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ORAL MICROBIOLOGY
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Differential expression of Toll-like receptors in chronic hyperplastic candidosis

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Introduction: The first step in the host defense against oral candidosis is the recognition of *Candida albicans* through a set of germ-encoded pathogen recognition receptors, e.g. Toll-like receptors (TLRs). In man, 10 types of such receptors have been identified so far, of which only TLR2, TLR4, and TLR6 have been linked to mediating candidal ligands, e.g. zymosan.
Methods: Biopsies from patients with chronic hyperplastic candidosis (n = 5), leukoplakia (n = 5), and healthy mucosa (n = 5) were immunohistochemically stained with antibodies to the TLRs (TLR1 to TLR9) to distinguish and compare the staining patterns of the epithelial layer in the three categories of tissues.
Results: On analysis, the epithelium of all tissues was divided into three layers: basal, middle, and superficial. Two of the five chronic hyperplastic candidosis sections showed high numbers of hyphae compared to yeasts, which paralleled a decrease in the expression of TLR2 and an increase in the staining intensity of TLR4. Leukoplakia and healthy tissue sections demonstrated stronger immunostaining of TLRs, except TLR9 which showed weaker staining in some sections of the former, and in the basal layers of some sections of the latter.
Discussion: This study supports the concept of negative regulation of TLRs that are either ligand-bound (e.g. in chronic hyperplastic candidosis), or not stimulated (in healthy tissue). It also augments the opinion that *C. albicans*, through its hyphae rather than blastospore, may utilize TLRs, i.e. TLR2, to evade the immune system of the host. Leukoplakia seems to be more immunologically alert, which reduces the chances of worsening the already-diseased tissue.

Key words: *Candida albicans;* innate immunity; leukoplakia; Toll-like receptors; zymosan

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As a constituent of the normal human microflora, the fungus *Candida albicans* is harbored in the mucous-lined body organs, e.g. oropharynx, gastrointestinal, and genitourinary tracts in about 40–60% of population (43). Although non-albicans *Candida* species are emerging as significant pathogens (e.g. *C. krusei*) (20), *C. albicans* still occupies the top of the list for causing all forms of candidosis, e.g. around half of all cases of blood-borne candidal infections (candidaemia) are caused by *C. albicans* (7, 26). Under normal conditions, *C. albicans* thrives peacefully inside its accommodating host in a commensal relationship that continues undisturbed as long as the immune status of the host remains intact. However, once the host (i.e. a human being) is afflicted with any immunocompromising condition, e.g. diabetes mellitus, neutropenia, acquired immunodeficiency syndrome, it will become more liable to candidosis (32). In addition, other factors that lead to a disturbance of the local environment of the oral cavity can predispose the individual to candidosis, e.g. broad-spectrum antibiotics. The scale of candidosis may range from a simple superficial lesion to deep-seated, life-threatening hematogenous infections. The fact that *C. albicans* can cause disease mainly in patients whose immunity is weak has led to candidosis being given the epithet 'disease of the diseased' (8).

In the oral cavity, *C. albicans* expresses some features that contribute to the development of oral candidosis

when optimum conditions are met. Such features include the morphological transition of yeast to hypha, its adherence capacity to the oral cells, as well as its ability to secrete proteases to degrade the extracellular substance of the oral mucosa (33, 35, 41).

Oral candidosis can exist in many forms with distinct clinical as well as histopathological pictures. Many classifications have been suggested for oral candidosis but the one adopted by Axell et al. (2) is among the best known. Oral candidosis was classified into four major classes: namely, acute erythematous, acute pseudomembranous, chronic atrophic, and chronic hyperplastic (19). Chronic hyperplastic candidosis represents a chronic opportunistic candidal infection of the oral cavity characterized by host tissue hyperplasia and associated with the development of oral cancer, e.g. squamous cell carcinoma (3, 21, 42).

To invade a tissue, the pathogen has first to establish adherence to the uppermost layer of the tissue. Therefore, it is logical to assume that the innate immune system of the host should be equipped with a germline-encoded class of receptors to detect the presence of those unwanted intruders. Such receptors, which were then called Toll, were discovered first in *Drosophila melanogaster*, where their main functions are to orient the dorsoventral polarization of the organism during embryonic development and also to impart resistance against fungi and gram-positive bacteria (14). Investigators extended their area of research and found that a similar set of receptors exists in mammals (including humans); these were given the name Toll-like receptors (TLRs). The TLRs represent a major class of transmembrane protein receptors mainly involved in recognition of a wide variety of microbial pathogens, including *C. albicans*. TLRs are expressed by many types of tissue cells, especially those that constitute physical barriers (such as epithelia of the skin and mucosa) against entrance of the floating (planktonic) as well as attached microfloral organisms. As they belong to the family of pattern recognition receptors, TLRs can recognize molecules that are broadly shared by pathogens but are distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). Ten types of TLRs have been characterized (TLR1 to TLR10) in man, each of which is devoted to a certain class of PAMPs, e.g. TLR2 recognizes bacterial lipoprotein, peptidoglycans, and leipoteichoic acid, TLR3 recognizes double-stranded RNA, TLR4 recognizes bacterial lipopolysaccharide and so on (39). They are believed to play a key role in eliciting the immune response against microbial infection.

Upon adhering to the host surface, some of the candidal cell wall components, i.e. zymosan, may bind to more than one TLR. This binding, in turn, ignites the host to deploy both non-specific and specific immune defense. Examples of non-specific defense measures involve resident epithelial cells, neutrophils, and mast cells, as well as the antigen-presenting dendritic cells. Specific immune cells comprise mainly the lymphocyte–plasma cell system. All the processes of communication between immune cells (which yield their recruitment and trafficking) are orchestrated through signaling interactions between these cells; these involve a group of chemicals known as cytokines and chemokines. The molecular details of the signaling pathways which are involved in TLR–ligand binding should be sought from other excellent reviews (36, 38).

