Properties of Lectins from Snails of the Genus *Helix* Probed by Monoclonal Antibodies

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Helix-Lectins, Monoclonal Antibodies against Lectins, Competition between Antibodies and Carbohydrates

Monoclonal antibodies were raised against the lectin of *Helix pomatia* (HPL). Besides antibodies bearing the more common γ and $\varkappa$ chains, antibodies with α, μ and λ_2 chains were elicited. The anti-HPL antibodies are expected to be useful in studies on HPL biogenesis and HPL substructure and in studies concerned with the binding of HPL to cell surfaces.

Binding of carbohydrates to HPL impaired the binding of anti-HPL antibodies. One to 3 mM GalNAc inhibited HPL-binding in two out of nine antibodies. None of the antibodies bound in the presence of micrograms per ml of the polyvalent blood group A-substance from hog stomach. Similarly, all anti-HPL antibodies were prevented from binding if non-inhibitory concentrations of A-substance were supplemented with GalNAc.

Lectins from *Helix aspersa* (HAL) and *Helix lucorum* (HLL) differed from HPL in antigenic properties. Only one anti-HPL antibody each bound these lectins as well as HPL. Binding of lectins of *Cepaea* and *Rapana* was scarcely detectable.

Most of the anti-HPL antibodies and the multivalent HPL-antigens formed precipitation lines in double diffusion tests. At least two antibodies (IgMs) did so with HLL but none with HAL.

The possibility that antibodies were selected because of unknown interactions between HPL and the carbohydrate moieties of certain fractions of antibodies was excluded by raising the antibodies in the presence of tunicamycin to inhibit N-glycosylation.

Introduction

Lectins are widely distributed among all organisms (compare refs. [1–4]). They are characterized by at least two sites at which mono- or oligosaccharides are specifically bound. Thus, proteins and cells bearing such groups may be precipitated or agglutinated, respectively.

Investigations on lectins center about three aspects: biogenesis and biological role, isolation and application, the structures responsible for their physiological importance.

The contribution of lectins to functions such as protection against microorganisms, cell-cell recognition and carbohydrate transport has been discussed [1, 2, 4]. The value of lectins for the biochemist and cell biologist is demonstrated by work on blood and cell typing, differentiation between normal and transformed cells, elucidation of cell surface architecture and the localization and characterization of carbohydrate-containing cell structures or components (numerous examples are given in ref. 3). The present communication is concerned with the third aspect.

We have raised monoclonal antibodies against the well-known lectin of *Helix pomatia* (HPL, for a synopsis see refs. [4–6], starting with the assumption that an antibody specific for the binding site of the lectin would be prevented from binding if the lectin had bound carbohydrate. Such an antibody would thus characterize this site and might therefore make it comparable to binding sites of other lectins with the same sugar specificity, namely GalNAc. The question was whether the known interspecific heterogeneity of functionally and evolutionarily related lectins also includes a heterogeneity of the carbohydrate-binding epitopes.

Moreover, anti-HPL antibodies were expected to be highly specific probes in studies on the biogenesis of HPL, on subunit characterization, and in studies concerning cell-lectin interactions. They were used to follow structural alterations of HPL caused by carbohydrate- or glycoprotein-binding and provided a means to compare surface features of lectins related to HPL.

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The study was stimulated by a report [7] showing a monoclonal antibody to be inhibited from binding the lectin of *Dolichos biflorus* by high concentrations of the appropriate carbohydrate (GalNAc), by the significant role of HPL in cell-typing studies (for examples see refs. [8–12]), and by our finding that monoclonal antibodies were efficient tools to compare structural alterations in homologous enzymes [13].

Materials and Methods

Reagents and substances

Lectin from *Helix pomatia* (HPL) was purchased from Pharmacia (Freiburg, W.-Germany), lectin from *Helix aspersa* and GalNAc-specific plant lectins from Medac (Hamburg, W.-Germany). Prof. Dr. G. Uhlenbruck (Köln) generousely provided preparations of lectins from *Helix aspersa* [14], *Helix lucorum*, *Cepaea nemoralis*, and *Rapana thomasiana* as well as samples of A-substance [15] from hog stomach (phenol-saline extraction of peptone).

