Ser	63.00	91.30		
Asialo-G_{M1} tetrasaccharide [β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-β-Gal*p*-(1→4)-Glc]	74.60	108.12	[c]	
Lacto-*N*-tetraose [β-Gal*p*-(1→3)-β-Glc*p*NAc-(1→3)-Gal*p*-(1→4)-Glc]	12.85	18.62		
β-Gal*p*-(1→3)-β-Gal*p*OMe	10.46	15.16	0.32	0.24
α-Gal*p*-(1→3)-α-Gal*p*OMe	0.80	0.80	7.48	5.58
β-Gal*p*NAc-(1→3)-α-Gal*p*OMe	0.19	0.28	489.77	365.50
β-Gal*p*-(1→3)-GlcNAc	1.70	2.46	0.04	0.03
β-Gal*p*-(1→3)-Ara	5.93	8.59	[c]	
β-Gal*p*-(1→3)-β-Glc*p*NAcOMe	1.09	1.58		

[a] The values for Jacalin were taken from refs. 11 and 14. [b] Galactose was used as the reference to calculate relative affinities. [c] No binding.

binding of asialo-G_{M1} tetrasaccharide (G_{M1}tetra) was ~1.5-fold higher than that of β-Gal*p*-(1→3)-GalNAc. The disaccharide, β-Gal*p*NAc-(1→3)-α-Gal*p*OMe, showed poor affinity for the lectin, the value of association constant being similar to that of GalNAc which is a very poor ligand for PNA (Table I). The disaccharide, β-Gal*p*-(1→3)-β-GalOMe, bound reasonably strongly to the lectin, the K_a value being 5.93mM. The disaccharides, β-Gal*p*-(1→3)-GlcNAc and β-Gal*p*-(1→3)-β-GlcNAcOMe, interacted with the lectin with comparable affinity. The tetrasaccharide, lacto-*N*-tetraose, was found to have an association constant of 15.8mM, which is about nine-fold higher than that of β-Gal*p*-(1→3)-GlcNAc, and is in contrast to an earlier study, where both these saccharides were shown to be nearly identical in their ability to inhibit the lectin activity in quantitative precipitin-inhibition assays[7].

DISCUSSION

In the present study, it has been possible to bring to light certain essential features of carbohydrate specificity of PNA, which on comparison with that of jacalin underline the necessity of such information when either of these lectins is used to probe the expression of T-antigenic determinant on cell surfaces. In an earlier study[11], we had shown that jacalin fails to bind to lactose and LacNAc, which is in contrast to PNA, which shows appreciable binding to both these disaccharides[9]. The same study had also demonstrated the absolute requirement of an axial orientation of OH-4 in the reducing hexopyranose residue of the T-antigenic disaccharide, β-Gal*p*-(1→3)-GalNAc, for binding to jacalin, in contrast to PNA, which binds to β-Gal*p*-(1→3)-GlcNAc also[7], which has an equatorial OH-4 group in the reducing sugar residue. A striking difference between PNA and jacalin, revealed here, is the failure of PNA to discriminate significantly between the α and β anomeric configuration of the reducing GalNAc residue of β-Gal*p*-(1→3)-GalNAc. This is evident from the high affinity of G_{M1}-tetra to PNA. Though studies showing the reactivity of PNA to G_{M1} and asialo-G_{M1} have been reported[23-25], no attempt was made to quantify this interaction. In the present study, we quantified the association of asialo-G_{M1} oligosaccharide with PNA, and found it to be the best ligand studied so far for the lectin. This is in marked contrast to jacalin which fails to recognize G_{M1}-tetra[14]. This difference may be explained by the β-anomeric configuration, in G_{M1}-tetra, of the GalNAc residue of the terminal disaccharide unit, β-Gal-(1→3)-GalNAc, as jacalin is known to show a remarkable discrimination between α-D and β-D anomers[11,14] (see also Table I). It is pertinent to mention here that only the α anomer of the T-antigen group, β-Gal*p*-(1→3)-α-GalNAc, is the cancer-related structure and not its β analogue[26]. In addition, certain anti-T monoclonals also fail to distinguish between the α and β anomers of the T-antigenic determinant group[25]. Thus, so far as the α and β anomers of the T-antigenic disaccharide group is concerned, jacalin offers a distinct advantage over PNA for monitoring the cell-surface expression of T-antigen. Another significant difference between PNA and jacalin, which may have bearing on their use as anti-T probes, is the failure of PNA to bind to GalNAc. This is in contrast to the avid binding of jacalin to α-GalNAc[14]. Not surprisingly, jacalin reacts