The aim of this work was to reveal, immunohistochemically, which TLRs could be involved in the immunodetection of *C. albicans*. Second, whether there were any differences in the immunostaining patterns of the TLRs (TLR1–9) in a group of sections from patients with chronic hyperplastic candidosis. The third aim was to compare the staining response (in terms of intensity and number of positive cells) of TLRs in chronic hyperplastic candidosis with sections from patients with leukoplakia and healthy tissue controls.

Materials and methods

Samples

The local ethical committee approved the study protocol. Biopsy samples of oral mucosal lesions were obtained from patients with chronic hyperplastic candidosis who were undergoing examination of mucosal lesions; while biopsies of *Candida*-negative leukoplakia were used as *Candida*-negative hyperplastic mucosal controls (Table 1). Chronic hyperplastic candidosis was defined as an invasive candidosis characterized by hyphal ingrowth into the oral epithelium leading to the formation of well-demarcated, palpable, and raised homogeneous or nodular lesions of the mucosal membrane varying from small translucent whitish areas to large opaque plaques. Leukoplakia, on the other hand, is defined as a white lesion of the oral mucosa that cannot be attributed clinically or histologically as any other known lesion. Histologically, the homogeneous lesions were characterized by hyperorthokeratinization or hyperparakeratinization, whereas the nodular lesions displayed a varying thickness of the hyperplastic surface epithelium, which was always characterized by parakeratosis and occasionally by hyperkeratosis or slight dysplasia. Chronic inflammatory cell infiltrates were found in the lamina propria.

Patients included in this study represented five consecutive patients in whom

Table 1. Clinical and demographic data of the patients with chronic hyperplastic candidosis, leukoplakia, and healthy controls

Number	Gender	Age	Location of the lesion	Clinical presentation	Hyphae (disclosed by PAS)
1 CHC	F	81	Cheek	Papular	+
2 CHC	M	44	Tongue	Ulcerative	+++
3 CHC	F	45	Tongue	Erythroplakia	+
4 CHC	F	55	Cheek/Commissures	Hyperplastic	+
5 CHC	F	53	Tongue	Nodular, indurated	+++
1 LP	F	55	Tongue	White plaques	−
2 LP	F	66	Palate	Striated	−
3 LP	F	50	Alveolar ridge	Exophytic	−
4 LP	F	48	Cheek	Striated	−
5 LP	F	64	Floor of the mouth	Homogeneous	−
			Location of the sample		−
1 HC	F	56	Upper left sulcus mucosa		−
2 HC	F	57	Upper left sulcus mucosa		−
3 HC	F	44	Lower right sulcus mucosa		−
4 HC	M	32	Upper right sulcus mucosa		−
5 HC	F	25	Upper posterior vestibular mucosa		−

CHC, chronic hyperplastic candidosis; F, female; HC, healthy control; LP, leukoplakia; M, male; PAS, periodic acid Schiff staining.
−, negative; +, some; ++, moderate; +++, high numbers of Candidal hyphae.

the clinical suspicion of chronic hyperplastic candidosis was confirmed in the biopsy specimen using hematoxylin & eosin and in whom the presence of candida was confirmed using periodic acid Schiff staining and a Dentocult CA® culture test (Orion Diagnostica, Espoo, Finland). Five samples of chronic hyperplastic candidosis, five of leukoplakia, and five of healthy controls were analyzed in the present study.

Data analysis

All sections were analyzed semiquantitatively using a light microscope and documented using a digital camera attached to the microscope. The main findings (positive cells) were graded in numerical order.

The differences in expression of the TLRs in the three epithelial layers (upper layer, middle layer, and basal layer) for the three groups (chronic hyperplastic candidosis, leukoplakia, and healthy mucosa biopsy samples) were analyzed separately with Pearson's chi-squared test in which the levels of the TLR expression of the epithelial cells were coded as (0 = no expression at all, 1 = 1–24% expression, 2 = 25–49% expression, 3 = 50–74% expression, and 4 = 75–100% expression) using the non-parametric Kruskal–Wallis test. Differences between the two groups (candidosis vs. control, candidosis vs. leukoplakia, leukoplakia vs. control) were assessed using the non-parametric Mann–Whitney *U*-test. All statistical analyses were performed using the SPSS version 14.0 program (SPSS Inc., Chicago, IL). Any *P*-values <0.05 (two-tailed test) were considered statistically significant.

Immunohistochemistry

Paraffin-embedded sections of chronic hyperplastic candidosis, leukoplakia, and healthy controls were deparaffinized in xylene, dehydrated through a graded ethanol series, and washed in distilled water. The antigen epitopes hidden in tissue sections by the fixation process were disclosed by using a heat-induced antigen retrieval protocol: sections were incubated in buffer (10 mM citric acid, pH 6.0) and heated in a special laboratory microwave oven (Milestone Mega T/T, Sorisole, Italy) at 94°C for 24 min. Tissue sections were stained using avidin–biotin–peroxidase complex staining. All sections were washed in 10 mM phosphate-buffered 0.1 mM saline, pH 7.4 (PBS), three times, for 5 min each time. Endogenous peroxidase activity was blocked by immersing the sections in 0.3% H_2O_2 in methanol for 30 min at room temperature. Non-specific binding sites were blocked with 1 : 50 diluted normal horse serum (Vector Laboratory, Burlingame, UK) for 1 h at room temperature. The sections were incubated with the primary antibodies of the TLRs in the following concentrations: 0.8 μg/ml rabbit anti-human TLR1 immunoglobulin G (IgG), 2.6 μg/ml rabbit anti-human TLR2 IgG, 2 μg/ml rabbit anti-human TLR3 IgG, 1.3 μg/ml rabbit anti-human TLR4 IgG, 1.3 μg/ml rabbit anti-human TLR5 IgG, 1 μg/ml goat anti-human TLR6 IgG, 0.8 μg/ml goat anti-human TLR7 IgG, 1 μg/ml rabbit anti-human TLR8 IgG, 0.5 μg/ml rabbit anti-human TLR9 IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were left overnight in a humid box at 4°C. The following day, sections were incubated in biotinylated secondary IgG antibody followed by avidin–biotin–peroxidase complex according to the manufacturer's instructions (Vector Laboratory). Finally, the sites of peroxidase binding were revealed with a combination of 300 μl 3% H_2O_2 and 0.023% 3,3′-diaminobenzidine tetrahydrochloride solution (35 mg of DAB in 150 ml PBS; Sigma Chemical Co., St Louis, MO). Control sections of both sets were treated with rabbit and goat IgG antibodies of the corresponding isotype against *Aspergillus niger* glucose oxidase (Dakopatts, Glostrup, Denmark), an enzyme which is not present or inducible in mammalian tissues. All sections were counterstained with Mayer's hematoxylin solution for 30 s, dehydrated in graded ethanol, cleared in xylene, and mounted in Diatex (Becker Industrifärg AB, Märsta, Sweden).

