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Lectins as targeting agents – the *in vitro* binding of lectins to lesions in the eye and mouth

C. Banchonglikitkul, J.D. Smart, R.V. Gibbs and D.J. Cook

School of Pharmacy and Biomedical Sciences, University of Portsmouth, White Swan Road, Portsmouth PO1 2DZ, UK.

Locating agents that bind to mucosal lesions of the eye and oral cavity would allow the selective delivery and retention of therapeutic or diagnostic agents. Lectins that bind specifically to the glycoconjugates present within a wound would provide an opportunity for such targeting.

Goldstein *et al*.[1] defined lectins as proteins or glycoproteins of non-immune origin that are capable of interacting with carbohydrates through at least two binding sites, agglutinating vegetable and/or animal cells, and precipitating polysaccharides, glycoproteins or glycolipids. The exact physiological role of lectins is unknown but they are implicated in many cell recognition and adhesion processes.[2]

Clear differences between cell-surface glycoconjugates on intact mucosal surfaces and diseased mucosa or cells from the underlying layers have been observed in previous studies. Differences in lectin binding were observed during the migration of the corneal epithelium,[3,4] in injured corneal endothelium during development,[5] in normal, scarred and lattice dystrophy corneas,[6] in keratoconus corneas,[7] and in normal, scarred and keratoconus corneas.[8]

Lectin binding has also been studied in oral squamous carcinoma,[9] in neoplastic and non-neoplastic oral white lesions,[10] in premalignant and malignant lesions of the oral cavity[11] and in regenerated junctional oral epithelium in the rat.[12]

A lectin that binds minimally to the intact mucosal surface might bind more avidly to the different glycoconjugates present in the underlying cells. Thus, the overall aim of this study is to investigate lectin binding to abrasions of the rat eye and oral mucosa, in order to identify a potential new method for targeting lesions with therapeutic and diagnostic agents. The current study uses an *in vitro* model of an abraded mucosal surface to investigate lectins identified from previous work[13-16] as binding both strongly and weakly to oral and ocular surfaces. Biotinylated lectins are employed and binding is detected using a streptavidin peroxidase/ diaminobenzidine staining procedure.

Biotinylated lectins from *Canavalia ensiformis*, *Erythrina corallodendron*, *Maackia amurensis*, *Lotus tetragonolobus*, *Narcissus pseudonarcissus*, *Solanum tuberosum* and *Sophora japonica* were purchased from Vector Laboratories (Peterborough, UK). The Diocleinae lectins (from *C. brasiliensis* and *Dioclea violacea*) were supplied as a gift by the University of Caera and biotinylated as described by Banchonglikitkul *et al*.[13] Sugar specificities for all lectins used in this study are shown in Table 1. Streptavidin peroxidase, 3′, 3′ diaminobenzidine tetrahydrochloride (DAB) and all other high-purity quality agents were purchased from the Sigma Chemical Company, Poole, UK. Lectin solutions were prepared as 10 mg/L in 0.05 mol/L Tris-buffered saline (TBS) containing 1 mmol/L $CaCl_2$ (pH 7.6).

Fresh tissues (eye balls, upper eyelids, buccal tissues and tongues) of Wistar rats, bred at the University of Portsmouth, were removed within two to three hours of death. All animals were reared in accordance with current UK government requirements.

All tissues were abraded by scratching with a blunt needle (1-mm diameter) across the central area of the tissue, in order to penetrate through the epithelium into the underlying connective tissue.

Table 1. Lectins used in the study

Source	Common name	Sugar specificity
C. brasiliensis	Brazilian bean	α-D-man, D-glc
C. ensiformis	Jack bean	α-D-man, D-glc
D. violacea	Brazilian bean	α-D-man, D-glc
E. corallodendron	Coral tree	β-D-gal-(1Æ4)-D-galNAc
L. tetragonolobus	Asparagus pea	α-L-fuc
M. amurensis	Maackia	Unknown
N. pseudonarcissus	Daffodil	Man
S. tuberosum	Potato	D-glcNAc oligomer
S. japonica	Japanese pagoda tree	β-D-galNAc, D-gal

Fig. 1. Control buccal mucosa counterstained with 2% methyl green (original magnification x400). N = normal area, L = lesional area.

