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Agaricus bisporus lectin binds mainly *O*-glycans but also *N*-glycans of human IgA subclasses

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The primary interaction between purified *Agaricus bisporus* lectin (ABL) and human IgA subclasses was studied by ABL-affinity chromatography, dot blot assay and competitive enzyme-lectin assay, considering that ABL could be an alternative tool for detection of IgA1 *O*-glycans. Both secretory IgA subclasses bound to ABL-Sepharose and the IgA2 subclass (which contains only *N*-glycans) was recovered with a high degree of purity when NH_4OH was used as eluent. ABL-Ig interaction was also observed by dot blot assays using ABL-peroxidase against monoclonal IgA1*k* Pan, IgA2m(1)*k* Gir, IgA2m(2)*k* Bel, secretory IgA2 and normal IgG (also contains only *N*-glycans). When these immunoglobulins were enzymatically treated with peptide *N*-glycosidase F (*N*-glycan hydrolysis), the ABL-IgA2 and -IgG interaction did not occur while IgA1 maintained a high degree of interaction with ABL. Also, the ABL-IgA interaction was observed by competitive enzyme-lectin assay, and when IgA1 subclass was treated with endo-*α*-*N*-acetylgalactosaminidase for *O*-glycans hydrolysis, the reactivity with ABL was very low. We conclude that the complementary use of ABL and peptide *N*-glycosidase F could be a useful tool to assess the *O*-glycosylation state of human IgA1 subclass, which is of relevant importance in the effector functions of immunoglobulins.

***Keywords*: *Agaricus bisporus* lectin, human IgA1 and IgA2, *O*- and *N*-glycans**

***Abbreviations*: ABL, *Agaricus bisporus* lectin; *α*1 and *α*2, heavy chains from human IgA1 and IgA2; C1-3*α*, constant domains (1–3) of heavy chains from human IgA; ECL, *Erythrina cristagalli* lectin; EEO, electroendoosmosis; EIA, enzyme immunoassay; ELA, enzyme lectin assay; Ig, immunoglobulin; HRP, horseradish peroxidase; ID_{50}, 50% inhibitory dose; Ka, affinity constant; O.D., optical density; PBS, phosphate buffered saline; PBS-t, phosphate buffered saline with Tween 20; PNGase F, peptide *N*-glycosidase F; RID, radial immunodiffusion; SD, standard deviation; TBS, Tris-HCl buffered saline; TBS-t, Tris-HCl buffered saline with Tween 20; T-disaccharide, Thomsen-Friedenreich disaccharide**

Introduction

The IgA represent the first immune barrier against transmucosal penetration of pathogens in its secretory version [1], and is also the second most abundant immunoglobulin in serum [2]. There are two human IgA subclasses, i.e., IgA1 and IgA2. The main difference between the primary structure of the constant region of α1 and α2 chains lies in the deletion of 13 amino-acids in the α2 hinge region [3]. As a consequence of this deletion, IgA2 lacks five serine residues as well as the *O*-linked galactosamine-containing oligosaccharide chains Gal*β*1-3GalNAcα [4, 5] that could be sialylated [6, 7]. Moreover, IgA1 contains two *N*-glycosylated sites per α-chain in the Fc region of the protein, located in C2α (Asn-263) and C3α (Asn-459) domains [8], whereas IgA2 contains four sites per α-chain with glycosylation in the C1α (Asn-166), C2α (Asn-263, -337) and C3α (Asn-459) domains [3]. The IgA2m(2) allotype contains a further glycosylation at Asn-211 in the C1α [9]. These oligosaccharide side chains are able to interact with carbohydrate-binding proteins (e.g. lectins). The *Agaricus bisporus* lectin (ABL) reacts mainly with Thomsen-Friedenreich disaccharide (T-disaccharide) namely Gal*β*1-3GalNAcα [10, 11] and with lower affinity to sialylated T-disaccharide [12]. In order to establish the binding specificities between ABL and human IgA subclasses, we have previously demonstrated that ABL precipitates human IgA1 but not IgA2 subclass [13]. The aim of the present work was to study the primary interaction between ABL and human IgA subclasses on account that ABL could be an alternative tool in the detection of IgA1 *O*-glycans.