Results

Routine histopathology

Candidal hyphae were revealed in chronic hyperplastic candidosis to be confined to the uppermost layers of the epithelia with varying degree of penetration, while in leukoplakia no hyphae were observed although some yeast cells were occasionally found on the surface of the mucosa in some of the samples. In chronic hyperplastic candidosis, candidal hyphae were usually growing in a perpendicular direction into the epithelium and were randomly distributed along the epithelial surface, colonizing some areas while being absent from others. Numerical grading was used to estimate the number of yeast cells and hyphae in individual cases (Table 2).

Table 2. Grading of Toll-like receptor 1 (TLR1) to TLR9 staining in the epithelium of chronic hyperplastic candidosis, leukoplakia, and healthy control

	TLR1			TLR2			TLR3			TLR4			TLR5			TLR6			TLR7			TLR8			TLR9		
Tissue	UL	SL	BL	UL	SL	BL	UL	SL	BL	UL	SL	BL	UL	SL	BL	UL	SL	BL	UL	SL	BL	UL	SL	BL	UL	SL	BL
CHC																											
1	++	++	+++	±	±	+	±	+++	+++	–	±	+	±	+++	+++	+	++	+++	++	+++	+++	±	+++	+++	±	+++	+++
2	±	++	+++	±	±	±	±	++	+++	±	+	+++	+	++	+++	±	±	+	+	++	++	+++	+++	+++	+++	+++	+++
3	–	+++	+++	±	+	++	–	+	+++	–	+	++	–	+++	+++	+	++	+++	++	+++	+++	++	+++	+++	+	+++	+++
4	±	++	+++	±	++	+	++	+++	+++	+	+++	+++	+	+++	+++	±	±	+	++	+++	+++	++	+++	+++	++	+++	+++
5	++	+++	+++	–	+	–	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++
LP																											
1	±	+++	+++	+	+++	+++	+	++	+++	+	+++	+++	+	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	+	+++	+++
2	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	±	+	++
3	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
4	+++	+++	+++	++	++	+++	++	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
5	+	++	+++	±	+	++	+	+++	+++	+	++	+++	+	++	+++	+	++	+++	++	+++	+++	+++	+++	+++	±	+	++
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3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4	++	++	+	+++	++	++	++	++	+	++	+	+	++	++	+	+++	++	++	++	++	+	+++	++	+	++	++	+
5	++	++	+	++	++	+	++	++	++	+++	++	++	++	++	+	++	++	++	++	+	+	++	+	++	++	++	+

CHC, chronic hyperplastic candidosis; LP, leukoplakia; HC, healthy control; UL, uppermost layer; SL, spinous layer; BL, basal layer.
–, no positive cells; ±, 0–25%; +, 25–50%; ++, 50–75%; +++, 75–100% of positive cells.

Staining of chronic hyperplastic candidosis sections with hematoxylin & eosin showed parakeratinized epithelium with clear, broad, and bulbous rete ridges. In some areas the keratin layer had eroded and the underlying epithelium was exposed. Individual neutrophils and microabscesses were often seen in the epithelia of chronic hyperplastic candidosis samples, whereas inflammatory cell infiltrates, composed of lymphocytes and plasma cells, were seen in the lamina propria in all sections.

Immunohistochemical staining

The three categories of samples (i.e. chronic hyperplastic candidosis, leukoplakia, and healthy controls) showed variable TLR immunostaining in terms of intensity and distribution of positive cells. All the epithelia were divided into three regions: lower (basal), middle (spinous), and upper (superficial). In most of the samples, TLR-positive epithelial cells were homogeneously stained along the same particular region of the epithelium with the strongest immunostaining being noticed at the basal layer. In chronic hyperplastic candidosis, the number of epithelial positive cells ranged from none (−) to high (+++) for different TLRs (Table 2). In sections of chronic hyperplastic candidosis where extensive candidal hyphae were seen by periodic acid Schiff staining (Fig. 1A), the whole epithelium was uniquely positive for TLR4, while it was only very faintly positive for TLR2, except for a very few cells at the very bottom (Fig. 1B,C, respectively). This difference in tissue response to TLR2 and TLR4 was noticed also in another sample (Fig. 1D,E, respectively). The uppermost layers of the epithelia of the chronic hyperplastic candidosis sections tended to be devoid of staining while the underlying middle layers varied in staining intensity from always weak (in the case of TLR2) to moderately immunopositive (in the case of TLR4), to heavy staining (the remaining TLRs), while the basal layer was always positive (Fig. 2A–I). In leukoplakia, TLR staining of the middle and basal epithelial layers was always positive except, in some sections, for TLR9, for which the staining was very weak or even negative (Fig. 3A–I). Wherever keratin was present (seen from routine histological staining), staining for TLR9 was evident, although this was lacking or greatly reduced several cell layers beneath the keratin (Fig. 3D–H).

