doi: 10.1111/j.1600-0714.2007.00527.x

J Oral Pathol Med (2007) 36: 329–36

www.blackwellmunksgaard.com/jopm

A possible CD1a Langerhans cell–mast cell interaction in chronic hyperplastic candidosis

Ahmed Ali[1,2], Riina Rautemaa[3], Jarkko Hietanen[4,5], Arzu Beklen[1,6], Yrjö Konttinen[7,8,9]

[1]*Department of Anatomy/Biomedicum, University of Helsinki, