Differential lectin binding to presumptive cortical cells of the wool follicle bulb

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Summary
An α-D-galactoside-specific lectin from *Bandeiraea simplicifolia* (BSLI) showed differential binding to cortical cells of the wool follicle bulb. The lectin bound to cells on one side only of the bulb and was completely blocked by α-D-galactose. The region of lectin binding extended from presumptive cortical cells at the base of the bulb to cortical cells at the top of the bulb, disappearing as cortical cells entered the fibre cortex. An orthocortex-specific monoclonal antibody was used to show that cortical cells recognised by the lectin lie directly below the fibre orthocortex and presumably give rise to the orthocortex. The results suggest that two distinct populations of presumptive cortical cells are present only two to three cell layers from the base of the bulb in a region where no morphological differences are detectable. The lectin-bound pre-cortical cells appear to give rise to orthocortical cells while cells not bound by lectin presumably give rise to paracortical cells.

Electron microscopy showed that the lectin bound to sites on the plasma membrane, probably on the extracellular surface. This suggests that the lectin target may be a membrane glycoprotein or glycolipid. Two polypeptides recognised by BSLI were separated from wool follicle extracts by SDS-gel electrophoresis. These polypeptides migrated at approximately 69 kDa and 17 kDa. However, only the 69 kDa molecule showed the expected loss of binding by BSLI in the presence of α-D-galactose. Further work will be required to determine if this glycoprotein is the bulb cell molecule recognised by BSLI. Dermal papilla cells were also bound by BSLI and may carry the same, or a similar, cell-surface molecule to presumptive orthocortical cells.

BSLI appears to be a useful marker for detecting patterns of cell differentiation in the bulb that correlate with patterns of cortical cell type in the wool fibre. The lectin may also be a useful tool for distinguishing cortical cells of different lineage in cell culture. Furthermore, the pattern of BSLI binding seen in small follicle bulbs appears to provide some support for a reaction-diffusion model of follicle differentiation.

Key words: wool follicle, cortical cell differentiation, *Bandeiraea simplicifolia* lectin, differentiation markers.

Introduction
The wool fibre originates in mitotically active cells in the lower half of the follicle bulb (Orwin, 1979). These cells surround the dermal papilla, a cluster of specialised fibroblast cells that are essential for fibre development (Oliver and Jahoda, 1989). As the basal bulb cells migrate up the bulb they differentiate into a number of cell types, including the orthocortical and paracortical cells, which eventually form the mature fibre cortex (Chapman and Gemmell, 1971a). These two types of cortical cell first appear above the dermal papilla approximately half way up the bulb. This is the region where keratin gene expression is first detected (Lynch et al. 1986; French and Hewish, 1986) and keratin microfibrils begin to accumulate (Chapman and Gemmell, 1971a). The orthocortical and paracortical cells at this level differ from each other in the content and arrangement of their microfibrils. Higher up the follicle in the emerging fibre cortex, the orthocortical cells can be distinguished by a monoclonal antibody produced against high-tyrosine type II proteins (Hewish and French, 1986). The final proportion of orthocortex and paracortex in the mature fibre varies between breeds of sheep and with nutritional status (Ahmad and Lang, 1957; Hynd, 1989). In general, fibres of smaller
diameter are segmented bilaterally into two discrete zones of orthocortex and paracortex while larger fibres can have a more complex pattern.

Although differentiation of orthocortical and paracortical cells in the upper half of the bulb is well documented, little is known of the events in the lower bulb that precede their appearance. Ultrastructural studies have shown that the precortical cells, which occupy most of the lower half of the bulb, are morphologically identical to each other (Chapman and Gemmell, 1971a). However, alkaline phosphatase (Lyne and Hollis, 1967) and nucleoside triphosphatase activities (Chapman and Gemmell, 1971b) both show an asymmetric distribution across the lower half of the bulb, suggesting differences within the precortical cell population that might be related to the later segmentation of the cortex.