In contrast, the epithelial layers of the healthy control tissue were always positive except, interestingly, for a few sections where the basal layers were very weakly stained or negative (Fig. 4A,B). No immunoreactive epithelial positive cells were observed in negative staining controls (Fig. 4C,D), which confirmed the specificity of the TLR epithelial staining.

Statistical analysis

From a statistical point of view, the lower expression of the TLR2 ($P < 0.05$) among the three epithelial layers was consistently statistically significant, both for candidosis vs. control and for candidosis vs. leukoplakia comparisons. In addition to that, in the upper layers in the candidosis vs. normal status there was also significantly lower expression of TLR3 to TLR6 ($P < 0.05$), and in the candidosis vs. leukoplakia there was significantly lower expression of TLR6 and TLR7 ($P < 0.05$).

Interestingly, there was statistically significant higher expression of the TLR4 and TLR6 ($P < 0.05$) in the leukoplakia basal layer vs. controls. On the other hand, in the upper and middle layers the expression of these two TLRs was observed to be significantly lowered for candidosis vs. leukoplakia or candidosis vs. controls.

Lower or higher expression ($P > 0.05$ but <0.066) was also observed for different TLRs among the three different epithelial layers (Fig. 5).

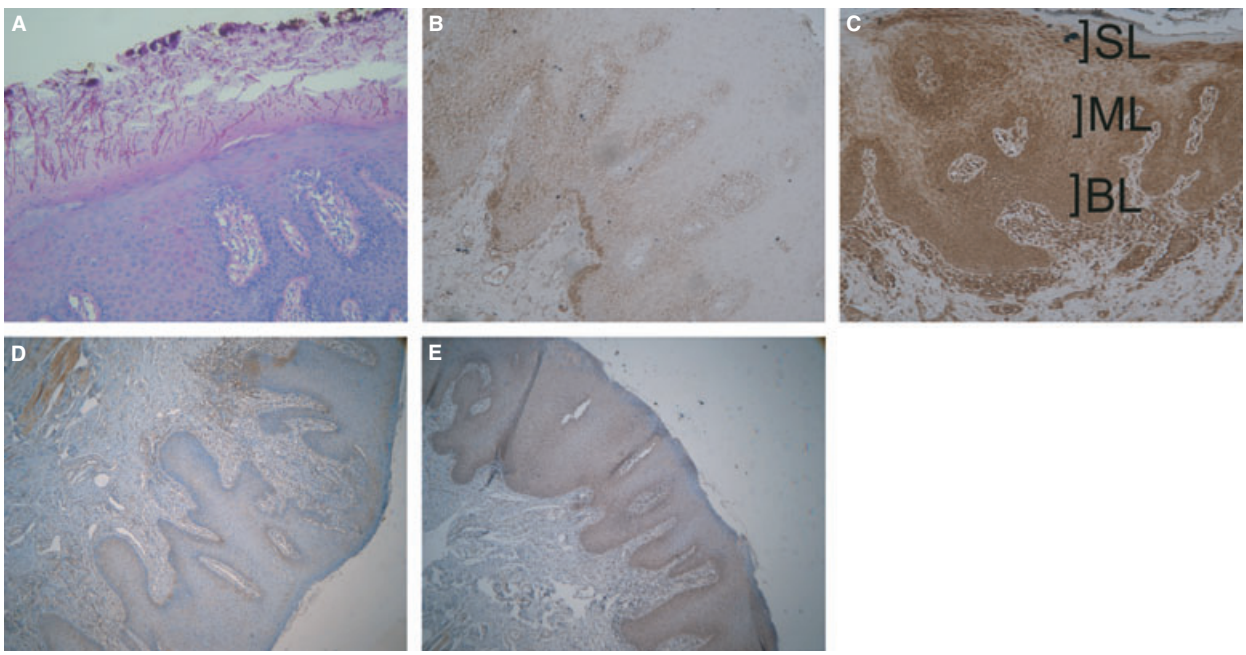

Fig. 1. The numerous candidal hyphae in chronic hyperplastic candidosis (A), in which Toll-like receptor 2 (TLR2) staining was very faint except few cells at the very basal layer (B) while the same section showed strong staining for TLR4 (C). The same finding was noted in another section where TLR2 was almost absent (D) while TLR4 was very intense (E). BL, basal layer; ML, middle layer; SL, superficial layer. (For A–C: original magnification ×100; for D,E: original magnification ×40).