Materials and methods

Materials

Agarose (Type II: Medium EEO), cyanogen bromide-activated Sepharose 4B, horseradish peroxidase (Type VI-A),

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Galβ1-3GalNAc, Galβ1-4GlcNAc (*N*-acetyllactosamine), bovine serum albumin (BSA) essensially globulin free, *N*-acetyllactosamine-BSA (~15–20 mol disaccharide per mole BSA), KSCN, gelatin, peptide *N*-glycosidase F (from *Flavobacterium meningosepticum*), neuraminidase (from *Clostridium perfringens*), endo-α-*N*-acetylgalactosaminidase (from *Diplococcus pneumoniae*), biotinylated monoclonal antibody to human IgA1 (Clone A1-18), avidin-horseradish peroxidase and *Erythrina cristagalli* lectin-horseradish peroxidase (ECL-HRP) were purchased from Sigma Chemical Co. (St Louis, USA). Human monoclonal IgA1*k* Pan, IgA2m(1)*k* Gir and IgA2m(2)*k* Bel were a gift from Dr P. Aucouturier (Poitiers University Hospital, France). Human monoclonal IgA1*k* Zal, normal IgG and ABL were purified as previously reported [13]. ABL was conjugated to horseradish peroxidase (ABL-HRP) by using $NaIO_4$ [14].

Partial purification of secretory immunoglobulins

Human milk (third day postpartum) was collected by breast pump, immediately refrigerated and processed as previously described [15]. Milk (1.5 ml) was diluted with an equal volume of PBS (10 mM potassium phosphate, pH 7.2, 150 mM NaCl) and centrifuged at $12\,000 \times \mathbf{g}$ at 4 °C for 1 h. The clear middle layer was collected by a Pasteur pipette inserted through the top lipid layer. Centrifugation was repeated to remove the remaining lipids. Approximately 2 ml of clarified milk were then acidified with acetic acid to pH 4.2 and the precipitated casein was removed by centrifugation at $15\,000 \times \mathbf{g}$ at 4 °C for 1 h. The pH of the clear supernatant was neutralized with NaOH and the immunoglobulins were then precipitated with an equal volume of slowly added saturated ammonium sulfate under constant mixing conditions [16]. The precipitate was collected by centrifugation, dissolved in distilled water and dialysed overnight at 4 °C against PBS. The dialysed sample was centrifuged and 450 µl of secretory IgA were recovered (1.05 mg ml^{-1} IgA1 and 0.85 mg ml^{-1} IgA2).

Agarius bisporus lectin-affinity chromatography

Agarius bisporus lectin (8.0 mg) was coupled to 2.5 ml of cyanogen bromide-activated Sepharose 4B [17]. The yield of coupling was 0.92 when total proteins was controlled in coupling buffer by the method of Lowry *et al.* [18]. The column was sequentially washed with PBS, PBS-1 M NaCl-0.1% Tween 20 (PBS-t), 100 mM NH_4OH (pH 11.0) 150 mM NaCl and equilibrated with PBS. Affinity chromatography using the ABL-Sepharose 4B column (1.0 × 3.0 cm) was performed at 2.0 ml h^{-1} flow rate and fractions of 0.7 ml were collected. Partially purified secretory immunoglobulins were applied on ABL-Sepharose 4B and the column was further washed with PBS, PBS-t, PBS and it was eluted successively by using 100 mM NH_4OH (pH 11.0) 150 mM NaCl. The tubes corresponding to protein peaks were pooled, neutralized with 1 M acetic acid and dialysed against PBS. Pools were concentrated by ultrafiltration in an Amicon device equipped with a PM10 membrane and analysed for human IgA1 and IgA2 subclasses.

Measurement of human IgA subclasses

Radial immunodiffusion (RID) for measuring IgA1 and IgA2 was performed as suggested by manufacturer (The Binding Site Inc., San Diego, USA) and the concentrations were determined from standard curves. IgA1*k* Pan, IgA2m(1)*k* Gir and IgA2m(2)*k* Bel were assayed as specificity controls.