In the present investigation we set out to identify cell-specific markers for early stages of cortical cell differentiation in the lower bulb. Glycoproteins were considered to be likely candidates for such markers. Different stages of cell differentiation are often associated with the appearance of new or altered cell-surface glycoproteins, which may vary in carbohydrate content as well as amino acid sequence (Feizi, 1985). Numerous studies have demonstrated the usefulness of lectins for locating cell-specific glycoproteins (e.g. see Skutelsky and Farquhar, 1976; Brabec et al. 1980; Lis and Sharon, 1986). We screened a range of lectins for evidence of differential lectin binding to pre-cortical cells. We report here that Bandeiraea simplicifolia lectin I (BSLI), which is specific for α-D-galactosyl residues (Hayes and Goldstein, 1974), distinguishes presumptive orthocortical cells close to the base of the bulb.

Materials and methods

All experiments involving animals were approved by the local animal ethics committee and were conducted in accordance with the Commonwealth and NSW Government codes of practice.

The sheep used in this study were Merino and Romney ewes, fed a daily ration of 1 kg of wheat chaff: lucerne (60:40, w/w) containing a supplement of cotton seed meal (17.5%, w/w). Bovine serum albumin (Amresco, Solon, Ohio, USA) was polymerised for 56 h at 50°C. Finally the blocks were embedded in Araldite resin, which was polymerised for 56 h at 50°C.

BSLI staining of skin sections

Sheep were anaesthetised with Xylocaine (Astra Laboratories, North Ryde, NSW) and skin samples were taken from the mid-flank using a 10 mm diameter trephine. Biopsies were fixed in Bouin’s fixative for 4 h, embedded in paraffin and serial 8 μm vertical or horizontal sections were cut (Adelson et al. 1990).

Rehydrated sections were blocked in TBS (10 mM Tris-HCl, 0.15 M NaCl, pH 7.5) containing 3 mg ml⁻¹ albumin for 1 h. BSLI was diluted immediately before use to a concentration of 4 μg ml⁻¹ in TBS containing 1 mg ml⁻¹ albumin. Blocked sections were incubated with the diluted lectin for 1 h, washed thoroughly in TBS and incubated for a further 1 h in avidin-alkaline phosphatase solution. The latter was diluted according to the manufacturer’s instructions, except that TBS containing 1 mg ml⁻¹ albumin was the diluent. The sections were washed again in TBS, followed by 0.1 M Tris-HCl, pH 9.5, and incubated with an alkaline phosphatase substrate in 0.1 M Tris-HCl, pH 9.5 for 1 h. The substrates used were Vector Black substrate (Vector Laboratories) or Histomark Red substrate (Kirkegaard and Perry, Inc; Gaithersburg, Maryland). Levamisole was included in the incubation according to the manufacturer’s instructions. After colour development, the sections were cleared in xylene and mounted in Depex. Sections were examined with a Zeiss Axiophot using bright-field or differential interference contrast optics. Counter-staining of sections was not necessary.

Control experiments for lectin binding included (i) omitting BSLI from the staining procedure and (ii) diluting BSLI in the presence of 0.5 M α-D-galactose.

Combined staining with BSLI and HiT96 antibody

Rehydrated skin sections were first treated with 1% (v:v) H₂O₂ in methanol for 30 min. Staining with BSLI was carried out using alkaline phosphatase and Histomark Red Substrate. After colour development, sections were washed in TBS and stained with HiT96 monoclonal antibody using a horseradish peroxidase detection system as previously described (Adelson et al. 1990). HiT96 culture supernatants were a kind gift from Dr Dean Hewish.

Gold-labelling studies

Small samples of sheep skin were fixed for 10 min in dilute fixative (1% paraformaldehyde/1.25% glutaraldehyde in 0.067 M sodium cacodylate, pH 7.4), then for 2 h in concentrated fixative (4% paraformaldehyde/5% glutaraldehyde in 0.067 M sodium cacodylate), followed by 2 h in 0.5 M NH₄Cl in 0.067 M sodium cacodylate. The tissue was then dehydrated in a graded ethanol series followed by propylene oxide. Finally the blocks were embedded in Araldite resin, which was polymerised for 56 h at 50°C.

