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Lectins in ocular drug delivery: an investigation of lectin binding sites on the corneal and conjunctival surfaces

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Abstract

Ocular drug delivery is limited both by patient acceptability and by the limited time that the dosage form is retained within the precorneal region. The aim of this investigation was to identify lectin receptors within the precorneal region as potential targets for a lectin containing ocular dosage form, thus facilitating prolonged drug delivery. Initial ex-vivo studies evaluated the binding, after 15 min contact with intact (unfixed) rat corneal and conjunctival epithelia, of a panel of 36 lectins of varying sugar specificities. Tissues were sectioned and examined using light microscopy. Lectin binding was identified using a standard histological procedure involving the avidin—biotin reaction, and the formation of a brown precipitate from a 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution. All lectins were observed to bind to both the cornea and conjunctiva to varying degrees. There was little evidence of a specific receptor or structural type of lectin that particularly favoured binding. The lectins from *Solanum tuberosum* (potato) and *Helix pomatia* (edible snail) which are specific for the terminal sugar residues *N*-acetyl-*D*-glucosamine and *N*-acetyl-*D*-galactosamine respectively, appeared to be the most promising and displayed clear visual evidence of binding to ocular tissues after only 10 s contact. The stability of binding of these two lectins was assessed using a video microscopy method of evaluation, and binding in a second species investigated using unfixed porcine cornea. Both lectins were found to show rapid, substantial and stable binding ex-vivo, and these will be taken forward in future work for quantitative in vivo studies and toxicological evaluation.

Keywords: Lectins; Ocular; Epithelium; Cornea; Conjunctiva; Drug delivery

1. Introduction

The delivery of aqueous solutions of ophthalmic drugs is limited by the efficient removal mechanisms that exist within the precorneal area. A maximum retention time of 15 s for an aqueous

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isotonic eye drop solution has been reported (Zaki et al., 1986). Ocular drug absorption requires good corneal penetration along with a prolonged contact time with the corneal tissue (Middleton et al., 1990). For this reason several approaches have been considered to extend the residence times of topically applied medications in the precorneal region and various formulations such as suspensions, ointments, inserts, and aqueous gels have been investigated (Greaves, 1989). These delivery systems offer some advantages over conventional solutions, but blurring of the vision (ointments) and lack of patient compliance (inserts) are major disadvantages (Zaki et al., 1986). Some limited success in enhancing drug delivery has been achieved using bioadhesive polymers (Davies et al., 1992; Hui and Robinson, 1985; Middleton et al., 1990; Thermes et al., 1992).

Lectins are defined as proteins or glycoproteins of non-immunoglobulin nature capable of specific recognition of and reversible binding to carbohydrate moieties of complex glycoconjugates, without altering the covalent structure of any of the recognised glycosyl ligands (Makela, 1957). Although lectins are structurally diverse, it is possible to group many of them into distinct families of homologous proteins that share common structural properties (Sharon, 1993). The largest and best characterised belong to the family of legume lectins, which show extensive homologies in their primary sequences and express three-dimensional structural similarities. The association constant, K_a, of lectins with monosaccharides is usually in the range of 10^3–10^4 (Lis et al., 1994). The exact physiological role of lectins is unknown but they are implicated in many cell recognition and adhesion processes (Zanetta et al., 1992). 'Lectin like' molecules that bind to cell surface glycoconjugates have been shown to be important in the adhesion of micro-organisms to mucosal surfaces (Calderone and Wadsworth, 1993; Goldhare, 1994; Hsu and Raine, 1982; Rudner et al., 1992; Sandin et al., 1982). Lectins have been described as 'second generation mucoadhesives' (Kompella and Lee, 1992), and their potential use as a means of enhancing drug delivery within to the gastrointestinal tract has been investigated in other studies (e.g. Irache et al., 1994; Lehr and Lee, 1993; Naisbett and Woodley, 1995).