Peroxidase-labelled HPL (HPL-Pox) was prepared essentially as described by Weil *et al.* [16] or was a gift from these authors. Substrate for peroxidase was 2,2′-azino-di(3-ethyl-benzthiazoline)-sulfonic acid-6 (ABTS, Sigma, Taufkirchen, W.-Germany) plus 0.005% H_2O_2 in 0.1 citrate buffer pH 5.

[^{125}I]labelled chain-specific anti-mouse antibodies were generous gifts of Drs. H. Tesch and A. Radbruch (Köln).

Immunization

Three mice of the inbred strain BALB/c were immunized with HPL. Different routes of immunization were employed. One mouse received a single i.p. injection of 1 mg of HPL precipitated by alum and suspended in 0.5 ml of PBS. This mouse was sacrificed 8 days after injection. A second mouse was injected i.p. with 100 µg of HPL in 250 µl of PBS emulsified with an equal volume of Freund's complete adjuvant. Two weeks later 50 µg of HPL were administered in the same way except that Freund's incomplete adjuvant was used. Finally, 2 weeks after the second injection and 3 days before cell fusing 50 µg in 250 µl of saline were applied, one half i.p., the other half i.v. A third mouse was treated as the second, but received 4 more injections (50 µg of HPL in Freund's incomplete adjuvant) in two week intervals.

Cell fusion and cloning

The spleens of the mice were isolated and the cells of each spleen fused separately with 10^8 BALB/c strain-derived X 63-Ag 8.653 myeloma cells [17, 18]. After fusion the cells of each spleen were spread in 8×24 wells fo Costar 3534 plates filled with RPMI medium supplemented with HAT [19].

HPL-positive clones were recloned 2 to 4 times by limiting dilution. Large amounts of antibodies were raised by injecting cloned cells into Pristane-primed BALB/c mice.

Selection of HPL positive clones

Wells of immunoassay plates were coated with 50 µl of rabbit anti-mouse antibodies (2 µg · ml^{-1}), incubated at RT for 3 h and washed 5 times under tap water. Residual binding sites of the wells were saturated by PBS-Tween (0.05% Tween-20 in PBS). After 30 min this solution was replaced by 25 µl of PBS-Tween and 25 µl of the culture supernatants.

After 18 h at 4 °C the wells were washed as before and filled with 50 µl of a 1:1000 dilution of peroxidase-labelled HPL (HPL-Pox, $A_{280/403} = 0.73/1.56$) in PBS-Tween. After 3 h at RT and 5 washings, 50 µl of ABTS substrate were added to the wells. For quantification the A values were read in a Titertek Multiskan at 405 nm.

Quantitative determination of HPL, comparison of other lectins and inhibition of antibody binding by carbohydrates

The test system for quantification of HPL and for a comparison of other lectins was essentially the same as for the selection of postive clones, except that 25 µl of a dilution series of HPL or another lectin were added together with 25 µl of HPL-Pox to the antibodies bound to the wells of the immunoassay plates.

In experiments analysing the inhibition of aHPL antibody binding by carbohydrates, dilution series of carbohydrates were substituted for the unlabelled lectins.

Determination of affinity constants

Affinity constants were determined by a solid surface assay similar to the methods just described. A dilution series of HPL-Pox was added to constant amounts of antibodies bound to the wells of immunoassay plates. After 16 h at 4 °C the amounts of HPL-Pox bound and unbound were quantified by ABTS conversion. Affinity constants were then calculated from the linear slopes of Scatchard plots. Deviations from straight lines were observed at low concentrations of HPL-Pox. (The absolute values should not be overestimated. Different species of HPL-Pox may constitute a conjugate, HPL-Pox may have properties different from HPL and the free exchange of reaction partners may be impaired by the solid surface assay.)

Biosynthesis and analysis of anti-HPL antibodies lacking carbohydrates

Approximately 2 to 5×10^7 cells of a hybridoma clone were collected, washed with PBS and preincubated for 1 h at 37 °C in RPMI 1640 medium lacking methionine but containing 8 $\mu g \cdot ml^{-1}$ tunicamycin. The medium was then replaced by RPMI 1640 containing [^{35}S]methionine (0.93 MBq per sample) and tunicamycin. Controls were treated in the same way, except that tunicamycin was omitted. After (6) 18 h the supernatants were collected and the cells lysed [20]. The binding properties of glycosylated and unglycosylated antibodies were analysed by adsorption on HPL-Sepharose and by their reaction with HPL- and anti-mouse Ig coated wells of immunoassay plates. HPL-Sepharose-bound antibodies were subjected to SDS-PAGE [21] and autoradiography.