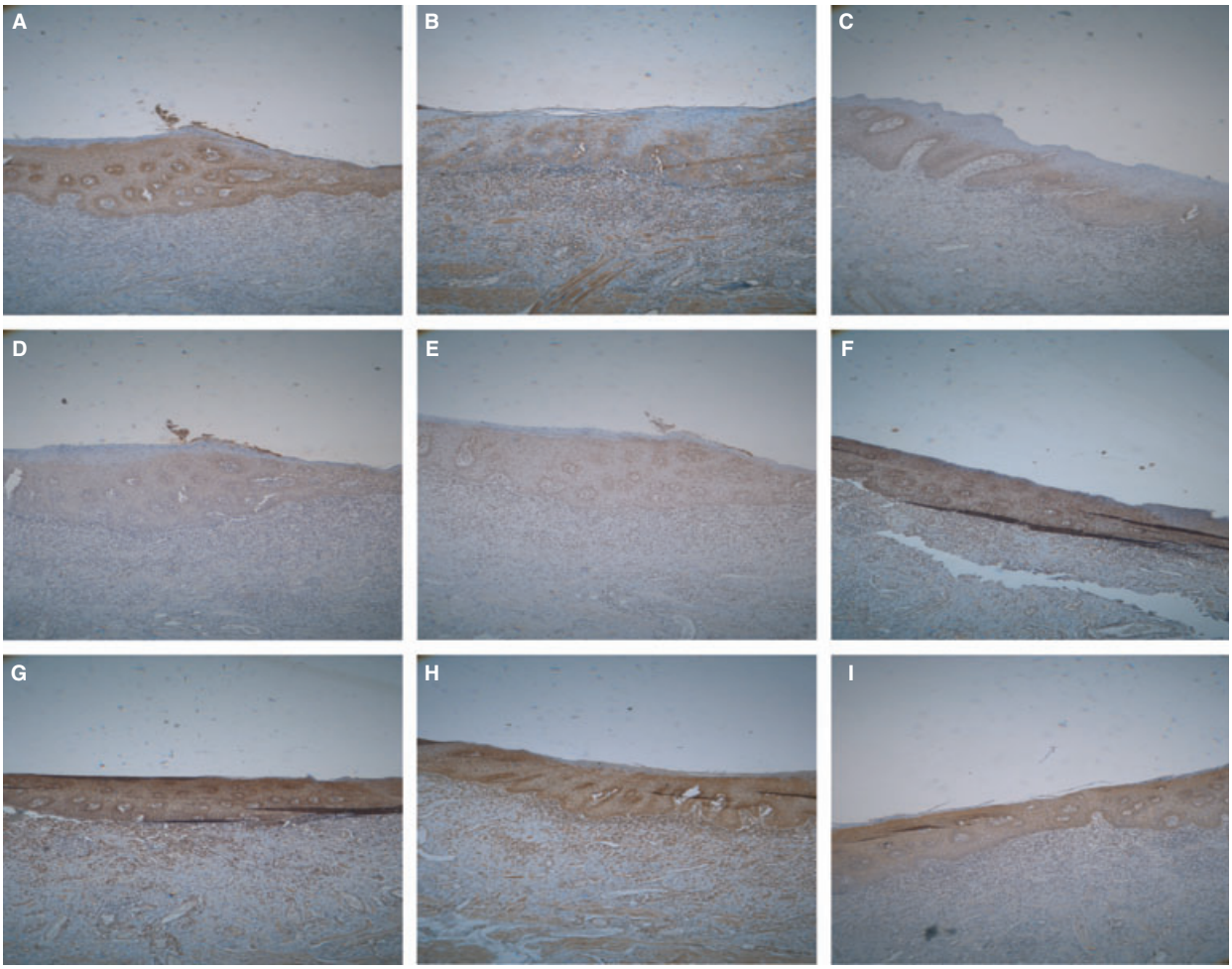

Fig. 2. The set of Toll-like receptors (TLRs) 1 to 9 (A–I, respectively) staining in chronic hyperplastic candidosis. (For A–I: original magnification ×25).

Discussion

In this work, we have investigated the immunohistochemical expression of nine classes of TLRs (TLR1 to TLR9) in a series of sections from chronic hyperplastic candidosis, leukoplakia, and healthy tissue. The interaction between *C. albicans* and the host (i.e. oral mucosa) is a complex process regulated mainly by the host immunity and the oral microenvironment, e.g. local pH, temperature (13). When candidal infection ensues, the host employs different parts of the immune system, composed mainly of cells and cytokines, to stand in the way of the attacking microbe. Cytokines play a major role in the defense mechanisms against *Candida* infections. There are two branches of cytokines in terms of enhancing or suppressing the host's immune protection against infection. Proinflammatory cytokines, e.g. interleukin-1β (IL-1β), IL-6, IL-12, and tumor necrosis factor-α, are part of the T helper type 1 (Th1) division, and play an important part in the host defense against infections, while anti-inflammatory cytokines, e.g. IL-4, IL-10, direct the immune status towards Th2, which, in contrast, has a suppressive effect. Body epithelia, e.g. oral, respiratory, intestinal, or genitourinary, serve as a first line of defense against the entry of any infectious invaders and therefore; it is logical that these organs are equipped with a cocktail of pathogen recognition receptors, such as C-type lectin receptors (e.g. mannose receptors), and TLRs. The step-by-step scenario of the pathogenesis of oral candidosis seems to be as follows: when the immune system of the host becomes weak or the local environmental *Candida* growth-promoting factors are favorable, *C. albicans* attempts first to establish a firm adherence to the oral epithelial cells. Having succeeded in this, *C. albicans*, by virtue of its contact-sensing ability i.e. thigmotropism (34), searches for any portal in the underlying epithelium through which it can gain entrance to the deeper layers. The uppermost layers of the epithelium do not remain passive but instead alarm both divisions of the immune system: innate and adaptive. Generally speaking oral mucosal cells perform three major protective roles against intruding microbes; two being indirect and one direct. The indirect roles reside in recognition and chemotaxis and both are arranged in a cascade order, i.e. when the first starts it stimulates the second. To continue this theory, epithelial cells, as a part of the innate branch of immune defense, seem to design the best strategy for the host immunity to deal with the incoming pathogen by recognizing it first, and this is mediated by TLRs. Once epithelial cells recognize *C. albicans* (usually through its cell wall components, i.e. zymosan), a set of intracellular signaling cascades starts to operate, which end in the production of proinflammatory cytokines and chemokines. IL-8 exemplifies the second job of epithelial cells by recruiting the impending neutrophils to be deployed