Lectin binding to the corneal and conjunctival surfaces and constituents of the tear film has been demonstrated in processed histological sections (Bishop et al., 1991; Brandon et al., 1988; Holmes et al., 1985; Panjwani et al., 1986). Schaeffer et al. (1982) pre-treated the rabbit corneal surface with the lectin from *Triticum vulgaris* prior to application of liposomes containing mixed brain gangliosides. The lectins were seen to facilitate liposome binding which had the effect of increasing the flux of carbachol across isolated rabbit corneas. It would therefore seem a reasonable proposition to incorporate lectins into drug carrier systems in order to prolong their residence in the precorneal region. The overall aim of this study is to formulate a lectin-drug carrier complex (a macromolecular carrier or microsphere) in which the lectin 'anchors' the dosage form to epithelial surfaces for a prolonged period, thus allowing extended ocular drug delivery.

The objective of this initial study was to use standard histological procedures to determine the lectin receptors present on the corneal and conjunctival surfaces, and to identify the types of lectins, from the large range available commercially, that will bind optimally to these. Previous studies used fixed sections, but histochemical fixation and processing is known to alter the cell surface by sequestering glycoconjugates and dissolving out fat-linked sugars (Leathem and Atkins, 1983). Therefore in this work the binding of a range of lectins to unprocessed ocular tissue was investigated. The speed and stability of binding of the most promising lectins were also considered with regard to the selection of a suitable lectin candidate for further investigation.

2. Materials and methods

2.1. Materials

Highly purified biotinylated lectins were purchased from either Sigma Chemical Company, Poole, UK, Vector Laboratories, Peterborough UK, or Bradshaw Biologicals Ltd, Loughborough, UK. The counterstain, methyl green, and mounting medium DPX, were supplied by Merck

LTD, Lutterworth, UK. All other agents were of analytical or high purity quality, and purchased from Sigma Chemical Company, Poole, UK. Upper eyelids and eyeballs were obtained from male Wistar rats of approximately 200 g weight, bred at the University of Portsmouth.

2.2. Reagent preparation

Lectins at a concentration of 10 mg l^{-1} were prepared in 0.05 M tris buffered saline (TBS) containing 1 mM $CaCl_2$. Streptavidin peroxidase, as used for the avidin-biotin complex (ABC) technique, was made up to 5 mg l^{-1} with 0.125 M isotonic TBS (containing 0.347 M NaCl) at pH 7.6. The chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used at a concentration of 0.05% (w/v) in 0.05 M isotonic TBS in the presence of 0.15% (v/v) H_2O_2.

2.3. Methods

2.3.1. Screening of lectin candidates

The method used was based upon standard histochemical procedures as described by Bishop et al. (1991) but modified for use with fresh tissues. Upper eyelids and eyeballs were removed from recently killed rats. These were placed in contact with 2.5–3.0 ml of 0.05 M isotonic TBS containing 1 mM $CaCl_2$ and 10 mg l^{-1} biotinylated lectin for 15 min, washed in 0.05 M isotonic TBS containing 1 mM $CaCl_2$, then blotted to remove excess buffer. The tissues were then incubated in 2.5–3.0 ml of the 5 mg l^{-1} streptavidin peroxidase solution for 1 h, washed in 0.05 M isotonic TBS, then blotted and placed in 2.5–3.0 ml of the DAB solution for 10 min. For fixation the tissues were placed in 4% phosphate buffered formaldehyde for 24 h.

Dehydration of the tissues was completed in a Histokinette (type E7326, British American Optical Company Ltd., Slough, UK) using graded alcohols followed by three changes of 1,1,1,trichloroethane and immersion into wax. Tissues were then vacuum embedded in paraffin wax using a Hearson Vacuum Embedder for 1–1.5 h. Sections (5 μm) were cut from the centre of the wax block using a microtome (Slee Medical Equipment Ltd, London, UK) and dried onto microscope slides at 45°C for 12 h. Paraffin sections were dewaxed in xylene and passed through graded alcohols to distilled water, then counterstained with 2% methyl green for 30 min. The tissues were dehydrated through graded alcohols, placed into xylene and mounted in DPX. Results were evaluated qualitatively using a light microscope (model YS2-H, Nikon, Tokyo, Japan) at × 400 magnification. The DAB deposit was observed and scored as follows; deposit intensity, absent to heavy (0/+ + + + +); surface cover, no coverage to complete coverage (0–5). The assessment was based on tissues obtained from three rats.