with GalNAc–HSA conjugate, albeit weakly, where the α anomer of GalNAc is linked to the protein[27]. This showed that a Tn antigen on the cell-surface [α-GalNAc-(1→3)-Ser] may be mistaken for T-antigen when jacalin is used as a probe, and therefore in such cases, the use of PNA or some other anti-T probe in conjunction with jacalin may prove useful.

The other significant observation resulting from this study is regarding the specificities of the different subsites in the combining region of PNA. The binding behaviour of the disaccharides, β-Gal*p*-(1→3)-β-Gal*p*OMe, β-Gal*p*NAc-(1→3)-α-Gal*p*OMe, and β-Gal*p*-(1→3)-Ara, was consistent with an extended combining region for PNA, proposed in an earlier study[6], where the terminal β-D-galactopyranosyl group in disaccharides such as β-Gal*p*-(1→3)-GalNAc and β-Gal*p*-(1→4)-Glc was shown to provide the major contribution to the total binding energy. Thus, the combining region of PNA is made up of two subsites, and the terminal galactosyl groups occupies the primary subsite. The disaccharide, β-Gal*p*-NAc-(1→3)-β-Gal*p*OMe, binds very weakly to PNA as compared to Gal or α-GalOMe, ruling out the possibility of the lectin binding to the α-GalOMe portion of the disaccharide. The weak binding of the disaccharide also indicated that for a disaccharide to interact with PNA it must contain a D-galactopyranosyl group at the nonreducing end. Presence of an acetamido group at C-2 of the nonreducing end group, as in β-Gal*p*NAc-(1→3)-α-Gal*p*OMe, presumably sterically hinders the access of the disaccharide to the combining site of the lectin.

From the foregoing, it is apparent that subsite A is highly specific for Gal and is of primary importance for ligand binding to PNA. Hence, it can be designated as the primary subsite (Subsite A). The other subsite, *i.e.*, the subsite where GalNAc of β-Gal*p*-(1→3)-GalNAc is expected to bind, can be designated as subsite B, or the secondary subsite. The increased affinity of asialo-G_{M1} tetrasaccharide over β-Gal*p*-(1→3)-GalNAc or its derivatives, β-Gal*p*-(1→3)-Gal*p*NAcOMe and β-Gal*p*-(1→3)-α-Gal*p*NAc-(1→3)-Ser, may be interpreted as resulting from additional interactions of the internal β-Gal residue, which is (1→4)-linked to the penultimate GalNAc residue. Such additional interactions are expected to be very small in view of the marginal (1.5-fold) increase in the affinity of G_{M1}-tetra over β-Gal*p*-(1→3)-GalNAc. This possibility could be examined further when the binding of β-Gal*p*-(1→3)-β-Gal*p*NAcOMe and β-Gal*p*-(1→3)-β-Gal*p*NAc-(1→4)-Gal to PNA will be studied.

The appreciable binding of β-Gal*p*-(1→3)-β-Gal*p*OMe, which is 15.2- and 8.9-fold greater than that of Gal and β-GalOMe, respectively, provided further evidence for the requirement of a D-galactopyranosyl configuration at the primary subsite, as its high affinity for PNA appears to result from the simultaneous interaction of both sugar units with the lectin-combining region.