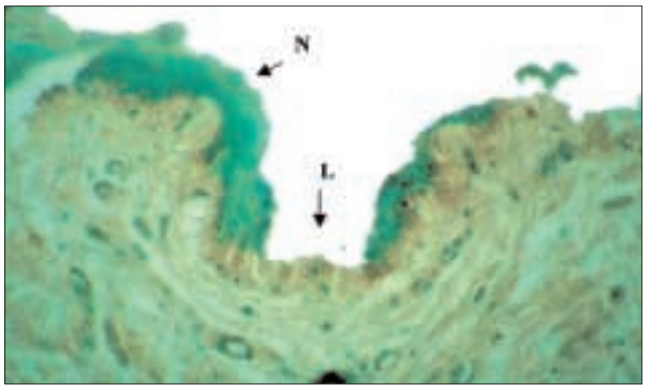
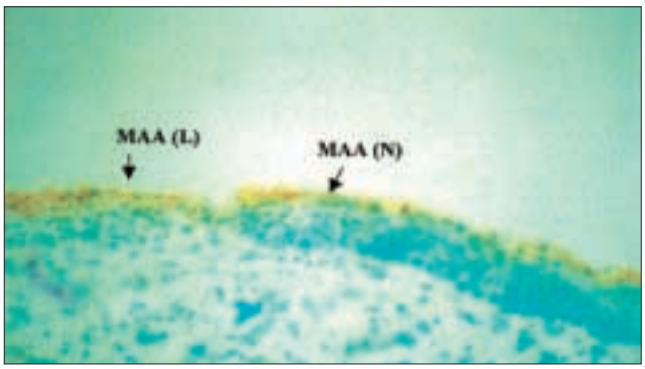


Fig. 2. The DAB deposits of M. amurensis lectin (MAA) on normal (N) and lesional (L) surfaces of conjunctival tissues counterstained with 2% methyl green (original magnification x400).



The lectin binding technique used in this study was that described by Nicholls *et al.*[15,16] and Banchonglikitkul *et al.*[13] Briefly, fresh tissues (upper eyelids, eyeballs, buccal tissues and tongues) were exposed to solutions containing 10 mg/L biotinylated lectin and 1 mmol/L $CaCl_2$ for 15 min. These were then washed in TBS, incubated in 3-5 mL of a 5 mg/L streptavidin peroxidase-containing solution for 1 h, washed again (x2), placed in 3-5 mL DAB solution for 10 min, then fixed by placing in 4 % phosphate-buffered formaldehyde. Paraffin sections (5 µm) were then prepared, cut perpendicular to the line of abrasion.

On microscopic examination, the DAB deposit intensity was scored as absent to strong (-/+++++) and the surface cover scored between no coverage and complete coverage (0 - 5). Three eyes were evaluated for each lectin and controls were completed where the exposure to lectin solution stage was omitted.

A semi-quantitative image analysis technique was also used. The sections were viewed using a Leitz Laborlux microscope fitted with a Panasonic video camera. The video signal was directed into a Watford video digitiser attached to an Archimedes 3000 computer. A suitable line (5 pixels wide), transecting the DAB deposit of both intact tissue and lesion, was selected. A computer programme written by one of the authors sampled along the selected line to measure the stain intensity (number of pixels per unit area). Maximum intensity of deposit was measured as the difference between the background and the highest reading found within the deposit along the chosen line. Data points ($n = 15$) were taken at regular intervals across both lesion and intact tissue for each of the three eyes.

Abrasions were easily identifiable and could be seen to cross the epithelium and enter the lower connective tissue (Figure 1). No evidence of DAB precipitate was seen in the lectin-free controls. Evidence of selective lesion binding was seen with the *D. violacea* and *M. amurensis* lectins only (Figures 2 and 3, Table 2). The semi-quantitative assessment of relative stain intensity is shown in Table 3, and confirmed that a degree of selectivity occurred with these lectins.

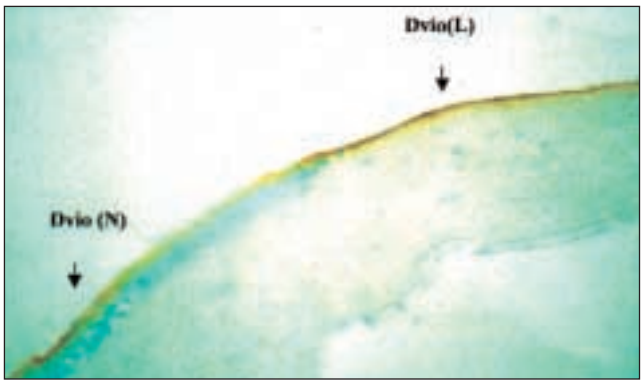
This study aimed to investigate lectins as a means of targeting therapeutic or diagnostic agents to mucosal lesions. Lectins have been shown to act as stimulators or inhibitors of cell proliferation, depending on the tissue and lectin used,[17,18] and thus potentially could be used as therapeutic agents in their own right. Lectins that only weakly bound to intact mucosae in previous studies[13-16] were tested using the abraded tissue model, and the relative binding to damaged and undamaged tissues was evaluated.