Immunoadsorption

Immunoadsorbents were prepared according to Jungfer [22].

Results

General properties of the anti-HPL antibodies

Nine stable hybridoma clones secreting aHPL antibodies were isolated from three fusions between spleen cells of immunized BALB/c mice and the myeloma cell line X63-Ag 8.653. The aHPL antibodies expressed α, γ, μ, $\varkappa$ and λ_2 chains. All antibodies with the more infrequently expressed α, γ_3, μ and λ_2 chains stemmed from a single fusion with spleen cells of a mouse immunized according to the immunization schedule 2 (see methods) (aHPL-11: α, $\varkappa$; aHPL-31: μ, λ_2; aHPL-133: γ_3, $\varkappa$; aHPL-173: μ, $\varkappa$; aHPL-62), although one would have expected to obtain IgMs from a short time immunization. The latter, however, yielded antibodies which could be inhibited from binding HPL by GalNAc (aHPL-75, aHPL-165). Only one clone secreting aHPL antibodies (aHPL-135) was isolated after a prolonged immunization period. An initial reclone of aHPL-62 used for ascites production showed a γ_3 chain, while later reclones expressed γ_1. Most of the aHPL antibodies formed precipitation lines with HPL in double diffusion tests. Their affinity constants ranged between 5×10^8 to 2×10^9 M^{-1} the highest being observed for aHPL-122 and aHPL-135.

Interdependence of carbohydrate and antibody binding

Since it was expected that conformational changes of HPL caused by binding of carbohydrates would influence the antigenic properties of HPL, the binding of aHPL antibodies to HPL (HPL-Pox) was assayed in the presence of appropriate carbohydrates.

Two antibodies, aHPL-75 and aHPL-165, were found to be prevented from binding by GalNAc (Fig. 1). The respective concentrations (average of

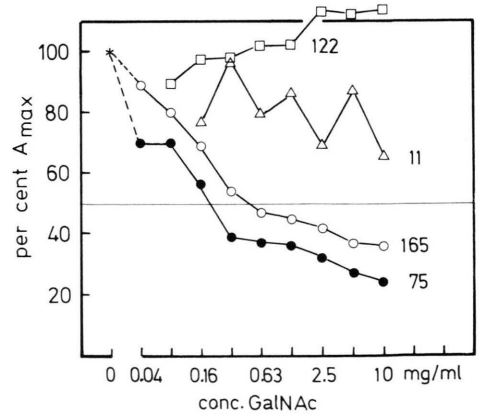

Fig. 1. The influence of GalNAc on the binding of aHPL antibodies. With the exception of aHPL-75 and aHPL-165, antibody binding was scarcely affected by GalNAc. Anti-HPL-11 and aHPL-122 represent the series of negative controls.

Table I. The inhibition of aHPL antibody binding by carbohydrates. Concentrations of carbohydrates and the polyvalent glycoprotein A-substance, respectively, necessary to give 50 percent inhibition.

Antibody	GalNAc [mM]	GlcNAc [mM]	Gal [mM]	Glc [mM]	A-subst. [$\mu g \cdot m^{-1}$]	A-subst. 0.5 $\mu g \cdot ml^{-1}$ GalNAc [mM]
aHPL-75	1.2	81	1660	2050	< 2	0.36
aHPL-165	3.2	326	2050	2370	< 2	0.36
aHPL-31	–	–	–	–	< 2	0.41
aHPL-135	–	–	–	–	< 2	0.90
aHPL-122	–	–	–	–	< 2	0.99
aHPL-62	–	–	–	–	3.1	0.77
aHPL-11	–	–	–	–	3.6	0.90
aHPL-173	–	–	–	–	4.8	1.22

3 experiments) were 0.26 and 0.70 mg GalNAc per ml (1.2 and 3.2 mM, respectively) for 50 percent reduction of HPL-Pox binding. The curves, however, show that higher concentrations of GalNAc were not sufficient to completely inhibit the binding by antibodies.

