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Lectin Histochemistry of Oral Premalignant and Malignant Lesions: Correlation of JFL and PNA Binding Pattern with Tumour Progression

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The expression of glycoconjugates specific to Jack fruit lectin (JFL) and peanut agglutinin (PNA) in various clinicopathological stages of tumour progression in the oral mucosa were studied. These included various clinical forms of dysplastic and non-dysplastic oral leucoplakias, carcinomas, normal keratinising (gingiva) and non-keratinising (buccal mucosa) epithelia. It was seen that the binding patterns of PNA and JFL in the epithelial cells of various types of oral lesions were more or less similar. Normal non-keratinising epithelium showed mild membrane staining only in the spinal layers, while normal keratinising epithelium showed a moderate membrane staining and mild cytoplasmic staining in all layers. Moderate membrane and mild cytoplasmic staining were observed in leucoplakias, irrespective of various clinical or histological types. In carcinomas, the intensity of lectin binding was high, particularly in the membrane of differentiated cells. Correlation analysis of the binding pattern of PNA and JFL showed significant correlation in the membrane and cytoplasm of all layers with histological stages of tumour progression. The present study thus showed that PNA and JFL may be used as cytochemical probes in differentiating malignancy from benign lesions of the oral mucosa.

Keywords: lectin histochemistry, JFL, PNA, oral leucoplakia, oral cancer

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INTRODUCTION

There is increasing evidence that the carbohydrate components of the cell membrane are associated with cellular differentiation, maturation and neoplastic transformation [1]. The biosynthesis of these membrane components involves a step by step build up with a specific glycosyl transferase catalysing the addition of monosaccharides to a growing chain. The resultant structural complexes along with a precursor–product relationship make carbohydrate antigens excellent markers for various cellular phenomena [1, 2]. A recent approach in the analysis of glycoconjugates of the cell surface is the application of lectins as probes [3]. The definition of this cytochemical reagent includes sugar binding capacity without enzymic activity together with activity in cell agglutination assays and/or precipitation of glycoconjugates [4]. Lectins were thus specified in terms of their ligands. High monosaccharide specificity of the lectins can be useful in recognising the fine differences in more complex structures of the cells.

Neoplastic transformation is accompanied by a variety of cell membrane changes, such as loss of adhesion, decreased contact inhibition, poikilocytosis, invasiveness, altered antigenic expression and escape from immunological surveillance [5], and is mainly due to the aberrant glycosylation in the membrane proteins [6]. This altered expression in the glycoconjugate composition also exhibits differences in lectin binding patterns from their normal counter parts [3, 7]. Many studies have used different types of lectins as cytochemical probes to differentiate normal, benign, preneoplastic and neoplastic lesions of a variety of organs, including the respiratory tract [8], breast [9], salivary glands [10], thyroid glands [11], colon [12], prostate [13] and uterine cervix [14].

Cancer of the oral cavity constitutes approximately 30% of all malignancies in India [15]. Tobacco chewing proved to be the major risk factor for buccal mucosal and gingival cancers. In the long incubation period between the beginning of carcinogenic tobacco habits and the development of invasive cancers, the oral epithelium first probably goes through a series of preneoplastic stages. The most common oral premalignant lesion is oral leucoplakia, which often has varying clinical features [15]. Clinically, oral leucoplakias can be classified into homogenous leucoplakia (simplex) and non-

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homogenous leucoplakias which include verrucous, speckled or nodular and erosive or erythroleucoplakia [16]. Histologically, leucoplakias were subdivided into dysplastic and non-dysplastic types, and have shown that those with dysplastic changes have higher malignant potential than non-dysplastic types [17]. Cytochemical studies have recently been attempted to define the characteristics of those leucoplakias which have a greater frequency to become malignant and various markers have been reported for assessing the malignant potency of the leucoplakias [18]. We have recently reported the variation in the AgNOR counts and scanning electron micoscopy of different types of oral leucoplakias and carcinomas [19, 20]. In the present study, we investigated the diagnostic potential of two common plant lectins, Jack fruit lectin (JFL) and peanut agglutinin (PNA) in different types of oral leucoplakias and carcinomas, both in terms of their clinical and histological terms.

MATERIALS AND METHODS

Lectin cytochemical studies were performed on a total of 167 formalin-fixed paraffin embedded oral biopsies. These included normal non-keratinising (buccal mucosa) and keratinising epithelia (gingiva), oral leucoplakias and oral carcinomas. The oral leucoplakias were categorised clinically [16] and subdivided histologically into dysplastic and non-dysplastic types [17]. Histopathological classification of the oral carcinomas was based on WHO criteria [21].