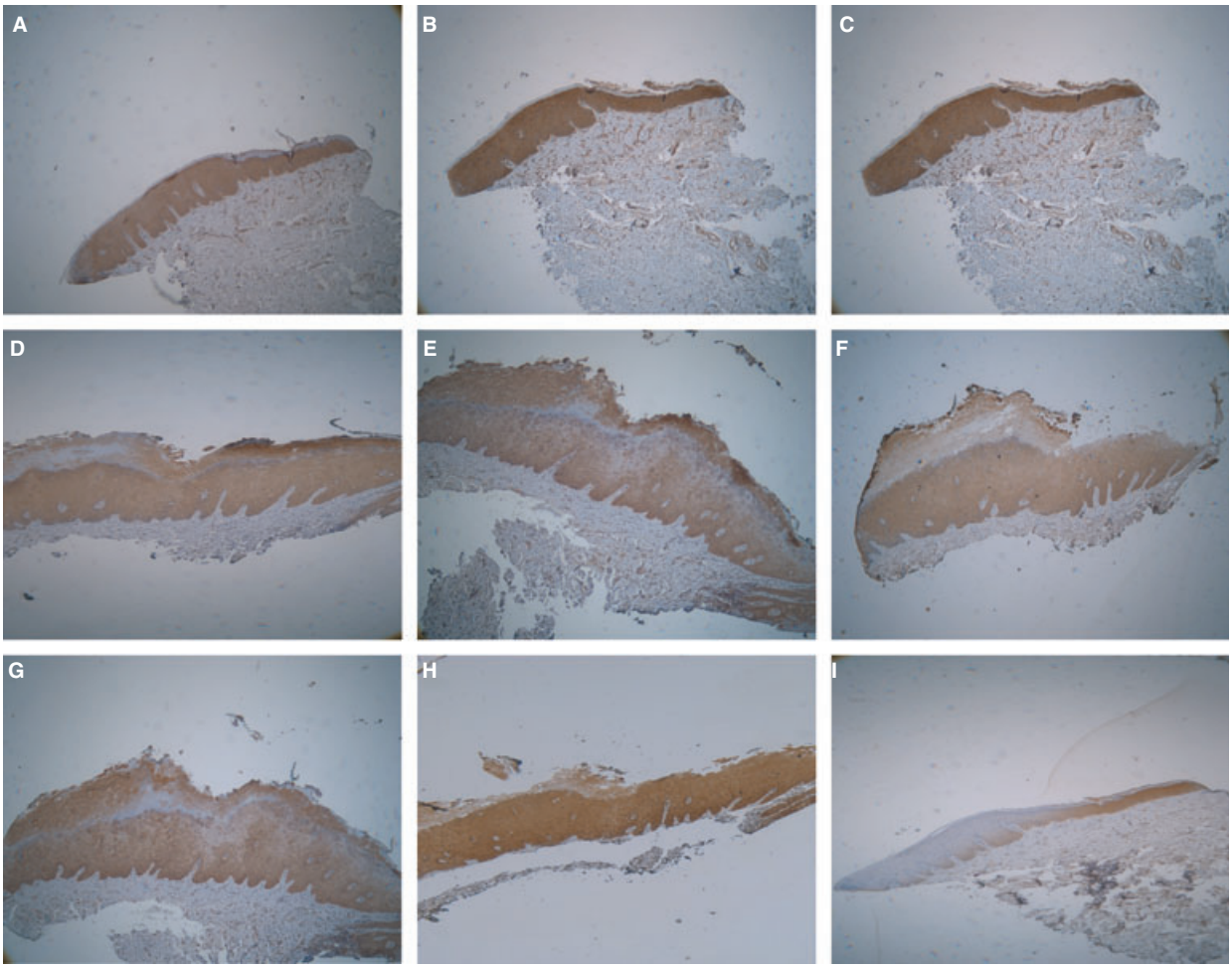

Fig. 3. The set of Toll-like receptors (TLRs) 1 to 9 (A–I, respectively) staining in leukoplakia (For A–I: original magnification ×25).

to the site of infection (1). The direct role of oral epithelial cells against *C. albicans* has been shown to have 80–90% *in vitro* inhibitive activity compared with other mucosal cells, e.g. vaginal (9). Taking into account the obvious versatility of their roles in immune defense mechanisms, we concentrated our work on the role of epithelial layers in the process of anti-*Candida* immunity in terms of their response to TLR immunostaining, which may provide a clear picture of the existence and amount of such novel receptors at the protein level. In this report, we compared the immunochemical staining of all known TLRs (except TLR10) in sections from chronic hyperplastic candidosis, leukoplakia, and healthy tissues. Chronic hyperplastic candidosis is a form of oral candidosis that is characterized by invasion of the oral epithelium by *Candida* hyphae, followed by a reactive tissue hyperplasia (4). It has been shown that candidal cell wall components constitute PAMPs for certain TLRs, e.g. zymosan is recognized by TLR2/TLR6 heterodimers while mannan is a ligand for TLR4 (22, 28). To analyze our results, the epithelium was divided into three layers: namely, uppermost, middle, and basal. We found that all TLRs were expressed by the epithelial cells of chronic hyperplastic candidosis sections with some variations. We found all TLRs, except those that are claimed to recognize *C. albicans*, i.e. TLR2, TLR4, and TLR6, to be strongly positive, especially in the middle and basal layers. This may indicate that when epithelial TLRs (i.e. TLR1, TLR3, TLR5–TLR9) are not stimulated by a particular PAMP (i.e. *Candida* zymosan or phospholipomannan), they tend to be constitutively expressed to detect any occasional intrusion by any foreign particle. This assumption is supported further by the staining pattern of the healthy tissue to the TLRs (TLR1–TLR9) and by a previous report, which has documented the expression of TLRs by a variety of oral mucosal cells (18). Regarding TLR2 and TLR4 staining, the situation is a bit different and needs more clarification. Before staining chronic hyperplastic candidosis sections using Immunohistochemical techniques, we verified the existence of candidal hyphae by periodic acid Schiff staining. Two sections out of the five (samples 2 and 5) showed high numbers of candidal hyphae compared to unicellular yeasts, and when stained with TLR antibodies, they showed very little staining for TLR2, while TLR4 staining was comparatively strong (Table 2, Fig. 1). In the other sections, in which hyphae were scarce, however, TLR4 was weaker. Considering the dogma that states that a process of negative regulation could be responsible for the reduced expression of a particular TLR expression despite the presence of its ligand in a known lesion (12), then we can suppose that TLR2 is more actively involved in recognizing and mediating the candidal ligand (and thus more down-regulated) in the hypha-rich sections. The concept of TLR-negative regulation,