Biotinylated lectin binding was visualised at the light microscope level on 1 μm Araldite sections using a silver-enhanced anti-biotin gold probe. The sections were etched in a saturated ethanol solution of NaOH diluted 1:1 (v/v) in absolute ethanol for 10 min, then rehydrated through a graded ethanol series. The sections were blocked for 1 h in TBS containing 1 mg ml⁻¹ albumin, then incubated for 1 h in 50 μg ml⁻¹ BSLI dissolved in the blocking buffer. After washing in TBS, the sections were blocked for 30 min in 1 mg ml⁻¹ cold-water fish gelatin (Aurlon) in TBS, then they were incubated for 1 h at 4°C in goat anti-biotin/15 nm gold (Biocell Research Laboratories) used at 1/20 dilution in 2% glutaraldehyde in TBS for 20 min. The sections were examined using the epi-polar illumination system of the Zeiss Axiophot microscope.

The pattern of biotinylated lectin binding at the ultrastructural level was investigated using the 15 nm anti-biotin gold probe on etched 80 nm Araldite sections. Sections were
graded ethanol series, dipped in a 2% solution of fresh 

The separated proteins were transferred to nitrocellulose 

Electrophoresis of follicle extracts was carried out on 12% 

follicles were cut off with a sharp blade and stored at -70°C. 

Solubilised wool follicle extracts 

Wool follicles were stripped from the shaved flank of a 

Gel electrophoresis and protein blotting 

Electrophoresis of follicle extracts was carried out on 12% 

Results 

BSLI binding to skin sections 

BSLI bound to every wool follicle examined. Vertical 

Wool cortical cell differentiation
began to appear. All follicles examined have consistently shown this labelling pattern.

**Gold labelling studies**

**Light microscope level**

In the follicle bulbs, an asymmetric distribution of binding was observed, similar to that seen with the Histomark Red system (Fig. 2). However, with the enhanced resolution of the silver-gold probe, it was now clear that the signal was concentrated on the plasma membranes of the bulb cells (Fig. 3A). It was necessary to etch the resin sections with ethanolic NaOH to obtain
Fig. 2. Double staining of wool follicles with BSLI and an orthocortex-specific antibody, HiT96. Sections were stained as described in Materials and methods. Red staining is due to binding of BSLI. Black staining is due to binding of HiT96 and serves to identify the orthocortical side of the wool follicle. (A) Vertical section showing that BSLI binds to the same side of the follicle as HiT96. Bar, 100 μm. (B) Higher magnification of A showing that binding of BSLI and HiT96 overlap in cells at the start of the fibre cortex. Arrows point to the edges of the fibre cortex. c, cortex; h, Henle's layer of inner root sheath; ors, outer root sheath. Bar, 50 μm.

Fig. 1. Binding of the lectin BSLI to wool follicles in fixed sections of adult sheep skin. Sections were incubated in the presence (A, B, C, D) of biotinylated BSLI. Lectin binding was visualised using an avidin-alkaline phosphatase detection system. (A) Vertical section through a Merino follicle; Nomarski optics. (B) Control in which BSLI was preincubated in 0.5 M α-D-galactose to compete for lectin binding, Nomarski optics. Note the black apparent staining in the left side of the follicle, this is due to the optical birefringence of the tissue in conjunction with the optics used and is not a staining effect. (C) Horizontal section through the bulb of a small Merino follicle; Nomarski. (D) Horizontal section through the bulb of a Romney follicle; Nomarski optics. Both horizontal sections were cut midway between the base of the bulb and the top of the dermal papilla. b, bulb; c, cortex; irs, inner root sheath cells; ors, outer root sheath cells; p, dermal papilla; pc, presumptive cortical cells. Bar, 50 μm.
Table 1. Location of binding sites for a range of different lectins in the wool follicle