On average, GlcNAc was about two orders of magnitude less effective than GalNAc and Gal and Glc almost 3 orders less effective (Table I). This finding could be expected because of the known carbohydrate-binding properties of HPL. The great difference between the efficiencies of GalNAc and GlcNAc, however, is in contrast to the relatively small difference in the association constants for methyl-α-GalNAc (5×10^3 M^{-1}) and methyl-α-GlcNAc (1.1×10^{-3} M^{-1}) [23].

None of the other aHPL antibodies behaved similarly to aHPL-75 and aHPL-165. If however, competition for HPL binding was carried out with the polyvalent A-substance, which was included in the experiments to mimic conditions on cell surfaces, all aHPL antibodies were prevented from binding (Table I). Complete inhibition was reached at concentrations between 2 and 50 μg of A-substance per ml.

Complete inhibition of antibody binding was also achieved if GalNAc was added to non-inhibitory concentrations of A-substance.

(In concentrations up to about 1 μg per ml A-substance served as a bridge between one HPL-Pox molecule bound by an antibody and several other HPL-Pox molecules, resulting in an amplification of the Pox signal.)

The high efficiency of A-substance in inhibiting aHPL antibody binding may be due to its multivalence. Hammarström [4] found very high association constants for HPL binding to A-erythrocytes.

Comparison of snail lectins by enzyme linked immunoassays

The test system was essentially the same as in the preceding experiments, except that lectins were substituted for carbohydrates. The experiments served to compare the antigenic properties of other lectins of snails to those of HPL.

The smallest amount of HPL which could be detected in this way was about 0.2 μg per ml or 0.01 μg per sample (inset of Fig. 2). On a molar basis these data mean that between 0.1 to 1.0 pmol of HPL could be quantified ($M_{r(HPL)} = 79\,000$).

The ability of lectins other than HPL to substitute for HPL in this type of experiments was limited (Fig. 2). *Helix aspersa* lectin (HAL), however, was strongly-bound by aHPL-135, while *Helix lucorum* lectin (HLL) was bound by aHPL-122. None of the aHPL antibodies showed a similar affinity for the lectin of *Cepaea nemoralis*.

In order to mimic the effect of HPL in inhibiting HPL-Pox binding to the antibodies exogenous HAL or HLL was required in 5 to more than 20 fold higher concentrations. In some cases, the available concentrations of HLL were not sufficient to obtain 50 percent inhibition of HPL-Pox binding. Nevertheless HLL seems to be more similar to HPL than HAL is. If the aHPL antibodies are ordered according to their capacity to quantify small amounts of lectins in the present assay, the resulting sequence of antibodies is similar for HPL and HLL but not for HAL (Fig. 2).

Inhibition of antibody binding was not observed if lectin of *Rapana thomasiana* of GalNAc-binding plant lectins (*Dolichos biflorus*, *Glycine max*, *Wisteria floribunda*) were applied (concentrations up to 250 μg per ml).