Table 2. Intensity of lectins binding to/surface coverage on intact and lesional areas of the eye and mouth mucosae of rats (n = 3)

Lectins	Intensity/surface cover of intact area (-/+++++, 0-5)				Intensity/surface cover of lesional area (-/+++++, 0-5)			
	Cor	Con	B	T	Cor	Con	B	T
Control	-/0	-/0	-/0	-	-/0	-/0	-/0	-/0
C. ensiformis	++/4	++/5	++/3	+/2	++/5	+/1	+/1	-/0
C. brasiliensis	+/1	+/2	+/2	+/4	++/3	-/0	+/3	-/0
D. violacea	+/2	+/4	+/3	+/4	++/5	++/4	++/3	-/0
E. corallodendron	(+)/1	(+)/2	+/1	+/3	(+)/1	(+)/4	(+)/1	+/2
N. pseudonarciscus	(+)/1	(+)/1	(+)/2	(+)/2	(+)/2	(+)/2	+/3	-/0
S. tuberosum	++/3	++/5	++/2	+/2	++/5	++/2	++/4	-/0
M. amurensis	+/2	+/3	+/3	+/3	+/3	+/5	+/3	++/3
S. japonica	(+)/1	+/4	+/5	+/4	(+)/1	+/4	++/4	+/3
L. tetragonolobus	+/2	+/4	+/4	++/4	+/3	+/3	++/4	++/2

Cor = cornea, Con = conjunctiva, B = buccal tissue, T = tongue, - = no binding, (+) = very weak, + = weak, ++ = moderate

Fig. 3. The DAB deposits of *D. violacea* lectin (Dvio) on normal (N) and lesional (L) surfaces of cornea counterstained with 2% methyl green (original magnification x400).



DAB deposit was absent from controls (Figure 1), indicating that deposit formation was dependent on the presence of bound lectin, and the presence of endogenous biotin and peroxidase had little effect.

In contrast to a previous observation in our laboratory (unpublished) and evidence suggested by previous studies,[3-8] little selective binding to lesions was seen with the majority of the lectins evaluated. However, *D. violacea* and *M. amurensis* lectins did appear to bind preferentially to lesional areas.

DAB deposits produced with *D. violacea* and *M. amurensis* lectins were examined using image analysis, and the intensities of deposit obtained with *D. violacea* lectin on corneal, conjunctival and buccal lesions were significantly different to that obtained from the non-lesional areas ($P < 0.0001$, Student's *t*-test). In sections of tongue, binding of *M. amurensis* lectin to lesional areas was significantly greater than that to intact tissue ($P < 0.0001$, Student's *t*-test). Moreover, the significant difference between normal and wound areas in corneal ($P < 0.01$) and buccal ($P < 0.0001$) sections was evident in this work, indicating that these two lectins have some potential for targeted delivery of therapeutic or diagnostic agents.

Table 3. Mean DAB deposit intensity of *D. violacea* and *M. amurensis* lectins on eye and oral cavity tissues of three rats ($n = 45$)

Tissues	*D. violacea* (mean ± SD)	*M. amurensis* (mean ± SD)
Cornea		
Intact area	15.1 ± 5.8	11.6 ± 2.3
Lesion area	20.8 ± 7.9	10.4 ± 1.9
% Ratio lesion/intact	138	90
Conjunctiva		
Intact area	11.9 ± 1.8	13.8 ± 3.1
Lesion area	22.3 ± 5.5	13.9 ± 3.9
% Ratio lesion/intact	188	101
Buccal (lower lip)		
Intact area	10.7 ± 1.6	8.3 ± 2.6
Lesion area	17.8 ± 5.2	11.3 ± 3.7
% Ratio lesion/intact	166	136
Tongue		
Intact area	15.8 ± 0.8	12.1 ± 2.5
Lesion area	–	21.3 ± 7.6
% Ratio lesion/intact		176

This *in vitro* study allowed a screening of lectin binding to lesions in these mucous membranes. In viable tissue, however, a cascade of events would occur to restrict the damage and initiate tissue repair, which are less likely to have occurred in this *ex vivo* wound model. Therefore, further work would need to consider lectin binding to wounds in viable tissues.

Although the majority of the lectins studied showed little differences in their binding to damaged and intact surfaces, significant differences were observed with the *D. violacea* and *M. amurensis* lectins, indicating potential for wound targeting. These should now be investigated further in a model in which the physiological responses to tissue damage are active. □

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Lipoprotein (a) in an immigrant Indian population sample in Australia

B. DEVANAPALLI, S. LEE, D. MAHAJAN and M. BERMINGHAM
School of Biomedical Sciences, University of Sydney, Australia

People from the Indian subcontinent (South Asia) show a very high incidence of coronary heart disease (CHD).[1] This is seen both in India and in countries to which Indians have migrated in large numbers, such as the UK, USA and Trinidad.[2] Large differences in disease rates are seen for people of Indian origin when compared with the host population: myocardial infarction (MI) occurs at a lower age and mortality from MI is ten times higher.[2]

In a large survey of Indian subjects in South Africa, important risk factors noted were hypercholesterolaemia, diabetes and smoking in men, and hypercholesterolaemia and smoking in women.[3] In another study, dyslipidaemia was higher in Indian physicians living in the USA, compared with US-born physicians.[4] However, an earlier study reported normal to low levels of total cholesterol in Asian Indians, and McKeigue *et al*.[1] believed the excess cardiac mortality observed in this population could not be fully explained by conventional risk factors.