A series of controls was included in the initial investigations, to eliminate the possibility of background staining and endogenous enzyme activity.

2.3.2. The speed of lectin binding

The most promising lectins identified from the initial screening were evaluated for their rate of binding to corneal and conjunctival tissues. The

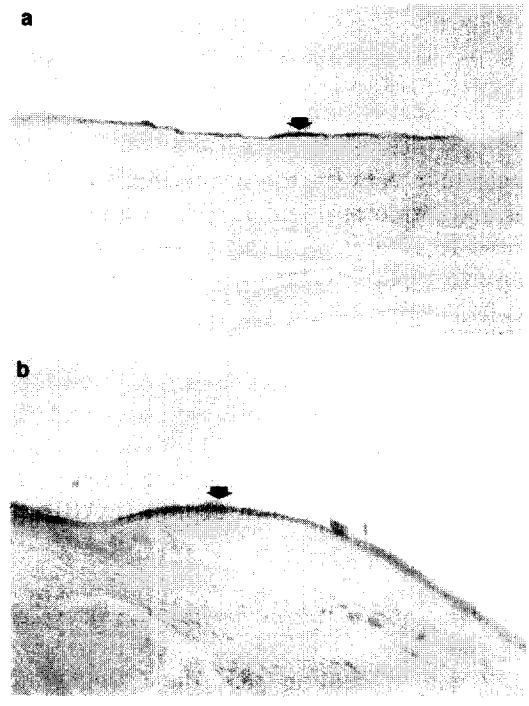

Fig. 1. The lectin from *Solanum tuberosum* binding to rat cornea (a) and rat conjunctiva (b) as indicated by the dark surface deposit of DAB (arrowed).

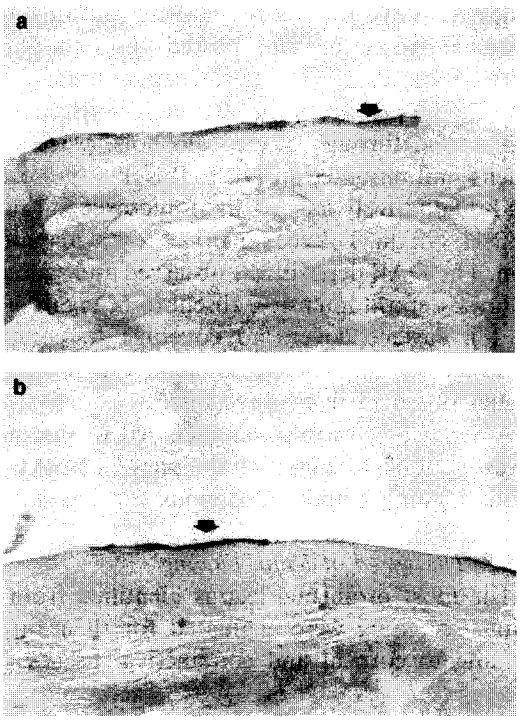

Fig. 2. The binding of lectins from *Trichosanthes kirilowii* to rat cornea (a) and conjunctiva (b) as indicated by the dark surface percipitate of DAB (arrowed).

contact time between the conjugated lectin solutions and the corneal and conjunctival tissues was reduced to 10 and 30 s, this being more relevant to in-vivo topical ocular drug administration.