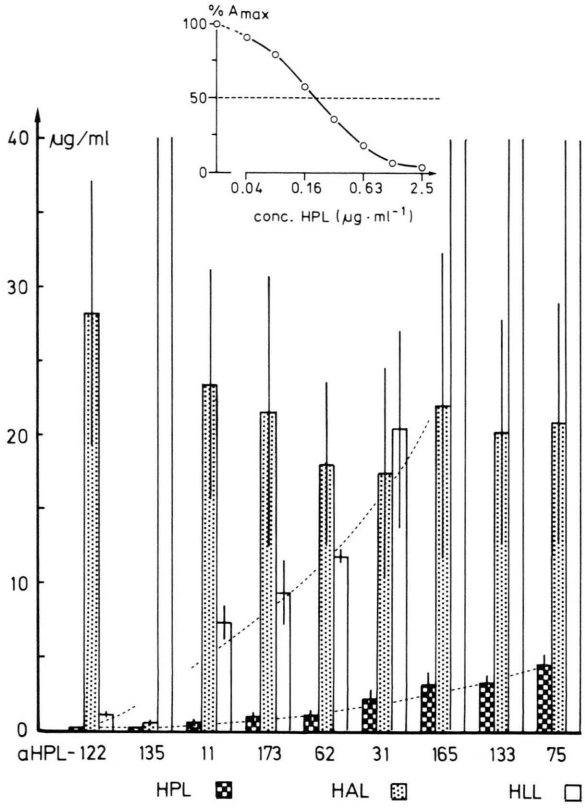

Fig. 2. Comparison of the antigenic properties of HAL, HLL and HPL. The columns give the concentrations of lectin required to compete out the binding of 50 percent of HPL-Pox to the antibodies. Each column represents the average of 5 to 7 independent experiments performed in duplicate. Vertical lines: standard deviation of the mean. Inset: Standard curve for the system HPL-Pox/HPL using aHPL-122. Dotted lines indicate a similar order of antibodies with respect to their apparent affinities toward HPL and HLL, respectively.

Although at least 5 to 7 independent experiments with duplicate samples were performed, the standard deviation remained high, if the lectins did not match the efficiency of HPL. An immunoadsorption step included beforehand to determine the amount of lectin in a preparation did not improve the result. Impurities in the commercial preparation did not influence the assay, since pure HPL added to impure HAL preparations showed the regular efficiency.

Comparison of Helix lectins in double diffusion experiments

Most of the aHPL antibodies formed precipitation lines with HPL (0.1 mg · ml^{-1}) if subjected to double diffusion tests in agarose. Even at the highest concentrations (~ 2 mg · ml^{-1}), aHPL-122 formed only faint lines. The other strongly-binding antibody, aHPL-135, which as aHPL-133, was only enriched about 30 fold from culture supernatants, did not visibly react with HPL.

No precipitation occurred if HAL was substituted for HPL even at concentrations of 1 mg · ml^{-1}. HLL formed precipitation lines with the two IgMs, aHPL-31, aHPL-173, and occasionally faint precipitation lines were observed with aHPL-11 and aHPL-62.

In this respect HLL appears once more to be more similar than HAL to HPL. Spurs were formed in double diffusion tests which allowed the comparison of precipitation lines of one aHPL antibody in relation to another one. The spurs were always observed as an extension of the heavier precipitation line. It may be assumed that these lines are formed before the weaker ones.

Spectral changes and changes in antigenicity of HPL due to carbohydrate binding: binding constants

Conformational changes of lectins due to carbohydrate binding can be monitored by absorbance changes [24, 25]. Spectral changes of HPL after binding of GalNAc were easily detectable. The difference spectrum had peaks at 298 and 286 nm (Fig. 3). Titration of HPL with GalNAc as monitored at 298 nm resulted in a saturation curve. If the respective data were transformed and plotted according to the transformed mass equation

$$\frac{[S]}{\Delta A} = \frac{1}{\Delta A_{max}}[S] - \frac{1}{\Delta A_{max} \cdot K}$$

(refs. [24, 25]), K was found to be 3.1×10^3 M^{-1}.

The binding of A-substance by HPL did not result in a pronounced difference spectrum; instead the absorbance increased in the shorter wavelengths range (light scattering by aggregated HPL-A-substance complexes?). The binding constant for GalNAc in the presence of 1 µg per ml of A-substance was 5.9×10^3 M^{-1}.

If data similar to those of Fig. 1 were treated in the same manner as in the experiments just described (except that A_{max} was the maximum decrease in A), a binding constant of 1.7×10^3 M^{-1} was