Horseradish peroxidase (HRP) conjugated PNA (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) and JFL were used in the present study. JFL was isolated, purified and conjugated as described elsewhere [22]. The lectin staining was employed as explained in our earlier studies [8]. For PNA binding the tissue sections were incubated with 1 U/ml neuraminidase from Vibrio cholerae (Boehring, Germany) for 30 min at 37°C.

Lectin binding in the cell membrane and cytoplasm was noted separately and graded as negative (−), mild (+), moderate (++) and intense (+++). The staining intensity was tabulated separately for each cell type as basal, lower spinal, upper spinal and corneal layers. In carcinomas, due to the loss of polarity and stratification, cells were grouped into basaloid, differentiated and well differentiated based on their differentiation.

Results were statistically analysed. The grades of lectin binding intensity were converted numerically as negative = 0, mild = 2, modcrate = 4 and intense = 6. Kruskal–Wallis one way ANOVA was used to see the significance of alterations in binding pattern of PNA and JFL between different histological groups. A Spearman's rank correlation analysis was also carried out to study the relationship between the lectin binding pattern and histological stage of tumour progression. For this, the cases were grouped as normal oral epithelia (stage 1), non-dysplastic leucoplakia (stage 2), dysplastic leucoplakia (stage 3) and carcinoma (stage 4). Results for all statistical analyses were only considered significant if $P < 0.05$.

RESULTS

The predominant lectin binding pattern of JFL and PNA in each clincopathological group was tabulated by layer/cell differentiation in Table 1. The staining pattern in the corneal layer of lesions was excluded due to their non-specific binding with lectins. In general, basement membrane and connective tissues showed a mild uniform staining in all types of lesions with both lectins. The staining pattern of PNA and JFL in the epithelial cells of various types of oral lesion were more or less similar. The normal non-keratinising epithelium (buccal mucosa) showed mild membrane staining with both lectins in the spinal layers only (Fig. 1). Whereas, normal keratinising epithelium (gingiva) showed a moderate membrane and mild cytoplasmic binding in spinal layers of the epithelium with

Table 1. JFL and PNA binding pattern in normal, premalignant and malignant epithelia of oral mucosa

	JFL						PNA					
	Basal/ basaloid		Lower spinal/ differentiated		Upper spinal/ well-differentiated		Basal/ basaloid		Lower spinal/ differentiated		Upper spinal/ well-differentiated	
Clinicopathological groups (*n*)	Mem	Cyto	Mem	Cyto	Mem	Cyto	Mem	Cyto	Mem	Cyto	Mem	Cyto
Normal non-keratinising (9)	−	−	+	−	+	−	−	−	+	−	+	−
Normal keratininising (5)	++	+	++	+	++	+	+	+	++	+	++	+
ND homogenous LKP (11)	−	−	+	+	+	+	+	−	+	−	+	−
ND verrucous LKP (5)	+	−	++	+	+	−	+	−	++	+	+	−
ND speckled LKP (4)	+	−	++	+	+	−	+	−	++	+	+	−
ND erosive LKP (5)	+	−	++	+	+	+	+	+	++	+	+	+
D homogenous LKP (14)	+	−	++	+	+	−	+	−	++	+	+	−
D verrucous LKP (5)	+	+	++	+	+	−	+	−	++	+	−	−
D speckled LKP (5)	+	−	++	−	+	−	+	+	+	+	++	+
D erosive LKP (6)	+	−	++	+	++	+	++	+	++	+	++	+
WDSCC (48)	++	+	++	++	+++	++	+	+	++	++	+++	++
MDSCC (30)	+	+	++	++	+++	++	+	+	++	+	+++	++
PDSCC (3)	+	+	+++	+	NP	NP	+	+	+++	++	NP	NP
Verrucous carcinoma (11)	+	+	+	+	+	+	+	−	+	+	+	+
Spindle cell carcinoma (6)	−	−	NP	NP	NP	NP	−	−	NP	NP	NP	NP

ND = non-dysplastic; D = dysplastic; LKP = leucoplakia; WDSCC = well-differentiated squamous cell carcinoma; MDSCC = moderately differentiated squamous cell carcinoma; PDSCC = poorly-differentiated squamous cell carcinoma; Mem = membrane; Cyto = cytoplasm, − = negative; + = mild; ++ = moderate; +++ = intense; NP = not present.