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Localisation of binding to wool follicle</th>
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<tr>
<td>Bandeiraea simplicifolia II</td>
<td>Outer root sheath</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>Entire bulb and base of fibre</td>
</tr>
<tr>
<td>Dolichos biflorus agglutinin</td>
<td>None</td>
</tr>
<tr>
<td>Erythrina cristagalli lectin</td>
<td>Upper bulb</td>
</tr>
<tr>
<td>Jacalin</td>
<td>None</td>
</tr>
<tr>
<td>Lens culinaris agglutinin</td>
<td>Occasional dermal papilla</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>Upper bulb and base of fibre</td>
</tr>
<tr>
<td>Phaseolus vulgaris erythrotagglutinin</td>
<td>Entire bulb (not dermal papilla)</td>
</tr>
<tr>
<td>Pisum sativum agglutinin</td>
<td>Occasional dermal papilla</td>
</tr>
<tr>
<td>Ricinus communis agglutinin I</td>
<td>Entire bulb and base of fibre</td>
</tr>
<tr>
<td>Sophora japonica agglutinin</td>
<td>Upper bulb</td>
</tr>
<tr>
<td>Soybean agglutinin</td>
<td>Upper bulb and base of fibre</td>
</tr>
<tr>
<td>Ulex europaeus agglutinin I</td>
<td>Upper bulb and base of fibre</td>
</tr>
<tr>
<td>Vicia villosa lectin</td>
<td>Entire bulb and base of fibre</td>
</tr>
<tr>
<td>Wheat germ agglutinin</td>
<td>Upper bulb</td>
</tr>
<tr>
<td>Suceviletae wheat germ agglutinin</td>
<td>Upper bulb</td>
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Tissue sections cut vertically through sheep skin were fixed and stained as described in Materials and methods, except that BSLI was replaced by each of the above lectins. Each was tested over a concentration range of 0.4 to 40 μg ml⁻¹ and wool follicles in the sections were carefully examined for staining. The table summarises the location of binding by the different lectins.

BSLI binding to wool follicle proteins

Detergent-solubilised extracts of follicles were analysed by SDS-electrophoresis and protein blotting to look for proteins recognised by BSLI. The lectin bound to two polypeptide bands, as well as to high molecular mass material at the top of the gel (Fig. 4, lane 1). The larger polypeptide apparently migrated at 69 kDa in a single, slightly diffuse band. The smaller polypeptide migrated at approximately 17-18 kDa but frequently appeared as a doublet. Galactose abolished all the lectin-dependent staining of the 69 kDa band and of the higher molecular mass material (Fig. 4, lane 2) but had no effect on the 17 kDa band. The faint band still seen at 69 kDa in the presence of galactose was also seen in the complete absence of lectin (Fig. 4, lane 3), suggesting weak binding of avidin-alkaline phosphatase directly to groups migrating in this band. The protein profile of the solubilised extracts is included for comparison with the blots (Fig. 4', lane 4) and suggests that the proteins recognised by BSLI are minor components of the follicle extract.

Discussion

The division of the upper part of the wool follicle bulb and of the wool fibre into orthocortical and paracortical regions is well documented (Chapman and Gemmell, 1971a; Orwin, 1979). The observations described in this paper provide evidence that this division is also present in the lower half of the bulb, well before cortical cell differentiation becomes visible. Pre-cortical cells in one region of the bulb can be distinguished by the presence of cell-surface molecules recognised by BSLI. These pre-cortical cells lie beneath, and presumable give rise to, the cortical cells from which fibre orthocortex eventually develops. This is suggested, firstly, by binding of both BSLI and an orthocortex-specific antibody to the same side of the wool follicle and, in the neck of the bulb, to the same cells. Secondly, cells that bind BSLI occur on the concave side of curved bulbs, which in laterally segmented follicles, corresponds to the orthocortical side (Fraser, 1964). Thus, BSLI binding sites appear to be a feature of both presumptive orthocortical cells and orthocortical cells in the bulb but not of the orthocortical cells in the fibre cortex. Their presence is particularly useful in distinguishing presumptive orthocortical cells from presumptive paracortical cells. Using BSLI staining these two cell populations can be distinguished two to three cell layers from the base of the bulb (Fig. 1A) even though no morphological differences are evident (Chapman and Gemmell, 1971a). The cell surface groups recognised by BSLI apparently persist throughout the orthocortical cell differentiation in the bulb, but disappear from the cortical cells as they enter the fibre cortex (Figs 1A, 2B).

