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Histochemical studies of lectin binding patterns in keratinized lesions, including malignancy

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Summary. Histochemical detection of lectin binding was carried out using the HRP-conjugated lectin method in hyperkeratinized lesions including leukoplakia, *carcinoma in situ*, Paget's disease, keratoacanthoma, and condyloma acuminatum. The lectins used for demonstrating sugar residues were: Con A (hexose), PNA and RCA-1 (Gal), DBA and SBA (GalNAc), UEA-1 (Fuc), and WGA (GlcNAc).

Lectin binding in normal squamous epithelium showed regional distribution patterns of keratinized, spinous and basal layer types. Histochemical localization of lectin binding was generally at the cellular surface and in the intercellular substance and sometimes in the cytoplasm of normal epithelial cells. Dysplastic cells or carcinoma cell, in contrast, displayed a loss of cellular surface and intercellular staining. Paget's cells were devoid of lectin staining. In keratoacanthoma and condyloma specimens, spinous cells, which were PAS-positive, showed an intense PA/Con A-HRP staining and moderate binding by other lectins, which was somewhat decreased when compared with that in the surrounding intact epithelium. The cytochemical distribution of epithelial lectin binding might be indicative of the expression of normal stratification and keratinocytic differentiation, and the disappearance of this typical epithelial pattern may suggest severe dysplasia and malignancy.

Key words: Lectin – Oral leukoplakia – Paget's disease – Keratoacanthoma – Condyloma acuminatum

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Abbreviations: HRP: horseradish peroxidase; PBS: phosphate-buffered saline; DAB: diaminobenzidine; Con A: Concanavalin A; PNA: peanut lectin, *Arachis hypogaea*; RCA-1: Caster bean, *Ricinus communis*; DBA: Horse gram, *Dolichos biflorus*; SBA: Soybean, *Glycine max*; UEA-1: Gorse seed, *Ulex europeus*; WGA: Wheat germ, *Triticum vulgaris*; Gal: D-galactose; GalNAc: N-acetyl-D-galactosamine; Fuc: L-fucose; GlcNAc: N-acetyl-D-glucosamine

It is reported that keratinocytes in the skin and oral mucosa bind to lectins which are specific for certain sugar residues. Nieland (1973) first pointed out the distribution of epidermal carbohydrates using FITC-conjugated phytohaemagglutinins and Con A binding. Hashimoto et al. (1974) have also reported that the cell surface, substances in the intercellular space, basement membrane, and epidermal glycocalx all showed positive binding by the FITC-Con A method. Recently, Dabelsteen et al. (1978a and b) have reported that lectin binding sites of Con A and *Ricinus Communis* (RCA) in human oral mucosa are mainly located on the cell surface in normal epithelium and that RCA binding disappeared in mucosal carcinomas. Brabec et al. (1980) also demonstrated differential lectin binding on the newborn rat epithelial cells. Dabelsteen et al. (1982 and 1983) pointed out that blood group antigens A (Gal and GalNAc) and B (Gal and GlcNAc) were undetectable in premalignant and malignant lesions. These findings suggest that there are alterative expressions of the epithelium and of intercellular materials related to epithelial adhesion and the previous studies have defined these expressions in terms of blood group antigen distribution.

The present study reports lectin-binding reactions in epithelial structures of the following hyperkeratinized lesions of skin and oral mucosa: oral leukoplakia including *carcinoma in situ*, Paget's disease, keratoacanthoma, and condyloma acuminatum.

Materials and methods

Materials

A toatal of 31 lesions were examined consisting of 17 cases of oral leukoplakia, 3 cases of keratoacanthoma, 3 cases of Paget's disease, and 8 cases of condyloma acuminatum. Of the 17 cases of clinical leukoplakia, 4 of them satisfied the criteria of *carcinoma in situ*. The tumor specimens were fixed in 10% formalin for 12 h, and 4 μ paraffin sections (10 serial sections) were made.

Lectin histochemistry

1. Concanavalin A-HRP (Con A-HRP) method. The paradoxical Con A-HRP method by Katsuyama and Spicer (1978) was used, with a minor modification. Prior to following this method, the deparaffinized sections were treated with a 0.3% an H_2O_2/methanol solution for 20 min in order to inactivate endogenous peroxidase activity.

(1) Con A-HRP Method: The sections were rinsed with PBS for 15 min and then reacted with 0.1% Con A/PBS solution for 30 min at 20° C. They were rinsed 3 times in PBS, immersed in a 0.0005% HRP solution for 30 min, rinsed again 3 times in PBS, and immersed in 100 ml of 0.05 M Tris-buffer (pH 7.6) containing 30 mg of DAB and 0.3% H_2O_2 for 10 min.

(2) Periodic Acid Con A-HRP Method (PA/Con A-HRP Method): The sections were oxidized, prior to the use of the Con A-HRP method by a 1% periodic acid solution for 30 min at 20° C. (3) Periodic acid oxidation, reduction and Con A-HRP method (PA/Red/Con A-HRP Method): The sections were immersed for reduction in a 0.2% borohydrate ($NaBH_4$) solution for 2 min after the periodic acid oxidation step.

2. Peroxidase-conjugated lectin method. After inactivation of endogenous peroxidase in the tissue sections, the slides were rinsed with PBS and placed in 1% bovine albumin/PBS for 5 min and then rinsed with PBS. The sections were then reacted with one of the conjugated

lectin solutions listed below for 40 min at 20° C, rinsed with PBS for 10 min, and immersed in 0.05 M Tris-buffer (pH 7.6) containing 0.03% DAB and 0.3% H_2O_2. The following conjugated lectins were used:

(1) D-galactose-binding lectins: (Gal-binding lectin)
Arachis hypogaea (Peanut lectin) PNA
Ricinus communis (Caster bean) RCA-1
(2) N-acetyl-D-galactosamine-binding lectins: (GalNAc-binding lectin)
Dolichos biflorus (Horse gram) DBA
Glycine max (Soybean) SBA
(3) L-fucose-binding lectins: (Fuc-binding lectin)
Ulex europeus (Gorse seed) UEA-1
(4) N-acetyl-D-glucosamine-binding lectin: (GlcNAc-binding lectin)
Triticum vulgaris (Wheat germ agglutinin) WGA

The concentrations used were 50 μg/ml for SBA, DBA, RCA-1, and PNA conjugates, and 100 μg/ml for WGA and UEA-1 conjugates. These lectins were purchased from E.Y. Laboratories.