Analysis of IgA1 subclass was performed by competitive enzyme immunoassay (competitive EIA): polystyrene microtitration plates (Corning, NY, USA) were coated with 2 µg ml^{-1} human IgA1*k* Pan (100 µl per well) in 0.1 M carbonate buffer (pH 9.6) overnight at 4 °C, and saturated with PBS-0.05% Tween 20 for 1 h at 37 °C. Standard (IgA1*k* Pan) and samples were preincubated with 1/80 000 biotin labelled anti-human IgA1 monoclonal antibody (Clone A1-18) for 1 h at 23 °C, before adding 100 µl per well. The plates were incubated 2 h at 23 °C and washed six times with PBS-Tween. After incubation with 1 µg ml^{-1} avidin-HRP for 20 min at 23 °C, six final washes with PBS-Tween were carried out. The colour reaction was developed by using 2 mg ml^{-1} *o*-phenylenediamine and 0.02% H_2O_2 in 0.1 M sodium citrate (pH 5.0) at 23 ° C for 30 min. Reactions were stopped by adding 100 µl of 2.5 M sulfuric acid and absorbance values were read at 492 nm with a microplate reader (model 450, Bio-Rad). Percent inhibition was calculated as follows:

$$100 \times \frac{(\text{O.D. 492 control} - \text{blank}) - (\text{O.D. 492 inhibited} - \text{blank})}{(\text{O.D. 492 control} - \text{blank})}$$

The mean and standard deviation (SD) of absorbance control values in all competitive assays correspond to 1.05 ± 0.11. No IgA1 was detected in human monoclonal IgA2m(1)*k* Gir, IgA2m(2)*k* Bel, normal IgG or gelatin samples.

Double diffusion gel and haemagglutination inhibition assays

Double diffusion gel was performed in 1% agarose in PBS. The diffusion was allowed to proceed for 24 h in a moisture chamber at room temperature. Haemagglutination and haemagglutination inhibition assays were performed according to Sueyoshi *et al.* [19].

Dot blot assays

Dot blot assays were performed as previously described [20]. Purified human serum immunoglobulins, secretory IgA2 fraction and gelatin (as negative control) were twofold serials diluted in PBS-100 mM glycine and 1 µl of each dilution was carefully applied (using a 10 µl Hamilton syringe) to nitrocellulose strips. After drying at 37 °C, the remaining binding sites on the membrane were blocked by incubation for 1 h in TBS (50 mM Tris-HCl, pH 7.5, 150 mM

NaCl) with 0.1% Tween 20 (TBS-t). The blocked strips were incubated with 0.7 μg ml^{-1} ABL-HRP or 5 μg ml^{-1} ECL-HRP in TBS for 1 h at room temperature. After five washes with TBS during 5 min and two washes with distilled water, the colour reaction was developed by using 0.5 mg ml^{-1} 4-chloro-1-naphtol and 0.02% H_2O_2 in methanol-TBS (1 : 5) during 30 min. Some dotted strips were subjected to an additional incubation with 1 U ml^{-1} peptide *N*-glycosidase F (PNGase F) in PBS during 40 h at 37 °C, before lectin-HRP addition. Some dotted normal IgG was boiled at 90 °C in PBS for 15 min [21]. In ECL assays, all strips were desialylated before conjugate addition by mild acid hydrolysis in 0.025 M sulfuric acid at 60 °C for 4 h [22], for improve the lectin binding. Finally the dots were measured by reflection at 580 nm in a Shimadzu Chromato Scanner CS-930. Graphs (dot blot assay and competitive ELA) were created by using Harvard Graphic soft and the graphed values represent the mean and SD of three determinations.

Competitive enzyme-lectin assay (competitive ELA)

Competitive ELA was performed as competitive EIA except that the plates were coated with 10 μg ml^{-1} IgA1*k* Pan, and 0.04 μg ml^{-1} ABL-HRP was used instead of biotin labelled antibody and avidin-HRP [23]. A sample of IgA1*k* Pan (1 mg ml^{-1}) was previously incubated in 20 mM sodium cacodylate (pH 6.0) with 1 U ml^{-1} neuraminidase during 24 h at 37 °C and then 0.5 U ml^{-1} endo α-*N*-acetylgalactosaminidase was added [24]. After incubation for 24 h at the same temperature, the reaction was stopped with the addition of 0.8 M potassium borate (pH 9.1). This sample was extensively dialysed against PBS and used as an inhibitor of ABL interaction. The data of saturated and competitive ELA were fitted with LIGAND soft [25] in the measurement of affinity constants (Ka) between ABL and glycoproteins [26, 27].

Results

Study of ABL-secretory IgA subclasses interaction by affinity chromatography

The chromatographic profile of human secretory globulins applied to ABL-Sepharose 4B is shown in Figure 1. The effluent volume as well as PBS-t fraction showed protein peaks, but they did not react with antibodies to IgA1 and IgA2 by radial immunodiffusion (RID) and competitive EIA. The fraction eluted with NH_4OH showed a recovery of 61% from the applied IgA2 but no IgA1 was detected by RID. Competitive EIA showed that IgA1 concentration was 556-fold lower than IgA2 concentration (Table 1). The purity of secretory IgA2 was evaluated by polyacrylamide gel electrophoresis (Figure 2).