2.3.3. Stability of lectin binding

The rat preocular tissues were incubated for 10 s with the conjugated lectin solution and then rinsed for 30 min in TBS. The tissues were assessed for lectin binding by the stain intensity and surface cover of the brown deposit (DAB) using light microscopy.

2.3.4. Inhibition of lectin binding by the specific sugar

In order to confirm that these lectins were binding to the ocular surfaces specifically by interaction with a sugar group, the binding of three lectins, *Canavalia ensiformis* and two of the most promising selected from above, was evaluated after premixing with 400 mg ml^{-1} of their hapten (specific inhibiting) sugar.

2.3.5. Lectin binding to porcine cornea

Whole eye balls were removed from a pig directly after slaughter and the cornea dissected into four portions; each of these was immediately incubated in a lectin solution for 10 s, immersed in streptavidin peroxidase followed by DAB and evaluated as before.

2.3.6. The effect of washing and semi-quantification of the DAB deposit width using a video microscopy technique

The thickness of the DAB deposit on the rat corneal and conjunctival surfaces was measured at 100 representative positions using video microscopy and DIGIT image analysis software, as a measure of the extent of lectin binding. Measurements were taken from sections of tissue

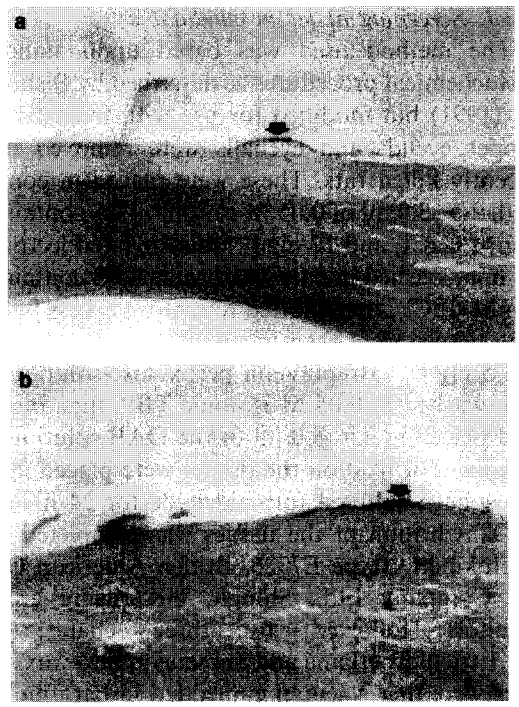

Fig. 3. The lectin from *Anguilla anguilla* binding to rat cornea (a) and conjunctiva (b) as indicated by the dark surface deposit of DAB (arrowed).

which had been incubated with appropriate lectins for 10 s and 30 s, or after 10 s exposure followed by 30 min wash in TBS.

3. Results

A series of controls was included in the initial investigations to eliminate the possibility of background staining and endogenous enzyme activity. Avidin has a high affinity and specificity for biotin and the minimal background staining observed emphasises the value of this technique. An endogenous peroxidase enzyme block was used initially in these investigations but was found to be unnecessary. Initial controls that omitted one component of the lectin/avidin—biotin/DAB reaction gave uniformly negative results, although a very faint brown precipitate was seen occasionally when the lectin was omitted. This was probably due to a small amount of residual biotin being present on the surface epithelia

All lectins bound to the corneal and conjunctival surfaces to some extent, with some showing much more intense and consistent binding than others (Figs. 1–3). The lectin from *Solanum tuberosum* (Fig. 1) showing greater surface cover and stain intensity than the lectins from *Tricosanthes kirilowii* (Fig. 2) and *Anguilla anguilla* (Fig. 3). There does not seem to be a particular type of lectin, in terms of sugar specificity and structural features that particularly favours binding (Tables 1–5). There were also no clear trends with regard to differences in corneal and the conjunctival binding.

Seven lectins were then selected for further study, one selection criteria being the extent of binding demonstrated. When the binding of these lectins after only 10 and 30 s was considered, the lectins from *Helix pomatia* and *Solanum tuberosum* appeared to bind most rapidly (Table 6).