The silver-enhanced gold-labelling studies showed that the molecules recognised by BSLI are located on the plasma membranes. BSLI binding could only be detected after etching of the embedding resins, sugges-
Fig. 3. Patterns of lectin binding in the follicle bulb revealed by gold-labelling. (A) Light micrograph of lectin binding with silver-enhancement. Labelling in the bulb cells is concentrated on the plasma membranes. Bar, 50 μm. (B)-(E) Electron micrographs of gold-labelled lectin binding in the extracellular spaces between the follicle bulb cells. Bar, 1 μm.

(B) Control section without BSLI treatment, showing binding of the gold probe to extracellular material around the dermal papilla and between two bulb cells (arrows), and the absence of labelling in the extracellular spaces. (C)-(E) BSLI-treated sections showing the affinity of the gold probe for the cellular material on the edges of the extracellular spaces. p, apposed plasma membranes.

The observations reported here suggest that the two cell lineages that give rise to orthocortical and paracortical cells begin to differentiate at the base of the bulb from a common precursor cell. This supports and extends earlier suggestions that the asymmetric distribution of alkaline phosphatase (Lyne and Hollis, 1967) and nucleoside triphosphatase activities (Chapman and Gemmell, 1971b) in the bulb are related in some way to
cortical segmentation. Initiation of this very early phase of ortho/para cortical cell differentiation may occur as dividing cells at the base of the bulb encounter asymmetrically distributed gradients of growth factors or other morphogens. The zone of presumptive orthocortical cells recognised by BSLI extends outwards from the dermal papilla (Fig. 1C, D) and could be influenced by factors secreted from the papilla. The existence of such factors is suggested by implantation studies with whisker papillae (Oliver and Jahoda, 1989) and by cell culture experiments (Reynolds and Jahoda, 1991), although no specific factors have yet been identified. BSLI bound strongly to dermal papilla cells, suggesting that these cells may carry the same, or a similar, cell-surface molecule to presumptive orthocortical cells. It is tempting to speculate that this molecule is a cell-surface receptor for an autocrine/paracrine factor produced by the papilla cells. A cell-adhesion molecule involved in cell recognition is another possibility.

Nagorcka and Mooney (1989) have developed a reaction-diffusion model for wool follicle development that is based on the hypothesis that two interacting morphogens can form a stable “prepattern” in the bulb. Together with a third factor from the dermal papilla, these morphogens are proposed to specify patterns of mitosis and cell differentiation in the wool follicle. The results presented here provide some support for this model. The pattern of BSLI binding seen in small follicles (Fig. 1C) is very similar to that found for alkaline phosphatase activity (Lyne and Hollis, 1967), and both patterns are of the same type as that predicted by the reaction-diffusion model for small follicles. Further work is planned to carry out a comprehensive analysis of wool follicles of varying size to see if additional morphogen “prepatterns” predicted by the reaction-diffusion model (Nagorcka and Mooney, 1989) can be visualised as patterns of BSLI staining.

In summary, the process of ortho/para cortical cell differentiation clearly commences in cells close to the base of the follicle bulb. The presence of BSLI binding sites serves to distinguish the pattern of cortical cell differentiation at a very early stage. It is not known yet whether BSLI binding is necessarily indicative of pre-cortical cell commitment to the orthocortical lineage. However, it is a useful marker for detecting patterns of cell differentiation in the base of the bulb, which apparently correlate to patterns of cortical cell type in the wool fibre. In addition, this lectin may prove useful as a tool for distinguishing between cortical cells of different lineage in cell culture.

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References


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