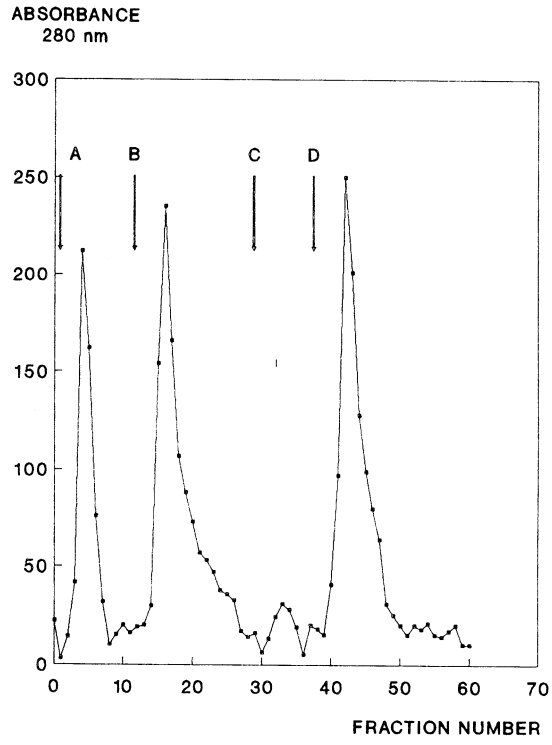

Figure 1. Affinity chromatography of secretory globulins from human early milk on ABL-Sepharose 4B column. (A) Sample applied to immobilized ABL and eluted with PBS. Column was (B) washed with PBS-1 M NaCl-0.1% Tween 20, (C) equilibrated with PBS and (D) eluted with 100 mM NH_4OH (pH 11) 150 mM NaCl.

Double diffusion gel and haemagglutination inhibition assays

The ABL ability for precipitating with several human glycoproteins was studied by double diffusion gel (Figure 3).

Table 1. Purification of human secretory IgA2 by ABL-affinity chromatography

Fraction	*Volume (ml)*	*Proteins*[a] *(mg ml^{-1})*	*IgA2 subclass (mg ml^{-1})*	*Purification (-fold)*	*Recovery (%)*	*IgA1 subclass (mg ml^{-1})*	*IgA2 Purification respect to IgA1 (-fold)*
Early milk	3.0	8.1	0.69	1.00	100	0.96	1.00
Ammonium sulfate precipitate	0.45	3.6	0.85	2.70	18.5	1.05	1.10
ABL-sepharose eluate	0.49	0.50	0.48	11.2	11.3	0.0012	556

[a] Proteins were measured by the method of Lowry *et al.* [18].

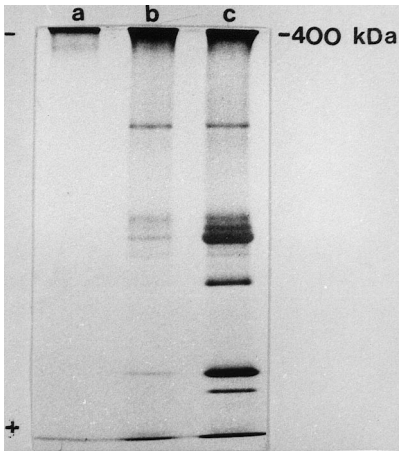

Figure 2. Polyacrylamide gel electrophoresis of samples from secretory IgA2 during the purification process. Electrophoresis was performed in 6–15% acrylamide gradient gel with sodium dodecyl sulfate without 2-mercaptoethanol [28]. All the samples analysed contained 1 μg of IgA2 subclass: (a) purified secretory IgA2 (dimeric form constituted by four *a*2 chains), (b) ammonium sulfate precipitate, (c) early milk. Proteins were detected by using silver staining [29].