The binding of lectins from *Helix pomatia*, *Solanum tuberosum*, and *Canavalia ensiformis*, to the corneal and conjunctival epithelium was substantially, or almost completely, inhibited in the presence of their specific hapten sugar, indicating

Table 1
N-acetyl glucosamine specific lectin binding to corneal and conjunctival surfaces of rats (n = 3)

Lectin origin	Biotin:lectin ratio	MW (kDa)	Intensity of stain (0/+++)		Coverage (0–5)	
			Cornea	Conjunctiva	Cornea	Conjunctiva
Datura stramonium	1:1	86	++	++	2	3
Lycopersicon esculentum	3.2:1	71	+++	+++	4	4
Triticum vulgaris	1:1	36	++	++	3	3
Solanum tuberosum	1:1	100	+++	+++	5	5
Phytolacca americana	2:1	32	++	+++	2	2
Urtica dioica	-	8–9	+	++	2	3

Table 2
N-acetyl galactosamine specific lectin binding to corneal and conjunctival surfaces of rats (n = 3)

Lectin origin	Biotin:lectin ratio	MW (kDa)	Intensity of stain (0/++++)		Coverage (0–5)	
			Cornea	Conjunctiva	Cornea	Conjunctiva
Helix pomatia	3:1	79	++	++++	5	3
Dolichos biflorus	5.3:1	135	++	++	5	5
Glycine max	4.4:1	110	++	++	3	2
Helix aspersa	-	79	+++	++	3	3
Sophora japonica	1.9:1	133	0/+	0	1	0
Vicia villosa	2.7:1	110	++	++	4	3
Tulipa sp.	-	-	++	++	3	3

that binding is by a specific lectin:sugar interaction.

Both lectins from *Helix pomatia* and *Solanum tuberosum* were seen to readily and avidly bind to porcine corneal surface (Table 7). When exposed to surface washing with TBS, both lectins appeared to be stable over 30 min (Table 8). Surprisingly, washing with TBS seemed to increase the width of the DAB deposit when examined by video microscopy, probably due to hydration and therefore expansion of the epithelial tissue in TBS. The lectin from *Helix pomatia* gave a greater overall stain width on both tissues than that from *Solanum tuberosum*, although a direct comparison was complicated by their different biotin:lectin ratios.

4. Discussion

The aim of this investigation was to examine lectin binding within the precorneal region, and this was subjectively assessed, using a standard histochemical technique, in terms of DAB precipitate intensity and degree of surface cover. The advantage of the avidin-biotin technique is that it is well established as a histochemical procedure to identify lectin binding, being highly sensitive, extremely specific, reliable, and comparatively rapid (Hsu and Raine, 1982; Philipp, 1992). It allowed the identification of lectin binding to cell surfaces and therefore the identification of the lectin receptors present.

Surprisingly the DAB deposit was present in

Table 3
Galactose specific lectin binding to corneal and conjunctival surfaces of rats (n = 3)

Lectin origin	Biotin:lectin ratio	MW (kDa)	Intensity of stain (0/+++)		Coverage (0–5)	
			Cornea	Conjunctiva	Cornea	Conjunctiva
Euonymus europaeus	1:1	140	+++	+++	3	3
Artocarpus integrifolia	-	18	++	++	4	3
Allomyrina dichotoma	-	65	++	++	4	2
Vigna radiata	-	53	++	+	2	1
Arachis hypogaea	3:1	120	++	++	3	1
Erythrina cristagalli	-	58	++	++	2	2
Erythrina corallodendron	-	60	+	+	2	2
Bandeiraea simplicifolia	3.8:1	114	++	++	2	3
Bauhinia purpurea	-	195	+	++	2	1

Table 4
Mannose/glucose and mannose specific lectin binding to corneal and conjunctival surfaces of rats (n = 3)