The affinity of β-Gal*p*-(1→3)-Ara is about 8.6-fold greater than that for Gal, suggesting that this disaccharide has additional favourable interactions with the lectin, despite the absence of a hydroxymethyl group at C-5 and an acetamido group at C-2 of the reducing end residue. This result points towards the broader specificity of the subsite B. The aforementioned evidence, combined with the observation that sugars such as lactose and *N*-acetyllactosamine bind to PNA with extended site interaction, despite the

Subsite	
A	B
Gal *β*-Gal*p*OMe	
	GalNAc *α*-Gal*p*NAcOMe
β-Gal*p*-(1 ⟶	3)-GalNAc
β-Gal*p*-(1 ⟶	3)-*β*-Gal*p*OMe
β-Gal*p*-(1 ⟶	3)-Ara
β-Gal*p*-(1 ⟶	3)-GlcNAc
β-Gal*p*-(1 ⟶	4)-Glc
	β-Gal*p*NAc-(1→3)-*α*-Gal*p*OMe

Scheme 1. A schematic representation of the combining region of PNA: The two subsites are named Subsite A and Subsite B. Subsite A is the primary subsite and is specific for the D-galactopyranose configuration. Subsite B can accommodate a variety of hexopyranoses, such as Gal, GalNAc, Glc, GlcNAc, arabinose, etc. Disaccharides with a *β*-D-galactopyranosyl group at the nonreducing end most probably have extended site interactions. Disaccharides having sugars other than Gal at the nonreducing end, such as *β*-Gal*p*NAc-(1→3)-Gal*p*OMe, fail to interact with subsite A and probably interact weakly with subsite B alone.

presence of a *β*-(1→4) linkage in these disaccharides — which causes significant conformational difference when compared to the *β*-(1→3) linkage in *β*-Gal*p*-(1→3)-GalNAc[6,11] — strongly suggested that, as compared to subsite A, subsite B is only of secondary importance.

The subsite specificity of jacalin is almost the reverse of that observed for PNA since the primary subsite accommodates the GalNAc residue of *β*-Gal*p*-(1→3)-GalNAc, whereas the terminal Gal group occupies the secondary subsite, which can also accommodate other monosaccharide residues such as Glc or GlcNAc[14]. This difference in subsite specificity is not without important consequences, at least two of which are not difficult to discern. Firstly, the fact that GalNAc is accommodated in the primary subsite makes jacalin also react with Tn-antigen, whereas its failure to be accommodated in the primary subsite of PNA makes it (PNA) capable of discriminating against this antigen. Secondly, the broad specificity exhibited by jacalin at its secondary subsite makes possible the recognition of globotetrasaccharide, although with a 12-fold lower affinity as compared to *β*-Gal*p*-(1→3)-*α*-Gal*p*NAc-(1→3)-Ser[14]. In contrast, owing to its failure to accommodate GalNAc in the primary subsite, PNA does not recognize the terminal disaccharide fragment of globoside, *β*-Gal*p*Nac-(1→3)-*α*-GalOMe, thus explaining its failure to react with globoside in the glycolipid-binding assay performed in an earlier study[23].

Recently, another anti-T lectin, amaranthin, isolated from the seeds of *Amaranthus caudatus*, which also recognizes the internal *β*-Gal*p*-(1→3)-GalNAc structure, has been described by Rinderle *et al.*[28]. This lectin, like jacalin, does not recognize lactose, *N*-acetyllactosamine, or *β*-Gal*p*-(1→3)-GlcNAc structures, but recognizes GalNAc in the *α* anomeric configuration and, therefore, is expected to recognize the

T-antigen, T_n-antigen, as well as cryptic T-antigen. However, peanut agglutinin does not recognize cryptic T-antigen and T_n-antigen. Thus, when amaranthin and jacalin are used to identify the expression of T-antigen on cell surfaces, it should be complemented by the use of PNA in order to exclude the possibility of any incorrect assignment of cryptic-T or T_n-antigens as the T-antigen.

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