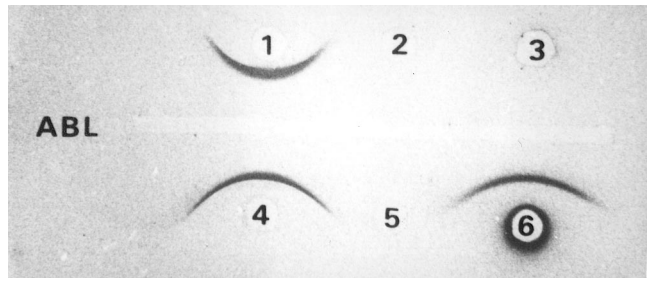

Figure 3. Double diffusion gel of human proteins with ABL in PBS. In the middle trough: 5 μg ABL. In wells: 10 μg each of (1) monoclonal IgA1*k* Pan, (2) monoclonal IgA2m(1)*k* Gir, (3) secretory IgA2, (4) monoclonal IgA1*k* Zal, (5) monoclonal IgA2m(2)*k* Bel and (6) 10 μl early milk (dil. 1/2 in PBS). The gel was stained for protein with Coomassie Brilliant Blue R-250.

Only monoclonal IgA1*k* Pan, IgA1*k* Zal and early milk precipitated with ABL. Monoclonal IgA2m(1)*k* Gir, IgA2m(2)*k* Bel and secretory IgA2 did not show a precipitation reaction against ABL. *Agarius bisporus* lectin-haemaglutinating inhibitory activity was not exhibited by secretory IgA2 up to 0.17 mg ml^{-1} (not available at higher concentration), monoclonal IgA2m(1)*k* Gir and IgA2m(2)*k*