Results

1. Leukoplakia and carcinoma in situ of the oral epithelium

Histological findings in clinical leukoplakia in the oral mucosa were lesions including hyperorthokeratosis and hyperparakeratosis with or without acanthosis, and varying degrees of inflammatory infiltrates in the submucosal connective tissues. Some of the cases indicated an epithelial downgrowth into the submucosa. Dysplasia or dyskeratosis were displayed as the main alterations, indicating *carcinoma in situ.*

The Con A-HRP staining in leukoplakia was negative in the superficial hyperkeratinized epithelial zone, strongly positive in the granular cell layer and moderately positive in the spinous and basal cell layers. The PA/Con A-HRP staining of the lesion indicated a positive distribution in the upper to lower spinous cell layer and was weak in the basal layer. PA/Red/Con A-HRP staining was generally negative or very weak in the spinous cells. Basement membranes and sometimes the cytoplasm of spinous cells, bound Con A.

The staining pattern of lectin binding in hyperkeratinized epithelium of the oral mucosa, including leukoplakia, was characterized by expression of special sugar residues in regional distribution patterns in the epithelial layers. The hyperorthokeratinized zone was devoid of any lectin binding activities, and the hyperparakeratinized zone showed varying intensities of lectin binding; the binding of PNA, RCA-1, DBA, SBA, WGA, and UEA-1 conjugates was moderate to strong in the intercellular spaces and cellular membranes and occassionally indicated irregular granular materials. The spinous cell layer displayed intense to moderate staining by PNA, RCA-1, DBA, SBA, and WGA conjugates, the distribution of which was particularly evident in the cellular membranes and intercellular gaps. The basal cell layer was generally only slightly stained by the lectins, whereas basement membranes and associated collagen fibers showed marked staining.

Table 1. Lectin binding sites of leukoplakia in oral epithelium

Lectins	Tissue and cells								
	Normal epithelium				Leukoplakia				
	Basal cell	Spinous cell	Granular cell	Horny cell	Basal cell	Acanthotic cell	Parakeratotic cell	Orthokeratotic cell	Dysplastic cell
Con A									
Con A-HRP	+2	+2~3	+3	0	+2	+2~3	+3~4	0	Reduce
PA/Con A-HRP	+1	+3	+3	0	+1	+3	+3~4	0	Reduce
PA/Red/Con A-HRP	0~1	0~1	0~1	0	0~1	0~1	0~1	0	Reduce
PNA	+1	+2~3	+2~3	0	+1	+2~3	+2~3	0	Irregular
RCA-1	+1	+2~3	+2~3	0	+1	+2~3	+2~3	0	Reduce
DBA	0~1	+2~3	+2~3	0	0~1	+2~3	+2~3	0	Reduce
SBA	0~1	+2~3	+2~3	0	0~1	+2~3	+2~3	0	Irregular
UEA-1	0	+2~3	+2~3	0	0	+1~2	+2~3	0	Irregular
WGA	+1	+2~3	+2~3	0	+1	+1~2	+2~3	0	Redue

* The grade of lectin binding intensity is arbitrary divided into 0~+4; 0 negative, +1 slight, +2 moderate, +3 strong, +4 strongest

Lectin binding in dysplastic lesions and *carcinoma in situ* of the oral epithelium was characterized by loss of staining in intercellular gaps and cellular membranes and sometimes disappeared completely in dysplastic areas. Epithelial lesions in which malignant transformation had occurred, such as in squamous cell carcinoma, showed greater reduction in lectin binding activities (Fig. 1a–d, 2a–d and 3a–b). (see Table 1).

2. Paget's disease

Paget's disease of the nipple and extra-mammary Paget's disease of the vulva were histologically similar and showed interpapillary processes of the epidermis accompanied by pale and vacuolated cells (Paget's cell) (Fig. 4a and 5a). There were many inflammatory cells and higher than normal vascularity in the subepithelial connective tissue. Paget's cells were strongly positive for the PAS reaction.

The Con A-HRP staining in Paget's cells, as well as in intact epithelial cells, was weak. Plasma cells and infiltrating cells were markedly positive by the Con A-HRP method. The PA/Con A-HRP staining was, however, strong in Paget's cells, revealing fine granules. PA/Red/Con A-HRP staining was variably positive in some of the granules in the Paget's cells (Fig. 4b and 5b).