Lectin origin	Biotin:lectin ratio	MW (kDa)	Intensity of stain (0/+++)		Coverage (0–5)	
			Cornea	Conjunctiva	Cornea	Conjunctiva
Canavalia ensiformis[a]	6:1	102	+++	+++	4	2
Pisum sativum[a]	5:1	49	+++	++++	1	3
Lens culinaris[b]	-	49	++	+++	4	3
Galanthus nivalis[b]	1:1	52	+++	++	3	3
Hippeastrum hybrid[b]	1:1	50	++	+++	1	2
Narcissus pseudonarcissus[b]	1:1.25	26	++	++	<1	<1

[a]Mannose/glucose.
[b]Mannose.

Table 5
Fucose, *N*-acetyl neuraminic acid and lactose specific lectin binding to corneal and conjunctival surfaces of rats (n = 3)

Lectin origin	Biotin:lectin ratio	MW (kDa)	Intensity of stain		Coverage (0–5)	
			Cornea	Conjunctiva	Cornea	Conjunctiva
Tetragonolobus purpureus[a]	7.5:1	120	+	+	1	1
Ulex europaeus I[a]	2.9:1	68	+++	+++	3	4
Anguilla anguilla[a]	-	72–84	++	+	1	2
Sambucus nigra[b]	1:1	150	+++	++	4	2
Maackia amurensis III[b]	1:1	75	+	++	2	1
Limulus polyphemus[b]	-	350–500	0/+	0/+	1	1
Colchicum autumnale[c]	-	100	++	+	3	2
Trichosanthes kirilowii[c]	-	56	+	+++	2	2

[a]Fucose.
[b]N-acetyl neuraminic acid.
[c]Lactose.

Table 6
The effect of shortened contact time of rat ocular tissues on lectin binding

Lectin origin	30s Cornea		30s Conjunctiva		10s Cornea		10s Conjunctiva	
	SC	SI	SC	SI	SC	SI	SC	SI
Lens culinaris	2	++	3	++	2	+	1	+
Solanum tuberosum	3	++	3	++	2	++	2	+
Lycopersicon esculentum	2	++	3	+++	2	+	2	+
Helix pomatia	3	++	1	+	2	++	1	++
Sambucus nigra	2	++	1	+	1	+	<1	+
Arachis hypogaea	<1	+	<1	++	<1	+	0	0
Dolichos biflorus	1	+	1	+	2	+	1	+

SC, surface cover of DAB deposit; SI, stain intensity of DAB deposit.

varying amounts on the surface epithelia of all tissues incubated with lectin solutions, but absent from the controls, and it was concluded that all the lectins studied bound to these surfaces to some extent. It was expected on the outset of this investigation that specific lectin receptors would be identified within the various areas of the precorneal region, towards which all further efforts could be concentrated, but this did not appear to be the case. It is unlikely however that lactose groups would be present on the precorneal surfaces but lectin binding was still evident (Table 5). The lectins from *Colchicum autumnale* and *Trichosanthes kirilowii*, although binding primarily to lactose residues, have a secondary weaker affinity for *N*-acetylgalactosamine and β-D-galactose respectively, and this may be reflected in the results obtained. Inhibition of binding by the hapten sugar indicated that binding was by a specific lectin/sugar interaction and not non-specific binding.

Similarly, no particular group of structurally homologous lectins seemed obviously to favour binding. For example, two of the leguminous lectins studied, from *Lens culinaris* and *Pisum sativum*, have molecular weights of 49 kDa, primary sequences which are 78% homologous (Pusztai, 1991) and are specific for α-mannose, and for α-mannose and glucose, respectively. The surface cover of the DAB deposit was similar on the conjunctival epithelial surfaces, but on the corneal surface was much less with the *Pisum sativum* lectin.