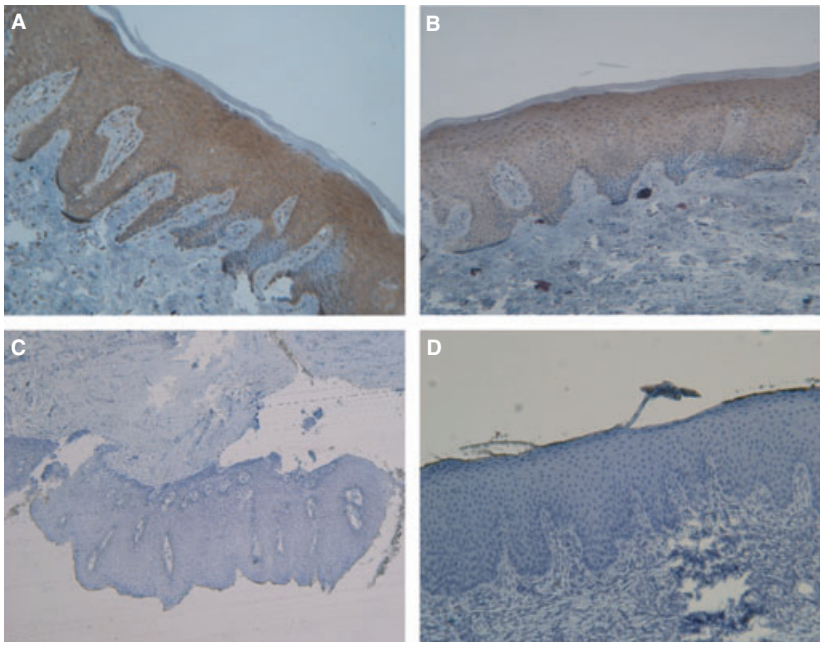

Fig. 4. The faint basal staining of healthy epithelium (A,B). (C,D) show the negative control staining. (For A, B and D: original magnification ×40; for C: original magnification ×25).

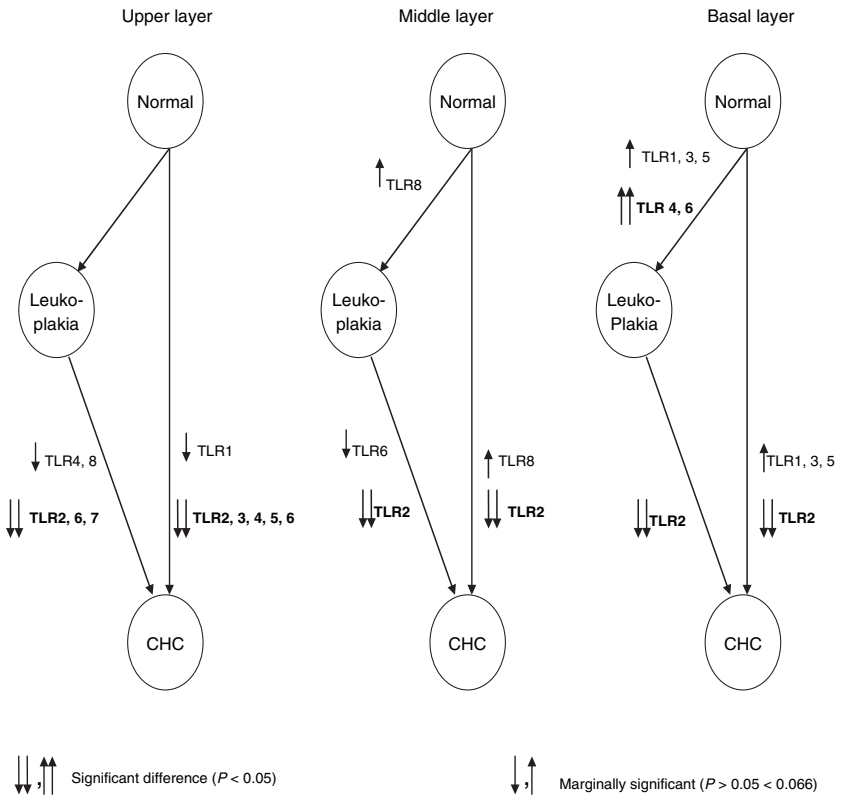

Fig. 5. The change in Toll-like receptor (TLR) expression in the three layers of epithelium, in chronic hyperplastic candidosis (CHC), leukoplakia, and healthy controls. The most significant change seems to be downregulation of TLR2 in all the three layers when the tissue condition (irrespective of its condition) shifts to candidosis.