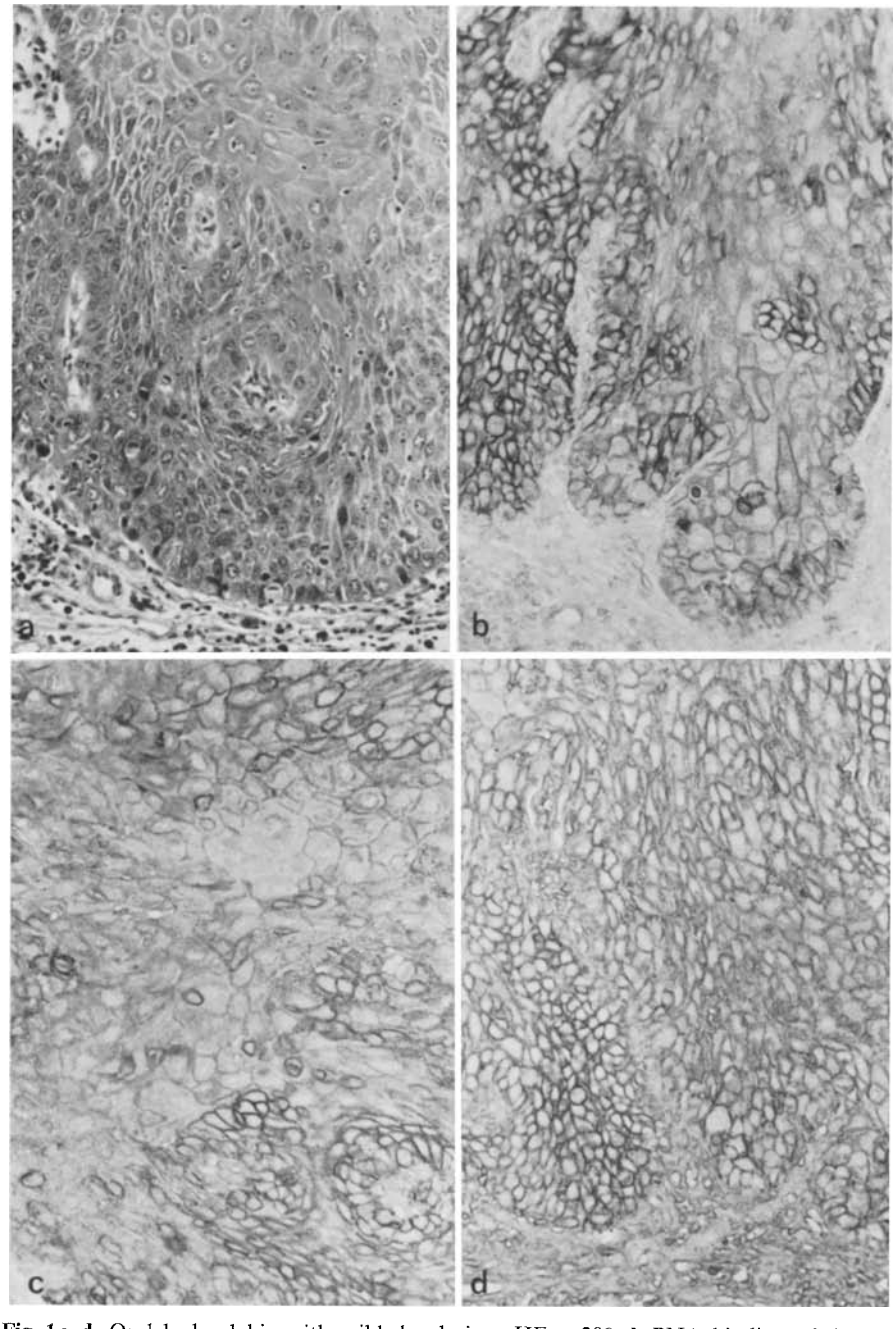

Fig. 1a–d. Oral leukoplakia with mild dysplasia **a** HE ×200. **b** PNA binding of dysplastic epithelia. PNA staining is regularly distributed in cellular surface and intercellular space of intact epithelial cells and irregularly present or almost absent in dysplastic or dyskeratotic cells. **c** SBA binding pattern is similar to that of PNA staining. **d** RCA-1 binding in leukoplakia with mild dysplasia is not so markedly changed