Table 7
Surface cover (SC) and stain intensity (SI) of the lectins from *Solanum tuberosum* and *Helix pomatia* on fresh porcine corneal epithelia after 10 s (n = 3)

Lectin origin	Cornea	
	Surface cover[a]	Stain intensity[a]
Solanum tuberosum	5 (2)	++++
Helix pomatia	5 (2)	+++
Control	<1 (0)	<+ (0)

[a]In brackets, results observed with rat corneal epithelium.

Apart from sugar specificity and structure, the binding of the lectin may also be affected by the binding strength and the size and shape of the lectin (i.e. its ability to gain access to partially hidden sugar groupings) and this may explain the lack of a clear trend.

The ability of all lectins studied to bind to some extent complicated the selection of candidates for further investigation. The relative quantification of binding using this technique is difficult because visual assessment, although widely used as a standard histochemical method (e.g. Kawano et al., 1988; Rittig et al., 1990) provides only a subjective evaluation of lectin binding, and the intensity of the DAB deposit depends on factors like the rate of reaction and the biotin/lectin molar ratio which are not constant. Given the somewhat complex nature and sometimes unknown molecular weight of many lectins it is normal practice in histochemistry to measure lectin concentration in terms of weight, as opposed to moles, per unit volume (e.g. Rittig et al., 1990; Philipp, 1992). The concentrations chosen in this study were those proposed by Brandon et al. (1988) to give an appropriate staining for a range of lectins. Preliminary studies indicated that these concentrations and volumes provided a large excess of lectin, thus saturating all the available receptors on the cell surfaces.

Lectins were therefore selected for further study on the basis of showing clear consistent binding, being present in the normal diet and therefore of relatively low potential toxicity, or in the case of the *Dolichos biflorus* lectin, being a well characterised lectin of particular scientific interest.

On administration in an ophthalmic solution, the lectin must show significant binding before being washed out in the tears. Therefore significant binding after 10–15 s is required. Only the lectins from *Solanum tuberosum* and *Helix pomatia* showed evidence of substantial binding after 10 s and it was decided that these were the most appropriate candidates for use in further studies. The substantial binding of these two lectins to fresh porcine corneal tissues (Table 7), indicated the relevance of this study to other species.

The video microscopy technique should, by the measurement of the DAB deposit width, allow a less subjective means of ranking lectin binding. There were changes in the DAB deposit width between 10 and 30 s on the corneal epithelia with both lectins. However, with the conjunctival epithelia, an obvious difference was only obtained with the lectin from *Solanum tuberosum* (Table 8). Surprisingly, washing with TBS seemed to increase the width of the DAB band, probably due to hydration and therefore expansion of the epithelial tissue in TBS.

Table 8
Mean stain width of the DAB deposit observed with the lectins from *Helix pomatia* and *Solanum tuberosum* on rat ocular tissues using video microscopy (n = 100)

Lectin origin	Tissue surface	Mean width after 10s contact	Mean width after 30s contact	Mean width, 10s contact, 30 min washing
Helix pomatia	Cornea	1.10 (0.39)	1.89 (0.75)[a]	1.70 (0.61)
Helix pomatia	Conjunctiva	1.36 (0.53)	1.23 (0.49)[a]	1.15 (0.38)
Solanum tuberosum	Cornea	0.87 (0.27)	1.57 (0.32)[a]	1.10 (0.40)
Solanum tuberosum	Conjunctiva	0.93 (0.32)	1.20 (0.43)[a]	1.20 (0.33)

Standard deviation in brackets.

This study represents the first attempt to evaluate the binding of a wide range of lectin candidates to a non-histologically processed mucosal surface, in order to systematically identify the appropriate receptors and lectin types for further studies. It was concluded that to only a limited degree was the extent of binding related to the structural type or sugar specificity of the lectin, and a wide range of lectin receptors must therefore be available. Of the lectins studied those from *Solanum tuberosum* and *Helix pomatia* showed the most promising binding properties.

Further work will more precisely locate the lectin binding site on the tissue surface and will quantify this binding. Lectin toxicity and in-vivo binding will then be considered prior to selecting the most promising candidates for formulation studies.

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