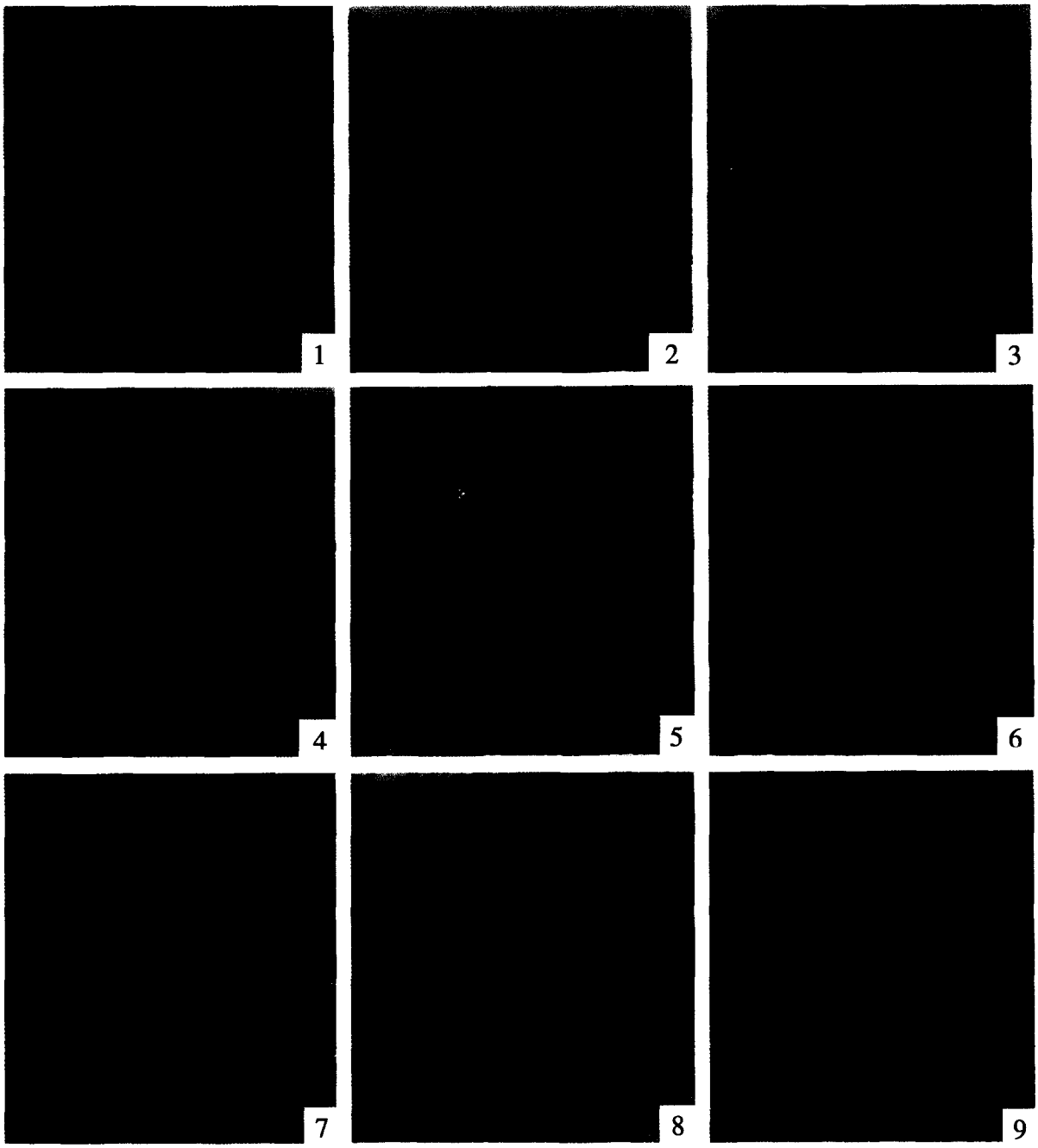

Fig. 1. Normal non-keratinising epithelium (buccal mucosa) shows mild membrane staining (arrow) in upper spinal cells with JFL (100 ×).

Fig. 2. Normal keratinising epithelium (Gingiva) shows moderate membrane and mild cytoplasmic staining in spinal cells with PNA (100 ×).