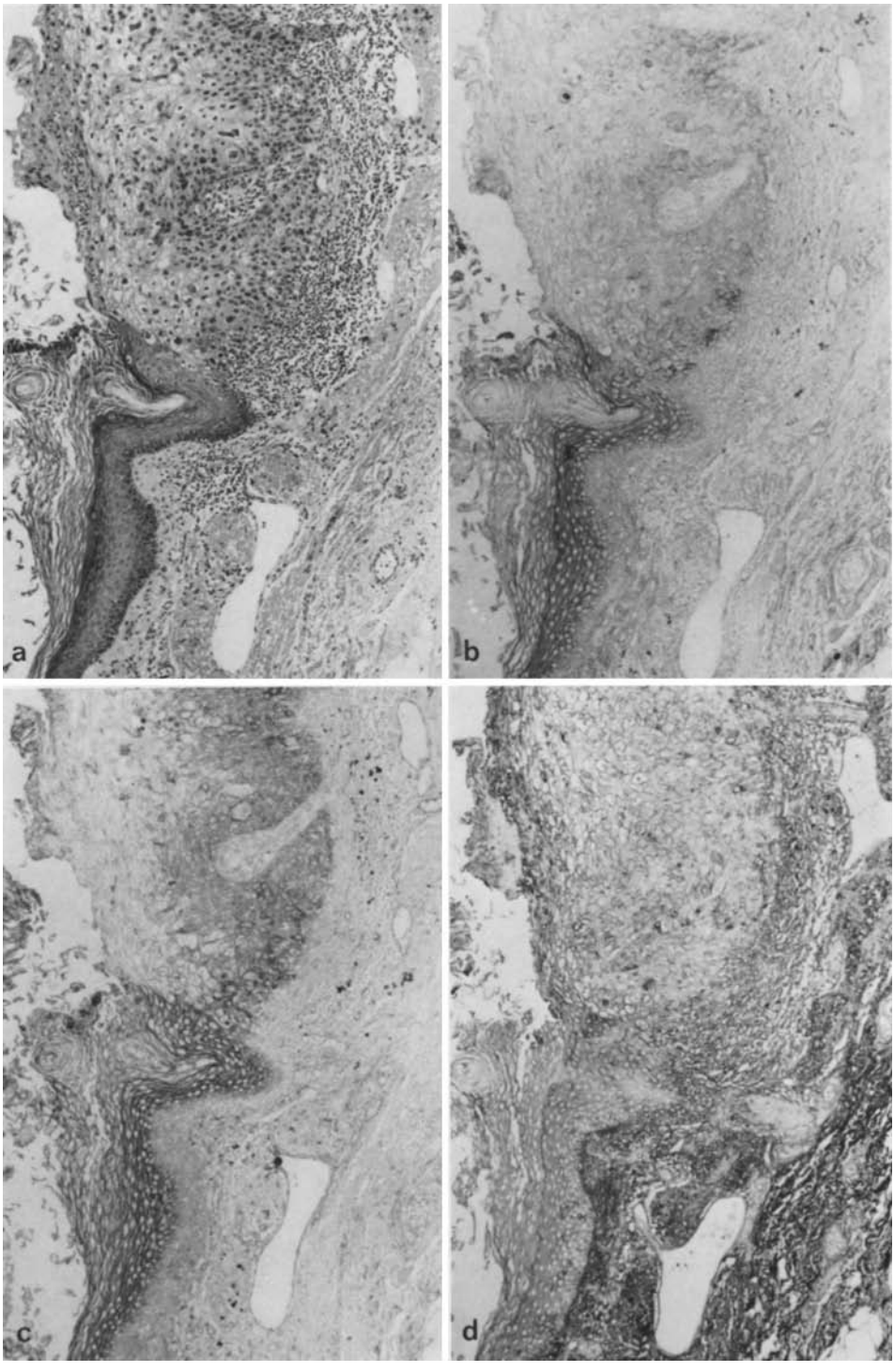

Fig. 2a–d. Squamous cell carcinoma and adjacent normal epithelia of lower lip mucosa (**a–d** $\times 40$). **a** Haematoxylin-eosin stain. The upper half reveals marked dysplastic epithelia with numerous infiltrating cells in submucosa. **b** PNA binding in normal squamous epithelium is markedly positive, in contrast, that in carcinoma cells is significantly reduced. **c** SBA binding in normal epithelia is mainly located in the spinous and basal layers, in contrast, that in squamous cell carcinoma is irregularly distributed. **d** RCA-1 staining as a regular distribution has also disappeared in neoplastic epithelial cells

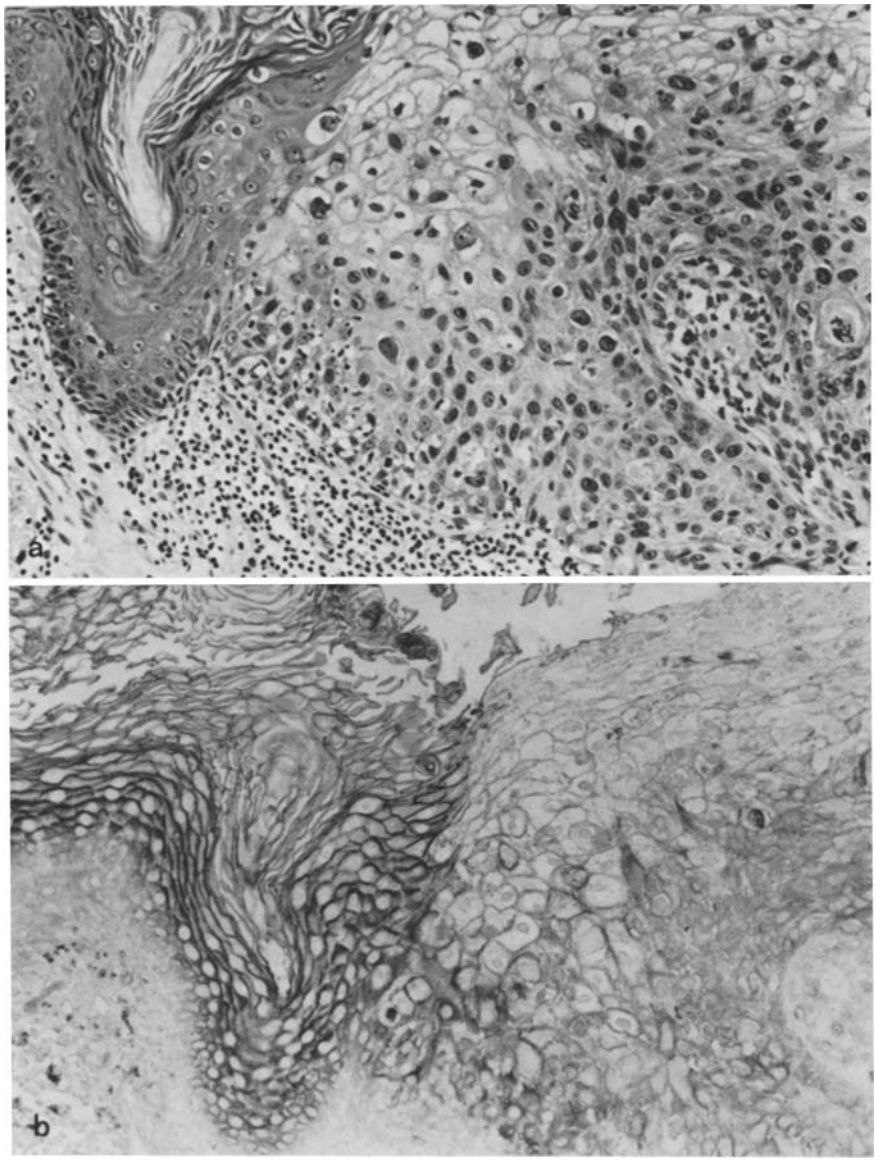

Fig. 3. a Haematoxylin-eosin staining of border area between normal mucosa and squamous-cell carcinoma (× 200). **b** Higher magnification of Fig. 2c, SBA staining in the normal epithelia is located in cellular surface and intercellular materials, in contrast, that in malignant epithelia is changed with decrease in density or dissappearance (× 200)

Lectin binding by Paget's cells was generally negative or weakly positive to PNA, RCA-1, DBA, SBA, UEA-1, and WGA conjugates, whereas that of normal epithelial cells was markedly positive (Fig. 4c, 4d, 5c and 5d). (see Table 2).