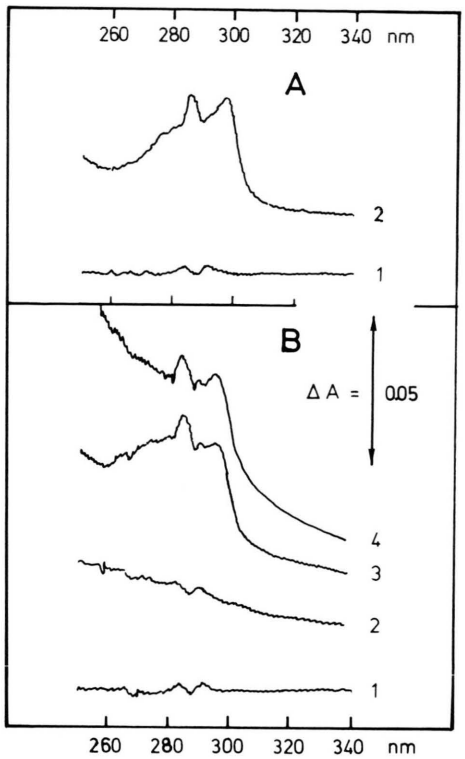

Fig. 3. Spectrophotometric changes of HPL caused by carbohydrate binding. A) Difference spectrum of HPL versus HPL saturated with GalNAc (0.5 mg HPL per ml PBS, light path 1 cm). 1: baseline, 2: spectrum HPL/HPL-GalNAc. B) Difference spectrum of HPL versus HPL plus A-substance (plus GalNAc). 1: baseline, 2: HPL + 1 μg · ml^{-1} A-substance, 3: 2) + saturating amounts of GalNAc, 4: 3) + 12 μg · ml^{-1} A-substance.

observed, irrespective of whether the experiments were performed with aHPL-75 or aHPL-165.

In another experiment where aHPL-75 was added to HPL (3 mg aHPL-75 and 0.6 mg HPL in 1.2 ml PBS) a precipitate formed immediately. However, solubilization of the precipitate was observed when diluted in PBS containing different amounts of GalNAc. Nephelometric *A* values transformed as before resulted in an association constant of $5 \times 10^3\ M^{-1}$. However, equilibrium was only reached after storing the samples for 3 days (at 4 °C).

The most reliable binding constant seems to be $3.1 \times 10^3\ M^{-1}$, because it was obtained in the absence of interfering substances, although the alterations of *K* caused by antibodies are relatively small. The *K* value for binding methyl-α-GalNAc is $5 \times 10^3\ M^{-1}$ [23]. The low value obtained using HPL-Pox may possibly reflect altered binding properties of HPL caused by conjugation with Pox. Nevertheless the experiments demonstrate that spectrometric detectable changes are reflected by changes in the antigenicity of HPL.

Binding properties of anti-HPL antibodies lacking the carbohydrate moiety

The possibility that antibodies were selected because of affinities between HPL and the carbohydrate portion of the antibodies was tested in experiments using aHPL antibodies lacking the carbohydrate moiety. Anti-HPL antibodies both with and without their carbohydrates were retained by HPL-Sepharose as detected by SDS-PAGE analyses of HPL-Sepharose eluates (Fig. 4).

No significant quantitative differences were detectable in individual aHPL antibodies in their ability to bind HPL and to be bound by anti-mouse Ig, whether or not the carbohydrate portion was present (data not shown). It may therefore be concluded that the selected antibodies are truly specific for the antigenic determinants of HPL.

Discussion

This study describes the production and analysis of two antibodies, aHPL-75 and aHPL-165, whose binding to the lectin of *Helix pomatia* (HPL) was specifically inhibited by exogenous GalNAc. Fol-

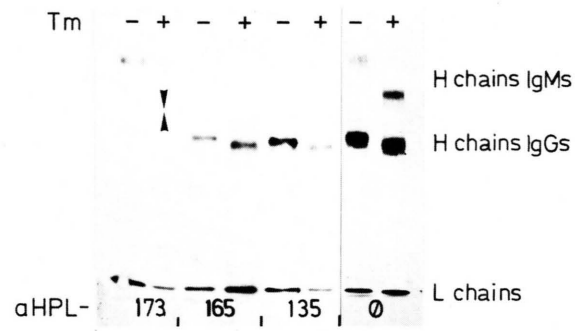

Fig. 4. Evidence that aHPL antibodies are not bound to HPL by their carbohydrate moieties. [^{35}S]labelled aHPL antibodies were biosynthesized in the presence (Tm^+ = unglycosylated antibodies) and in the absence (Tm^-) of tunicamycin, adsorbed onto HPL-Sepharose, eluted, reduced and subjected to SDS-PAGE. IgM secretion is strongly inhibited under the influence of tunicamycin (ref. 20). Arrow indicates the faint band of Tm^+ aHPL-173 antibodies. ∅: IgMs and IgGs from lysed cells. Six individual clones were assayed together. It is obvious that Tm-treated clones do not produce glycosylated antibodies.