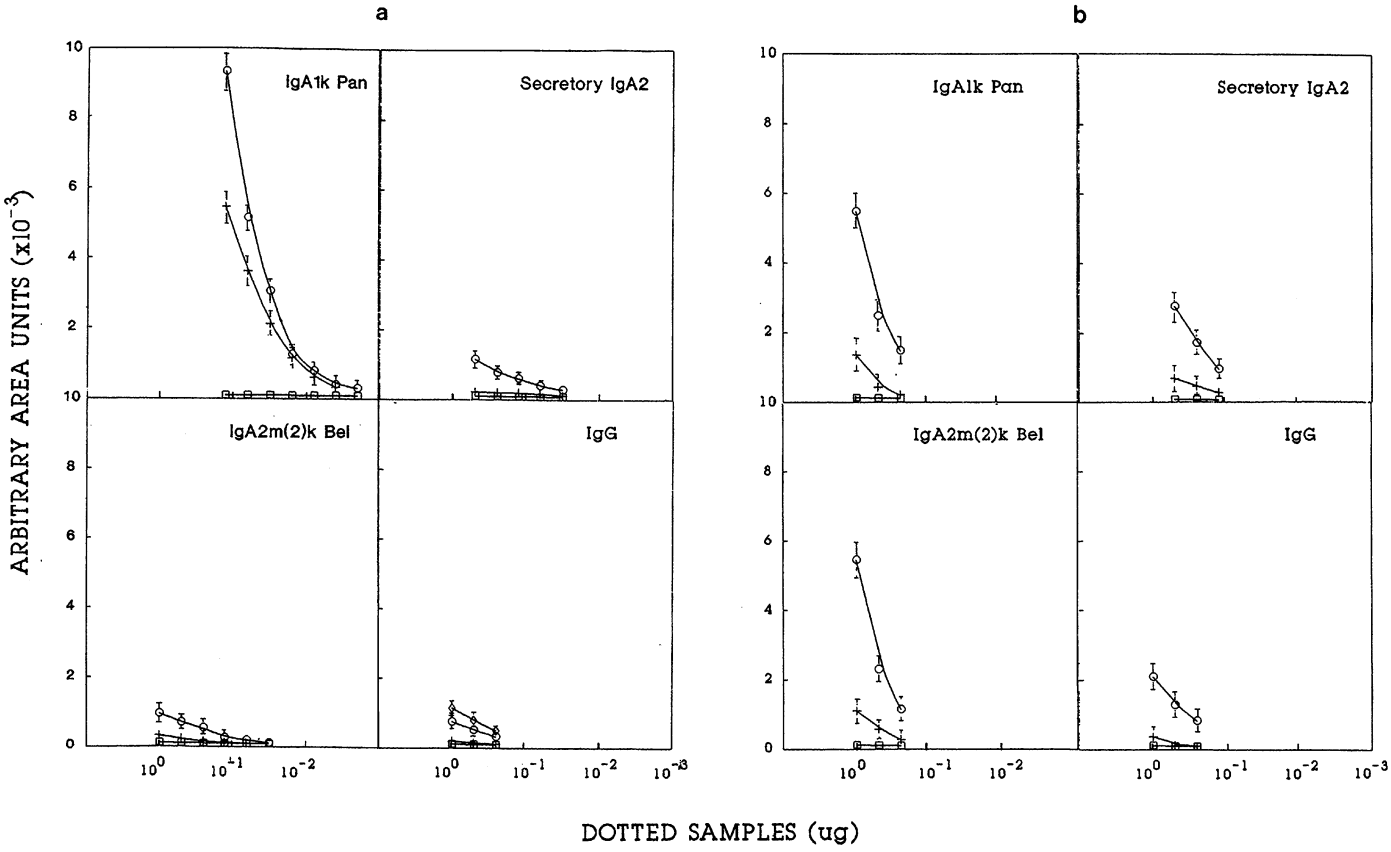

Figure 4. Dot blot assays of several human Igs against (a) ABL-HRP and (b) ECL-HRP measured by Chromato Scanner. Monoclonal IgA*k* Pan, secretory IgA2, monoclonal IgA2m(2)*k* Bel and normal IgG were assayed without (○–○) and after (+–+) treatment by peptide *N*-glycosidase F. Normal IgG was also boiled (◇–◇) to expose the sugars. Gelatin (□–□) was used as negative control.

Bel up to a final concentration of 2.7 mg ml^{-1}. However, this agglutination was inhibited when monoclonal IgA1*k* Pan and IgA1*k* Zal were tested at 0.02 and 0.04 mg ml^{-1}, respectively. Also, Galβ1-3GalNAc showed haemaglutinating inhibitory activity at a final concentration of 0.21 mM but it was not observed by *N*-acetyllactosamine up to 100 mM.

Dot blot assays

A strong binding of ABL to human IgA1*k* Pan and a weak but definite primary interaction with secretory IgA2, monoclonal IgA2m(2)*k* Bel and normal IgG is documented in Figure 4a. Moreover, monoclonal IgA2m(1)*k* Gir evidenced a weak interaction (data not shown). Normal IgG revealed an increased interaction with ABL-HRP when this dotted Ig was previously boiled. When secretory IgA2, monoclonal IgA2m(2)*k* Bel and normal IgG were treated with peptide *N*-glycosidase F (PNGase F), the ABL-Ig interaction disappeared while IgA1*k* Pan treated with PNGase F maintained a high degree of interaction with ABL (Figure 4a). The effect of this enzyme on human Ig *N*-glycans was confirmed by dot blot assays using *E. cristagalli* lectin-HRP (Figure 4b). A very low interaction between the lectin and Igs was observed after treatment with PNGase F.

Competitive ELA

Studies of the interaction between ABL and Igs by competitive ELA is shown in Figure 5a. Both monoclonal IgA1*k* Pan and IgA1*k* Zal strongly inhibited (ID_{50} 0.4 µg ml^{-1}) the binding of ABL-HRP to IgA1*k* Pan coated plates while IgA2m(1)*k* Gir, IgA2m(2)*k* Bel and secretory IgA2 yielded a lower but significant inhibition. Gelatin and IgG did not show inhibition. Treatment of IgA1*k* Pan with neuraminidase/endo-α-*N*-acetylgalactosaminidase significantly reduced its inhibitory potency (estimated ID_{50} 20 µg ml^{-1}). However its sample shows approximately tenfold higher inhibitory activity than IgA2, it could be to incomplete *O*-glycan removing due to the difficult access to Ig hinge region. Table 2 shows the different affinity constants between ABL and human IgA subclasses. An additional assay produced the evidence that *N*-acetyllactosamine-BSA inhibited the ABL interaction (Figure 5b) and the affinity constant measured by using LIGAND soft in analysis was 3.20.10^6 M^{-1}. This affinity constant was similar to ABL-secretory IgA2 interaction.