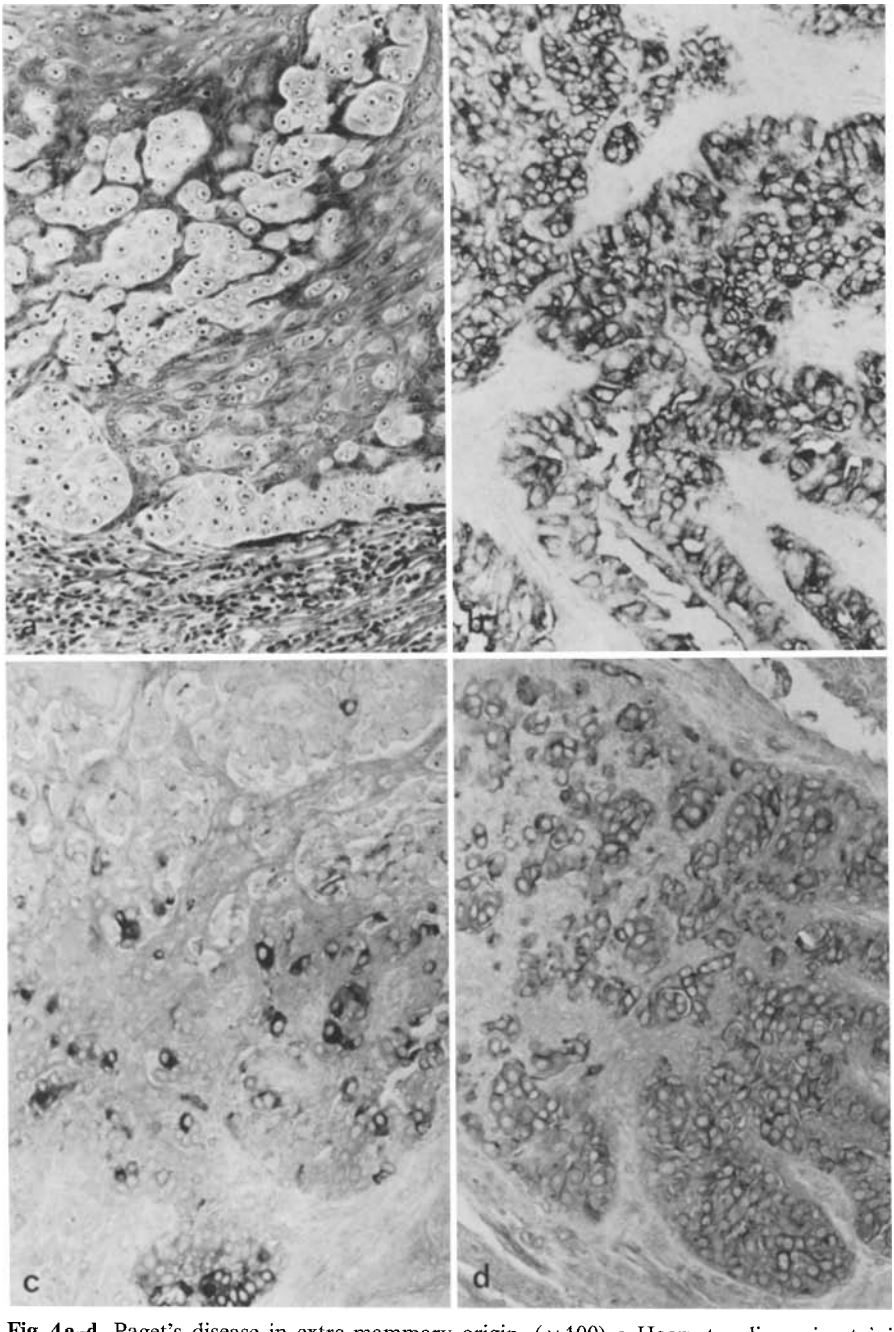

Fig. 4a–d. Paget's disease in extra-mammary origin. (×100) **a** Haematoxylin-eosin staining. There are numerous Paget's cells with pale cytoplasm (PAS positive). **b** PA/Con A-HRP staining is strongly positive in Paget's cells. **c** Lectin, PNA binding in the lesion shows strong positive scattered cells. Paget's cells are devoid of PNA staining. **d** DBA binding is localized in the epithelial cells of the Paget's disease