lowing the interpretation of Borrebaeck and Etzler [7], these antibodies interact with the GalNAc binding sites of the lectin or with a determinant conformationally interdependent with the active sites. This interpretation is corroborated by two additional findings from our investigations. Firstly, HPL-aHPL precipitates could be solubilized in the presence of GalNAc, and secondly, conformational changes of HPL following GalNAc binding were easily detectable. The concentrations of GalNAc necessary to cause 50 percent reduction in antibody binding (1 and 3 mM, respectively), however, were at least two orders of magnitude lower than those hitherto reported [7]. If aHPL-75 and aHPL-165 were directed against lectin-owned carbohydrates resembling GalNAc, a similar behaviour could be expected. But then one needs to explain why HPL does not bind to HPL.

Multiple binding, and therefore high association [4], may be responsible for the higher efficiency of A-substance in preventing antibodies from binding to HPL. In addition, it seems reasonable that the binding of A-substance covers a greater area of the surface of HPL, thus rendering the lectin generally inaccessible to antibody binding. This finding may be relevant in further studies in which lectins on a cell surface are detected by antibodies. However, HPL-aHPL-cell surface interactions may be different from those described here because of the lesser motility of HPL receptors on a cell surface relative to HPL receptors of A-substance. Further investigations will be necessary to allow a fully satisfying interpretation of the phenomena found.

Contrary to expectations the binding sites (presupposed aHPL-75 and aHPL-165 recognize these sites) of other lectins related to HPL did not show antigenic similarity to HPL exceeding that of other antigenic determinants when analysed for ability to bind aHPL antibodies. On the whole, lectins of snails related to *Helix pomatia* were 5 to more than 20 times less effective than HPL itself in competing for the binding of labelled HPL by the aHPL antibodies. However, one aHPL antibody (aHPL-135) showed a high affinity for HAL, while a second antibody (aHPL-122) bound efficiently to HLL. In contrast, lectin of *Cepaea nemoralis* (formerly *Helix nemoralis*) did not show a similar binding property. Assuming that the immunization regimes used were not sufficient to induce the production of an appropriate antibody, this result may reflect the greater evolutionary distance between these two snails.

On the basis of the present results HLL may be more closely related to HPL than HAL is. For example, there are similarities in the succession of aHPL antibodies if these are ordered according to their relative abilities to quantify HPL and HLL, respectively. In addition, HLL forms precipitates with aHPL antibodies while HAL does not. However, more information will be necessary on peptide and subunit composition of HLL and HAL to confirm the validity of the present conclusion. In this regard, reduced HPL on 17.5% SDS-PAGE is separated in two distinct bands, one running slightly ahead of cytochrome *c* ($M_r = 12\,300$), the other one slightly ahead of myoglobin ($M_r = 17\,800$).

The present data allow us to divide the aHPL antibodies into four functionally-different groups. The data concerning the binding of HLL and HAL show that aHPL-122 and aHPL-135 recognize different epitopes, which are in turn distinct from epitopes recognized by the remaining aHPL antibodies. Anti-HPL-75 and aHPL-165 may be distinguished from the other aHPL antibodies by their behaviour in the presence of exogenous GalNAc. The members of the remaining group are at least different with respect to their immunoglobulin chain classes.

The results may also be evaluated in another respect. There have been reports of lectins which selectively bind a certain percentage or group of antibodies from serum [26–29]. In a similar manner, HPL could have selected particular antibodies because of unknown affinities between HPL and certain fractions of antibodies. Although several data (*e.g.* binding constants, carbohydrate composition of known antibody classes, diversity of chain classes in the selected aHPL antibodies) discount this possibility, the most direct proof for the specificity of the aHPL antibodies is the fact that they remain able to bind HPL even after they have lost their carbohydrate moiety.

Such specific antibodies characterizing different epitopes will be useful in further studies on lectin substructure, lectin biogenesis and possibly on cell-lectin interactions. Provided a conformational alteration of HPL (and not steric hindrance or antibodies binding to HPL-owned carbohydrates) is responsible for the interdependence of GalNAc and antibody binding, these findings may indicate

similar possibilities in the analysis of other proteins, *e.g.* phytochrome, undergoing conformational changes during biological functioning.

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