Insulin resistance, leading to a cluster of metabolic factors, is thought to explain the early onset of CHD[5] in Asian Indians and this has been demonstrated in some Asian Indian groups.[6] However, while insulin resistance may be implicated in the early occurrence of risk factors and CHD in Indians, there is some evidence to suggest that other aspects of the lipid profile, such as the lipoprotein (a) (Lp[a]) level, influence risk in Indian heart patients.[7] One study found Lp(a) to be an independent risk factor for CHD in non-insulin-dependent diabetes mellitus (NIDDM) patients in South India.[8]

Berg first identified Lp(a) in 1963[9] as a cholesterol-rich lipoprotein similar to low-density lipoprotein cholesterol (LDL-C) in physical and chemical properties, with apolipoprotein B100 as its major protein constituent. Studies have identified increased levels of Lp(a) – usually regarded as >30 mg/dL – as an important independent risk factor for CHD.[10] A meta-analysis of 27 studies shows a clear association between Lp(a) and CHD.[11] Increased LDL-C, or increased Lp(a), increases the risk of CHD two-fold; but if the patient has both then the risk increases six-fold.[12] Most studies show that Lp(a) is largely under genetic control and is resistant to dietary or lipid lowering intervention,[13,14] other than treatment with niacin[15] or hormone replacement therapy.[16]

Levels of Lp(a) range from <0.1 mg/dL to >100 mg/dL[17] and are known to vary markedly with ethnicity.[18] Only limited studies have been carried out on Lp(a) levels in people of Indian origin, and include Indian groups in Singapore, the USA and in India itself. The values obtained range from means of 8.7 mg/dL and 9.2 mg/dL in the USA and India, respectively, to 20.1 mg/dL in Singapore and 34.1 mg/dL for one small group in the USA. Clearly, the distribution of Lp(a) in Indian population groups needs further investigation.

The aim of this study is to investigate the level and distribution of Lp(a) in an Indian immigrant population in Australia and to determine the stability of this Lp(a) after 12-months' storage at -80˚C.

The study sample consisted of 50 volunteers (25 men, 25 women; age range: 23-75 years) who had migrated to Australia from India over the past 20 years. Anthropometric measurements (weight, height, hip and waist circumference) were taken without shoes and over light clothing.

Following overnight fasting, venous blood samples were collected into EDTA tubes and centrifuged at 1500 rpm for 10 min. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides were analysed on a Reflotron reflectance photometric analyser (Boehringer Mannheim, Germany) within 2 h of collection. LDL-C was estimated using the Friedewald formula.[19] Immuno-turbidimetric analyses of apoprotein A1 (apo A1) and apoprotein B (apo B) were carried out on plasma using a Turbitime system (Behring Diagnostics, Australia).

Lp(a) was determined by a sandwich enzyme-linked immunosorbent assay (ELISA).[20] Nunc-Immuno microplates (Nalge Nunc International, Denmark) were coated with 150 mL sheep anti-human Lp(a) antiserum (7.6 µg/mL; Immuno AG, Vienna) in phosphate-buffered saline (PBS; pH 7.4) overnight at room temperature (RT) with gentle shaking. Unbound antiserum was tipped out and the wells washed (x4) with PBS containing 5 g/L Tween 20. All the free sites were blocked by treating the wells with 200 µL 5 g/L bovine serum albumin (BSA) in PBS for 1 h. The wells were then washed as before and then incubated for 2 h with controls and plasma samples (100 µL) diluted 1 in 10 000 with dilution buffer (PBS containing 5 g/L Tween 20 and 5 g/L BSA) and the appropriate standards. After 2 h the wells were washed as before and 100 µL rabbit anti-human Lp(a) serum (Dade Behring, Germany), diluted 1 in 1250 with dilution buffer, was added. The plates were incubated for 2 h at RT and then washed (x4). To each well was added 100 µL diluted (1 in 5000) horseradish peroxidase (HRPO)-conjugated goat anti-rabbit-IgG (Sigma-Aldrich, USA) and the plates were incubated for 2 h at RT.

After washing as before, the reaction was developed by adding 100 µL substrate (0.4 mg/mL OPD [Sigma-Aldrich, USA] in 0.05 mol/L phosphate citrate buffer [pH 5.0] containing 0.03% sodium perborate [Sigma-Aldrich, USA]) per well. The reaction was allowed to proceed in the dark for