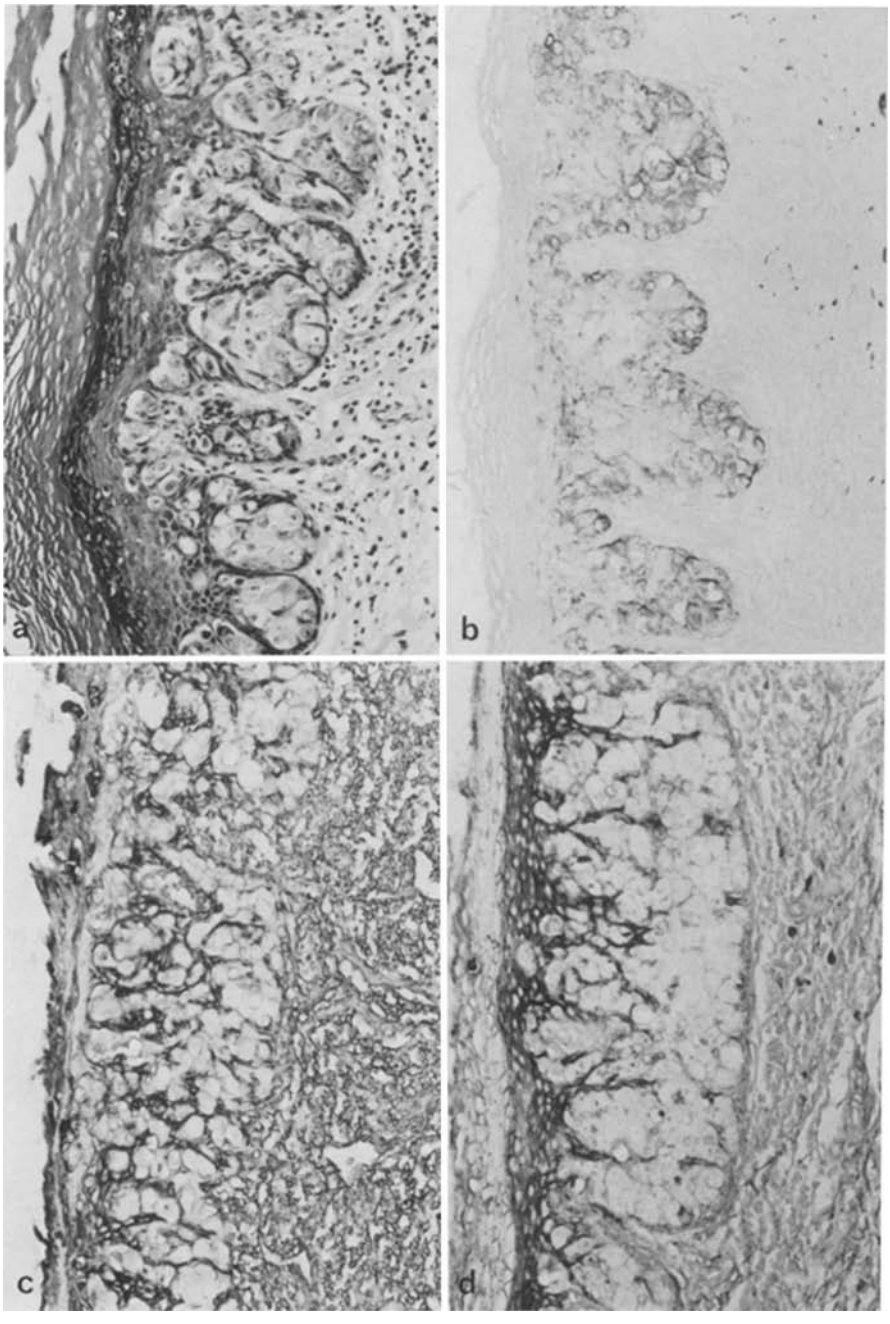

Fig. 5a–d. Paget's disease in nipple (×100). **a** Haematoxylin-eosin staining. The lesions composed of highly keratinized epithelia and Paget's cells in the lower layer. **b** PA/Con A-HRP staining is slightly positive in the Paget's cells. **c** RCA-1 binding is reactive in the squamous cells and negative in the Paget's cells. **d**, SBA binding is mainly distributed in squamous cells and almost negative in the lesion

Table 2. Lectin binding sites of Paget's disease

Lectins	Tissue and cells		
	Squamous cell	Hyperkeratinized cell	Paget's cell
Con A			
Con A-HRP	+2~3	0	0~1
PA/Con A-HRP	0~2	0	+2~3
PA/Red/Con A-HRP	0~1	0	0~1
PNA	+3	0	0~2
RCA-1	+3	0	0
DBA	+2~3	0	0~2
SBA	+2~3	0	0
UEA-1	+2~3	0	0~1
WGA	+2~3	0	0

* The grade of lectin binding stainabilities is arbitrary divided into 0~+4; 0 negative, +1 slight, +2 moderate, +3 strong, +4 strongest

3. Keratoacanthoma

The histological appearance of these lesions showed most intense keratinized zones invaginated of the epithelial foci or consisted of papillary proliferation as exophytic growths. The epithelial foci were well demarcated from the connective tissue stroma and had an intact basement membrane. The excessively keratinized lesion was composed of a hyperorthokeratinized zone as an upper layer and a well-developed spinous cell layer as the lower (Fig. 6a).

Con A-HRP staining was slightly positive, but sometimes negative, in the hyperkeratinized zone and moderately positive in the spinous cell layer (Fig. 6b). PA/Con A-HRP staining was negative in the hyperkeratinized zone; in contrast, it was abundant in the spinous cell layer and slight in the basal layer (Fig. 6c). The PA/Red/Con A-HRP method gave no staining in the hyperkeratinized zone, but resulted in conspicuously higher staining in the parakeratinized (granular cell layer) and upper spinous layers of the lesions.

Binding by PNA, RCA-1, UEA-1, and WGA conjugates was weak, but was moderate for DBA and SBA, in the hyperkeratinized areas. The upper spinous cell layer showed moderate binding to PNA, RCA-1, DBA, and SBA conjugates and the lower spinous or basal cell layer gave weak staining by DBA, SBA, UEA-1, and WGA conjugates (Fig. 6d). UEA-1 binding was positive in blood capillaries. (see Table 3)

4. Condyloma acuminatum

This lesion consisted of a parakeratotic superficial layer and was devoid of a completely keratinized layer. The spinous cells showed papillomatous growth with acanthosis and elongating rete pegs.