Discussion

We have previously reported that ABL reacts with human IgA1 but not with IgA2 by gel precipitation and

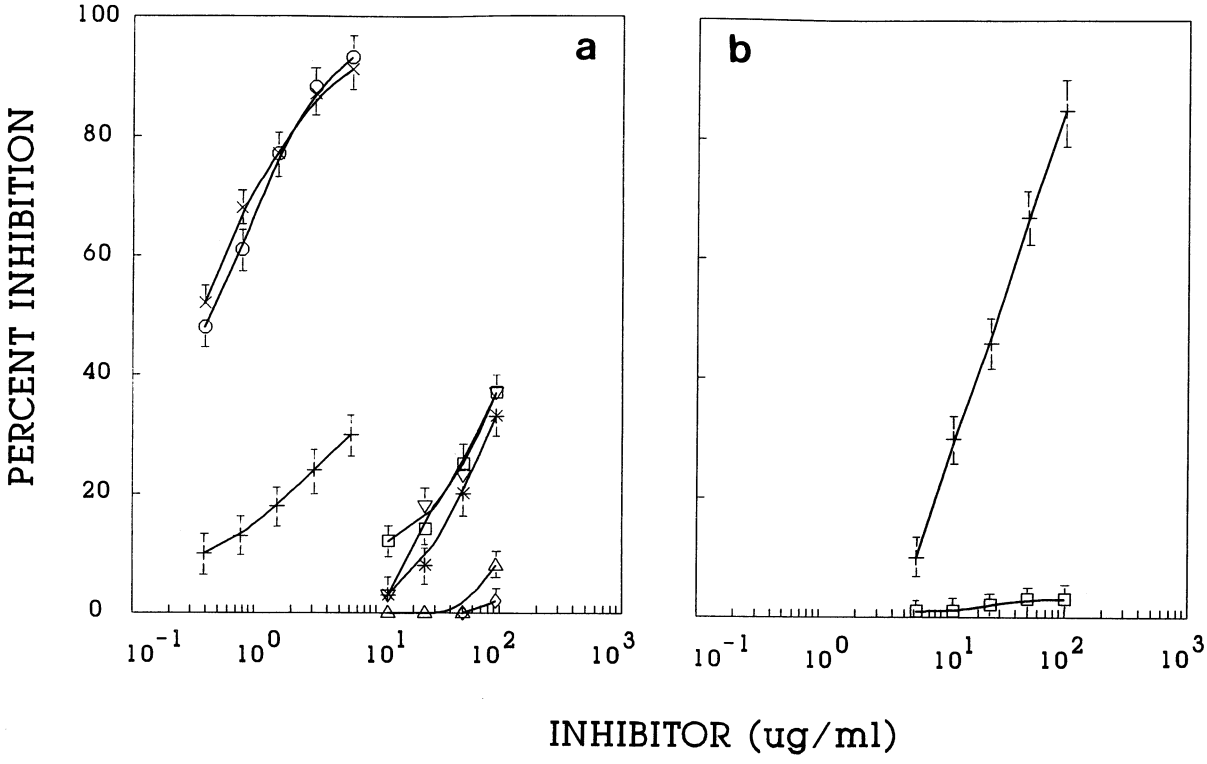

Figure 5. Competitive ELA using ABL-HRP against coated IgA1*k* Pan. Increasing concentrations of (a) human Igs and (b) glycoconjugate assayed as inhibitors. (a): Monoclonal IgA1*k* Pan (×–×), IgA1*k* Zal (○–○), IgA2m(1)*k* Gir (∗–∗), IgA2m(2)*k* Bel (□–□), secretory IgA2 (▽–▽), normal IgG (△–△) and IgA1*k* Pan treated with neuraminidase/endo-*α*-*N*-acetylgalactosaminidase (+–+). Gelatin (◇–◇) was used as negative control. (b): *N*-acetyllactosamine-BSA (+–+), BSA (□–□) was used as negative control.

Table 2. Affinity constants for the interaction of ABL with human IgA subclasses.[a]

Immunoglobulins	$10^{-6} \times Ka$ (M^{-1})
IgA1*k* Pan	220 ($\pm$ 32)[b]
IgA2m(1)*k* Gir	2.5($\pm$ 0.31)
IgA2m(2)*k* Bel	1.9($\pm$ 0.20)
Secretory IgA2	3.9($\pm$ 0.49)

[a] Values are calculated for 23 °C. [b] Parentheses indicates SD ($n = 3$).

haemagglutination inhibition assays [13]. In the present paper we studied the primary interaction of ABL with human IgA subclasses by affinity chromatograpy, dot blot assay and competitive ELA in an attempt to analyse the ABL specificity for IgA1 *O*-glycans.

Affinity chromatography showed that ABL binds both human secretory IgA1 and IgA2. Secretory IgA2 with a high degree of purity was recovered using NH_4OH as an eluent, but NH_4OH failed as an eluent of secretory IgA1. This result is in agreement with that of Sueyoshi *et al.* [12], who reported that glycopeptide with terminal Galβ1-3GalNAcα bound to an ABL isoform (ABA-I) column so tightly that it could not be eluted with 200 mM NH_4OH.