Fig. 3. Non-dysplastic erosive leucoplakia shows mild membrane staining in basal cells and moderate membrane staining in lower spinal cells with JFL (100 ×).

Fig. 4. Dysplastic erosive leucoplakia shows moderate membrane and mild cytoplasmic staining in spinal cells with PNA (100 ×).

Fig. 5. Poorly-differentiated squamous cell carcinoma shows moderate membrane and cytoplasmic staining in the differentiated cells with JFL (250 ×).

Fig. 6. Moderately-differentiated squamous cell carcinoma shows moderate membrane and mild cytoplasmic staining in the differentiated cells with JFL (250 ×).

Fig. 7. Well-differentiated squamous cell carcinoma shows intense membrane and moderate cytoplasmic staining in well-differentiated cells with JFL (250 ×).

Fig. 8. Verrucous carcinoma shows mild membrane staining in differentiated cells with PNA (100 ×).

Fig. 9. Spindle cell carcinoma shows no staining with PNA (250 ×).

Table 2. Statistical analysis of the relationship between the JFL and PNA binding pattern and stage of tumour progression in oral mucosa

		JFL				PNA			
		Kruskal–Wallis one way ANOVA		Spearman's rank correlation		Kruskal–Wallis one way ANOVA		Spearman's rank correlation	
Cell layers		Chi square	*P* value	Coefficient	*P* value	Chi square	*P* value	Coefficient	*P* value
Basal/basaloid	Cyto	23.3181	0.0000*	0.4070	0.000*	6.3617	0.0953	0.1972	0.018*
	Mem	30.4568	0.0000*	0.4177	0.000*	26.1320	0.0000*	0.4284	0.000*
Lower spinal/ differentiated	Cyto	24.4660	0.0000*	0.3738	0.000*	30.9637	0.0000*	0.4646	0.000*
	Mem	26.3588	0.0000*	0.3958	0.000*	21.3988	0.0000*	0.3776	0.000*
Upper spinal/ well-differentiated	Cyto	65.2728	0.0000*	0.5994	0.000*	49.5805	0.0000*	0.5752	0.000*
	Mem	67.2713	0.0000*	0.6048	0.000*	54.8583	0.0000*	0.5986	0.000*

* = Significant.

PNA and JFL (Fig. 2). The dysplastic and non-dysplastic epithelium did not exhibit much difference in binding by both JFL and PNA. However, in dysplastic epithelium, comparatively more spinal cells showed lectin binding than in non-dysplastic lesions (Figs 3 and 4). In carcinomas, intensity of binding was high with both lectins, particularly in the membrane of differentiated cells, irrespective of grade of differentiation (Figs 5–7). In squamous cell carcinoma, variants of the oral mucosa, verrucous carcinoma showed mild staining in differentiated cells (Fig. 8) and spindle cell carcinoma showed almost negative binding with both lectins (Fig. 9).

The results of the statistical analysis are shown in Table 2. Kruskal–Wallis one way analysis of variance showed significant *P* values between various clinicopathological groups in all staining grades for both the membrane and cytoplasm, except the PNA staining pattern in the cytoplasm of the basal cells. While comparing the binding pattern between individual groups by the pairwise multiple comparison test, only carcinomas, except the verrucous type, showed significant differences in the lectin binding pattern from various leucoplakias and normal mucosa, both in histological and clinicopathological grouping. However, the correlation analysis showed a significant correlation between JFL and PNA binding in the membrane and cytoplasm of all layers with histological stages of tumour progression.

DISCUSSION

The basic biology of initiation and progression of many of the cancers is still incompletely understood. Most of the invasive oral carcinomas are preceded by a precancerous non-invasive stage that may last for years. The progression of squamous carcinoma has been broadly classified into normal hyperplastic (non-dysplastic), dysplastic, carcinoma *in situ* and invasive carcinoma [23]. Preceding these cytological changes, aberrations will occur in the genetic as well as epigenetic organisation of the cells. These aberrations are quantifiable and often show marked differences between neoplastic and preneoplastic tissues [24]. The oral keratinocytes follow a well-defined pathway of differentiation from the basal progenitor region to the flattened, maturated surface layers of the stratified epithelium [1]. A wide range of antigenically distinct glycoconjugates, including glycolipids and glycoproteins, are present at the surface of the epithelial cells and showed variations in expression of these glycoconjugates during differentiation and maturation of the keratinocytes [25]. Changes in these surface components in preneoplastic and neoplastic epithelia appear to be particularly interesting in terms of their potential value in diagnosis [25, 26]. In this regards, lectin can play an important role. Lectins provide a range of readily available and well-defined potential reagents for the identification of various cellular phenotypes. The lectins employed in this study, PNA and JFL, are specific to galactose and *N*-acetyl-D-galactosamine, respectively. Thus, the present study analysed the expression pattern of galactosyl and *N*-acetyl-D-galactosamine residues in different clinicopathological stages of tumour progression of the oral mucosa.