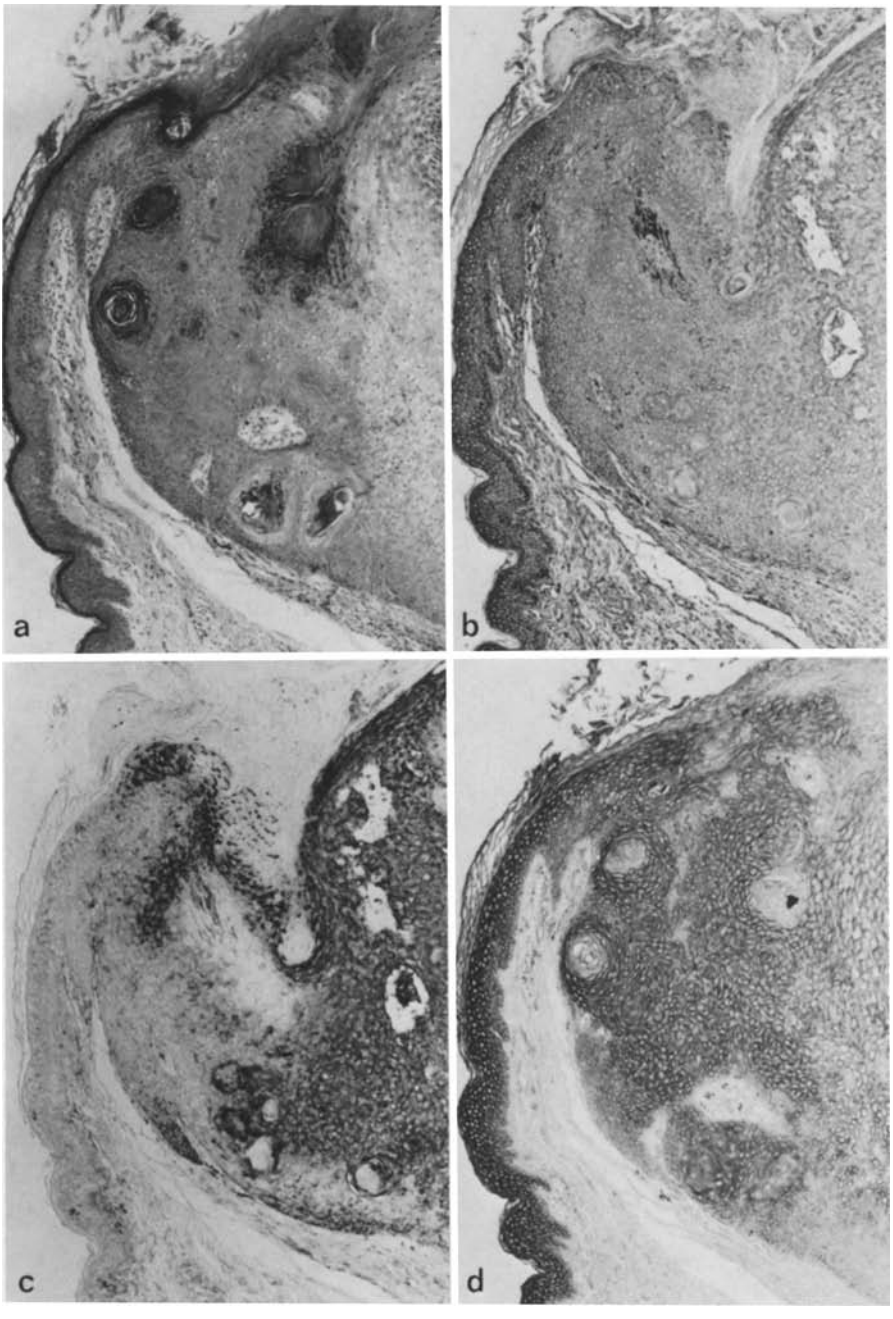

Fig. 6a–d. Keratoacanthoma (× 40). **a** Haematoxylin-eosin stain. **b** Con A-HRP staining is positive in the tumor epithelial elements and staining of surrounding intact squamous epithelia is little higher than tumor cells. **c** PA/Con A-HRP staining is characterized by strongly existence in the squamous-cells of neoplastic mass. **d** Lectin, PNA binding shows higher intensity in the surrounding normal epithelium and moderate in keratoacanthoma cells

Table 3. Lectin binding sites of keratoacanthoma and condyloma acuminatum

Lectins	Tissue and cells					
	Keratoacanthoma			Condyloma Acuminatum		
	Basal (Peripheral) cell	Spinous cell	Keratinized cell	Basal (Peripheral) cell	Spinous cell	Parakeratinized cell
Con A						
Con A-HRP	+2	+2	0~1	+2	+2~3	+2~3
PA/Con A-HRP	+1	+3~4	0~1	+1	+3~4	+3~4
PA/Red/Con A-HRP	0~1	0~1	0~1	0~1	0~1	0~1
PNA	+1	+2~3	0~1	+1	+3	+3
RCA-1	+1	+2~3	0~1	+1	+3	+3
DBA	0~1	+2~3	0~3	0~1	+3	0~2
SBA	0~1	+2~3	0~3	0~1	+3	+1~3
UEA-1	0~1	+2	0~1	0~1	+2~3	+2~3
WGA	+1	+2	0~1	+1	+2~3	+2~3

* The grade of lectin binding stainabilities is arbitrary divided into 0~+4; 0 negative, +1 slight, +2 moderate, +3 strong, +4 strongest

The Con A-HRP staining was slight to moderate in all the epithelial cells of this lesion, but was less intense in the basal layer than in the upper spinous layer. The PA/Con A-HRP staining was similar to that of Con A-HRP, being significantly higher in epithelial structures. The PA/Red/Con A-HRP staining resembled that of the PA/Con A-HRP technique. Almost all lectin binding was reduced in the epithelial component of the lesion when compared with the surrounding normal squamous epithelium which had a moderate or high degree of lectin binding. In the lesion, PNA, RCA-1, DBA, and SBA conjugates gave higher staining in the upper spinous layer, including the border zone between it and the parakeratinized layer, than in the lower spinous and basal layers. Binding activity of UEA-1 was positive in the blood capillaries in the peripheral stroma. (see Table 3)

Discussion

The stratified squamous epithelium of the skin and mucosa consists of different layers – the basal cells, spinous, granular, and keratinized layers – and these strata each have their own characteristic morphology from basal cells to keratinized cells. During keratinocytic stratification, enzymes and other biochemical components contained in the epithelial cells change in each layer. For example, polysaccharides are distributed in the spinous and granular cell layers, and the localization of various oxidative enzymes is restricted to specific layers. That is, the basal cell layer contains succinate dehydrogenase; and the superficial layer, glucose-6-dehydrogenase, however, lactate

dehydrogenase is found throughout all of the cell layers (Kawakatsu and Mori 1963; Ishihara et al. 1964; Mori et al. 1964). Recently, filamentous proteins, in the normal epithelial structure including keratin polypeptides, have displayed a regional distribution; i.e., keratins were strongly positive in the spinous, granular, and keratinized cells, in contrast, they were not detectable in basal and dyskeratotic cells, though staining for other types of keratin was slightly positive in the basal cells (Oberle et al. 1979; Sun et al. 1979; Lönig et al. 1980; Schlegel et al. 1980). This apparent distribution of cellular components in individual layers of the squamous cell epithelia is a sign of stratification during histodifferentiation and is the morphological expression of cell growth and maturation.