which was described in detail by Liew et al. (15), who categorized multiple levels at which negative regulation of TLRs can be achieved, is fascinating and logical because a TLR–ligand stimulation that was too lengthy would exhaust the immune system and promote excessive inflammation, especially in chronic lesions, e.g. chronic hyperplastic candidosis. The present data, therefore; lead to the suggestion that candidal hyphae direct the immune response towards stimulating TLR2 rather than TLR4, which would otherwise be faint as well. This differential pattern of TLR stimulation by candidal ligands has already been reported in the literature (40). In this context, our observations seem to be consistent with a previous work of Netea et al. (24) who have shown that the opportunistic pathogen *C. albicans* can exploit TLRs, i.e.TLR2, to evade the immune system. The concept of candidal evasion of the host immune system through TLR2 has been the subject of intense debate. First, Netea et al. (23) have demonstrated that TLR2 knockout mice had gained more resistance against systemic candidosis compared with wild-types, and this was attributed to less release of anti-inflammatory cytokines, e.g. IL-4 and IL-10 which are elaborated by TLR2 activation (23). This hypothesis was criticized by Gil et al. (10), who later published an opposing study in which they approved the role of only TLR2 (and *not* TLR4) in the protective cytokine production in immune defense against *C. albicans* (11). Indeed, this idea did not appeal to Netea et al. (25) and in their reply, they stressed the roles of both TLR2 and TLR4 in the recognition of *C. albicans.* Such conflicting opinions reflect the complexity of this issue and necessitate more work to unravel the still-hidden aspects of candida–TLR interactions.

C. albicans is a polymorphic microorganism existing in three basic morphological entities, namely, yeasts, pseudohyphal, and hyphal forms (41), and it expresses an array of pathogenic weapons by which it can invade the oral mucosa, e.g. adhesion, production of proteases. An important virulence feature of *C. albicans* is its switching between the unicellular (yeast) and the filamentous (hyphal) forms (30), which is further supported by many reports stating that non-filamentous mutants have less ability to cause infection (17, 31). In addition to that, stimulating two cardinal cells of the immune system, monocytes and dendritic cells with candidal hyphae, has shown the latter to be more immunogenic (than yeasts) to the host. Such *in vitro* experiments showed that monocytes fail to phagocytose candidal hyphae, and produce less IL-12 (16), while dendritic cells can phagocytose the hyphae but IL-12 is inhibited and IL-4 is stimulated, thereby

leading to the anti-inflammatory Th2 response (29).

Based on our results, we present a new possibility, rather than clear evidence, that candidal hyphae are more pathogenic than the yeast form, in the sense that they bring down the anticandidal Th1 cytokines (by evading TLR4), and simultaneously augment the anti-inflammatory Th2 process (through stimulation of TLR2). Therefore, this study broadens the results of Netea et al. (22) (who have used systemic candidosis) to the mucosal level. This selective strategy, in which *C. albicans* seems to evade the immune system, reflects the capability of the organism to thrive as a commensal without elimination by the host. On the other hand, the somewhat strong staining of TLR6 may dictate its limited role in taking part in the innate immune response in oral candidosis, at least compared with TLR2 and TLR4. The reason for this seemingly lower immune response of TLR6 (despite its well-known ability to recognize zymosan) could be that its recognition of some ligands is shared by TLR2 in a heterodimeric manner (27), although the load seems to be taken most by the latter.

In most, if not all, sections the basal layer of the epithelium showed wider staining for all TLRs than the more superficial layers, where the staining gradually decreased until the stratum corneum where the staining vanished completely. This higher content of TLRs in the basal layers has been explained by the possibility that TLRs are synthesized first in the basal layer and then, while transported upwards along the epithelial shedding cycle, become diminished for a variety of reasons, e.g. limited half-life, downregulation (Arzu et al. unpublished data).

In leukoplakia, we noticed strong epithelial staining, which extended to the overlying keratinous layer, for all TLRs except TLR9, for which it was less in some sections (Fig. 3). To reach a rational explanation for this unique observation, we need to refer to the classical definition of leukoplakia as a white lesion of the oral mucosa that cannot be attributed clinically or diagnostically as any other known disease (6). This definition provides us with two possibilities. First, leukoplakia is a lesion and as a result; the immune system has already been evoked and activated. This may explain the exaggerated expression of TLRs in epithelium to protect the already-diseased underlying tissue. Second, the etiology is not yet known and no microorganisms have, so far, been implicated in the pathogenesis of leukoplakia. Therefore, it is logical that we did not find any particular TLR (except TLR9) to be involved in long-term ligand binding and, so be downregulated. The endosomal location of TLR9 is parallel to its ability to recognize the unmethylated CpG DNA, which can be of bacterial or fungal origin (5, 37). We suppose that, in leukoplakia, the oral tissue becomes more liable to infection and so becomes a target for attack by the continuous bathing of oral microflora. The mucosal immune system seems to degrade the invading microbes, whether bacteria or fungi, by phagocytosis (e.g. by polymorphonuclear cells) or by direct lysis (by antimicrobial peptides). This should induce leakage of the microbial cell contents, including its genetic material, which would keep on stimulating TLR9 leading to its decreased expression. This assumption is not, however, a consistent feature of leukoplakia because it was found only in some sections. More studies are needed to clarify, more broadly, the role of keratin in TLR expression.

In healthy tissue, the strong expression of all TLRs, especially by the middle and superficial layers, confirms the importance of epithelium as a physically protective organ, checking the peripheral mucosal regions against attempts by any foreign body to gain access to the oral tissues. Our observation that the basal layers of some sections showed faint or even absent TLR staining may extend the concept of negative regulation to healthy tissue with unoccupied TLRs. Even in healthy tissue, TLRs are very likely involved in ligand binding but this interaction is seemingly temporary and does not last long enough to elicit the immune system. We believe that the basal layer, because of its close vicinity to the underlying lamina propria, is possibly the most involved epithelial layer in terms of activating the humoral and cellular immune systems. Therefore; it is the layer that tends to have a break-rest if the TLRs of the more superficial layers are not stimulated with their corresponding ligands for a considerable time.

This pilot study, although it is the first to assess the immunochemical localization of the first nine classes of TLRs in oral candidosis compared with leukoplakia and healthy control, remains merely descriptive and further work will be needed to shed more light on the labyrinth gateways of *C. albicans*–TLR interactions.

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