Primary interaction between ABL and human IgA2 subclass was also observed by dot blot assays using ABL-HRP against secretory IgA2, serum monoclonal IgA2m(1)*k* Gir and IgA2m(2)*k* Bel. Likewise, all human IgA2 assayed by competitive ELA inhibited ABL-IgA1 interaction. However, these human immunoglobulins did not react with ABL by gel precipitation and haemagglutination inhibition assays, in agreement with previous results [13].

In order to study the role of *N*-glycans in ABL-IgA interaction, the use of PNGase F was included in the assays. This enzyme has a unique ability for cleaving the *N*-glycan linkage of glycoproteins between asparagine and carbohydrate side chains [30]. The use of PNGase F on human Ig *N*-glycans was previously reported [31]. We used ECL to corroborate the PNGase F action on human Igs adsorbed to nitrocellulose considering that it is an *N*-acetyllactosamine-binding specific lectin [32]. *N*-acetyllactosamine as well as sialyl *N*-acetyllactosamine constitute the main terminal *N*-glycan from α-chains [7, 33, 34]. Experiments performed with PNGase F produced the evidence that ABL-IgA2 interaction is precluded while IgA1 maintains its capacity for ABL binding. It appears that the ABL-IgA2 interaction occurs essentially through the *N*-glycoside chains and their terminal residue (Galβ1-4GlcNAc) could be responsible for the ABL-IgA2 interaction. Although *N*-acetyllactosamine did not show significant inhibition of ABL haemagglutination, the reactivity of ABL with *N*-acetyllactosamine was demonstrated when *N*-acetyllactosamine-BSA was used as an inhibitor of ABL interaction in competitive ELA, and similar affinity constants between ABL-secretory IgA2 and ABL-*N*-acetyllactosamine-BSA interactions were obtained in broad agreement with the fact that both glycoproteins have similar oligosaccharide chain number per mole.

On the other hand, *N*-glycans with terminal Galβ1-4GlcNAc are also present on Asn-297 from human IgG [35], and a weak primary interaction was observed between ABL and this human immunoglobulin by dot blot assay. This interaction was increased if IgG was boiled, thus exposing their sugars [21], but did not occur when this Ig had been previously treated with PNGase F. However, ABL-IgG interaction was not observed by competitive ELA against coated IgA1. These results may arise from the fact that Activity Modulation assays (e.g. competitive ELA) have limited detectabilities in relation to excess of binding protein used in Activity Amplification assays and to the law of mass action [36]. The reason for the interaction between ABL and IgA2 but not IgG can be seen by competitive ELA could be due to the existence of 4-5 *N*-glycans per α2 in relation to only one *N*-glycan per gamma chain, as well as to the inaccessibility of glycosides from IgG [37], as observed by boiling the samples in dot blot assay.

The fact that the interaction between ABL and *N*-glycan from immunoglobulins is non-precipitating but can be evidenced by methods of primary interaction could be a consequence of a low affinity (Table 2). A similar phenomenon was reported for antibody-antigen interaction [38].

Moreover, ABL-IgA1 interaction seems to occur mainly through *O*-glycan chains because ABL-IgA1 interaction decreased $\sim$50-fold after neuraminidase/endo-α-*N*-acetylgalactosaminidase treatment. This result is in agreement with the ABL carbohydrate-binding specificity showed by haemagglutination inhibition assays [10, 11, 19].

Jacalin (*Artocarpus integrifolia* lectin) also binds mainly Galβ1-3GalNAc [39] and consequently it shows major binding specificity for human IgA1 than IgA2 subclass. However, general information on jacalin-IgA2 interaction is contradictory and not well explained [23, 31], and the affinity constants between this lectin and human IgA subclasses were not described yet.

Considering that the carbohydrates on Ig participate in several processes including: binding to Fc receptors on monocytes, T cells and eosinophils [40–42], activation of complement [43, 44], binding, internalization and catabolism by hepatocytes and other cells [45, 46], opsonization [47] and prevention of bacterial adherence [48], the complementary use of ABL and PNGase F could be a useful tool to establish the *O*-glycosylation state of human IgA1 subclass, as for recombinant molecules [49] or for a potential detection of defective galactosylation from IgA1 *O*-glycans, which could be a possible etiopathogenic factor in IgA nephropathy [50].

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