Recently, lectin binding activity has been well documented in mammalian tissues through the use of histochemical techniques. Several lectins have been reported to bind specific sugar residues in the cell coat, cytoskeleton, and intercellular materials. The lectin, Concanavalin A (Con A), is one of the powerful lectins and links specificially to hexose (glucose, mannose, fructose, and sorbose) in polysaccharides complexes and glycogen (Sharon 1975; Kennedy 1979; Debray et al. 1981). It has been pointed out that FITC-conjugated Con A binds to intercellular substances, cell surfaces, the basement membrane zone, and dermal collagen in normal skin and oral mucosa (Nieland 1973; Hashimoto et al. 1974). Dabelsteen et al. (1978a and b) have described that staining reactions of Con A and RCA conjugates in the oral epithelium occurred in cellular membranes, intercellular materials, and cytoplasm, with some differences. Brabec et al. (1980) also noted differential lectin binding in newborn rat epidermis. BSI-B_4 lectin (*Bandeiraea simplicifolia* isolectin B_4) mainly bound to basal cells; and Ulex lectin (*Ulex europaeus*), to the spinous cells; and BSII lectin (*B. simplicifolia* lectin II), to stratum corneum membranes. Dabelsteen et al. (1982 and 1983) have reported that blood group antigens in human oral epithelium were located in cellular membranes and that antigens A and B were missing in premalignant and malignant oral lesions. Those studies suggested that Gal and GalNAc (A-antigen), Gal and GlcNAc (B-antigen), and Fuc, Gal, and GlcNAc (H-antigen) were located on the epithelial cell surface. The immunogenicity of these blood type antigens is known to be associated with specific sugar residues on the blood cell surface. The present cytochemical experiment was done to determine if cell surfaces or intercellular materials in normal epithelium would interact specifically with certain lectins. The finding that blood group antigens A and B disappeared from cellular membranes in malignant epithelia (Dabelsteen 1982 and 1983) is similar to the present results in which decreased staining for PNA and RCA binding (Gal-linking) and DBA and SBA binding (GalNAc) in epithelial structures accompanied severe dysplasia or epithelia in which malignant change had occurred. The loss of staining reactions for PNA, RCA, DBA, and SBA in the intercellular material of dysplastic epithelia is an important feature of malignancy, because the malignant transformation of epithelial cells may affect the number of cellular aggregation processes, resulting in a significant decrease desmosomes and hemidesmosomes. The phenomenon of reduced lectin binding may be useful as a new diagnostic aid.

It has already been accepted that PAS-positive polysaccharides, (mainly glycogen) in epithelial cells are located in the spinous cell layer and not in the basal cell layer. This zonal distribution was also reflected by the staining pattern shown by the PA/Con A-HRP method. The distribution of binding sites for the PA/Con A-HRP staining in keratinized lesions was almost all in the spinous layer, including the upper spinous and its border zone with the granular cells. Paget's cells contain PAS-positive materials, and the present study has also shown this staining of Paget's cells to be coincident with PA/Con A-HRP staining. In the case of hyperkeratinized epithelial lesions, the spinous cell layer in keratoacanthoma cases displayed an intense staining by the PA/Con A-HRP method. Similar findings were found in condyloma acuminatum. The staining pattern observed by the PA/Con A-HRP method in epithelial lesions was also associated with PAS-positive material.

Lectin binding activities in the epithelium of keratoacanthoma and condyloma acuminatum specimens showed little reduced staining as compared to that of adjacent or surrounding intact epithelial cells. The distribution of lectin binding in keratinized tumors was similar to that of normal squamous epithelium. Lectin staining in the hyperkeratinized zone (keratoacanthoma) and in the parakeratinized zone (condyloma) were different; in general, the hyperkeratinized layer indicated moderate DBA and SBA binding and weak staining by PNA and RCA-1 conjugates, suggesting a higher concentration of GalNAc in hyperkeratinized cells. In contrast, the hyperparakeratinized layer, including the upper spinous cell layer, showed prominent staining by DBA and SBA reagents. The two lesions, keratoacanthoma and condyloma, are designated as having different types of keratinization, and their lectin-binding activities were also related to the degrees of stratification and to some extent to the level of differentiation of the epithelium. This regional relationship between epithelial structure and lectin binding may reflect the expression of epithelial stratification in mature epidermis.

It has been reported that epithelial antigens of the skin may be devided into two types – upper epidermal cytoplasmic (UEC) and general epidermal cytoplasmic (GEC) forms (Burnham 1974); and there are also two different types of epidermal cytoplasmic (CYT) antibodies – those reacting with the upper most layers of the epidermis (U-CYT) and those binding generally throughout the epidermis (G-CYT) (Abel and Bystryn 1976). Bystryn (1977), Bystryn et al. (1978) and Paluch & Bloch (1982) stated that three antigenic patterns existed in the normal epidermis. Also, U-CYT and BCL antigens were not expressed in malignant skin lesions. The anticytoplasmic antigens in those studies were obtained from patients with pemphigus vulgaris. They suggested that epithelial antigens were expressed during differentiation, with U-CYT and BCL antigens being cell-specific maturation antigens and G-CYT antigens tissue-specific antigens. The cytochemical distribution of epidermal antigens also resembled that of lectin binding in the present study, with regard to the cellular surface and intercellular spaces. The expression of these three types of epidermal antigens may be related to the specific patterns of lectin binding observed in this study.

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