The difference in expression of glycoconjugate between normal keratinising and non-keratinising types may be due to differences in the differentiation pathway of keratinocytes in these two types of epithelia [27]. Studies on normal epithelia with Con A and PNA also reported the differences in binding pattern between non-keratinised and keratinised cells [28]. The lectin binding pattern showed a relationship with the grade of differentiation of the cells. Differentiated cells showed a higher intensity in binding than the basaloid cells. An *in vivo* and *in vitro* study on lectin binding in keratinocytes showed that the lectin binding patterns of oral and skin keratinocytes are dependent on the functional status and degree of differentiation of the cells [29]. In various clinical types of dysplastic and non-dysplastic leucoplakias, PNA and JFL did not show much difference in binding pattern. Whereas, in carcinomas, both lectins showed denser binding than normal, non-dysplastic or dysplastic leucoplakias. Many studies using various lectins showed marked variations in the expression of glycoconjugates in neoplastic cells [3]. This study shows an increase in the expression of galactosyl and *N*-acetyl-D-galactosamine residues in the membrane and cytoplasm of malignant cells. The difference in binding may be due to the fact that JFL may be able to recognize incomplete non-sialated forms of membrane glycoconjugates which may be expressed at the surface of neoplastic cells, as reported earlier by Ross *et al.* [30]. Previous studies with JFL on premalignant and malignant lesions of the uterine cervix [14] and respiratory tract [8] also found intense binding in the membrane of the squamous cell carcinoma.

The histological variants of squamous cell carcinoma show a distinct lectin binding pattern from that of squamous cell

carcinoma. Histologically, verrucous carcinoma were considered to be a well-differentiated lesion, but this neoplasm showed comparatively less binding than well-differentiated squamous cell carcinoma. Toto *et al.* [31] reported that verrucous carcinoma showed a very close resemblance to the binding pattern of normal epithelium and benign epithelial hyperplasia with biotinylated PNA. In spindle cell carcinoma, a totally negative binding was observed with both lectins. This may be due to the undifferentiated status of the cells as seen in basal/basaloid cells of the leucoplakias and carcinomas studied. Our earlier studies have also demonstrated similar findings with JFL in large cell anaplastic carcinoma and oat cell carcinoma of the respiratory tract [8].

A few studies have only been reported on the lectin binding pattern in oral mucosal lesions [28, 32–34]. Such studies with PNA and JFL in the oral mucosa are limited. Toto *et al.* [31] studied the binding pattern of seven lectins in squamous cell carcinoma and suggested its significance as a marker for invasive potential. Vigneswaran *et al.* [33] compared the binding pattern of five lectins in normal mucosa, leucoplakias with or without dysplasia, papillomas and carcinoma. Among the five lectins, PNA showed binding in the suprabasal cells of keratinocytes of normal oral epithelium. While a decrease in staining intensity was observed in dysplastic leucoplakias and carcinomas in contrast to non-dysplastic lesions. A similar study by Saku and Okabe [32] on the oral mucosa reported the result to be just the reverse. They observed an increase in binding of PNA corresponding to the degree of atypia. Our results showed a significant difference in lectin binding between normal and malignant cells. However, most of the preneoplastic lesions, including non-dysplastic (hyperplastic) and dysplastic epithelia, did not show a significant difference from that of their normal controls. Statistical analysis also showed similar observations. Even though the correlation analysis showed a significant coefficient between the binding pattern of these lectins with tumour progression, univariate analysis did not see any significant binding variation between the normal and non-dysplastic or dysplastic leucoplakias. Comparing the binding pattern of PNA and JFL in various histological lesions did not find any prominent differences. The study with frozen sections also did not find significant differences in PNA binding pattern between various histological grades of premalignant lesions of the oral mucosa [28]. Thus, the present study shows that PNA and JFL have values in differentiating neoplastic changes from benign lesions of the oral cavity, but have little support in predicting the biological behaviour of potentially malignant oral lesions.

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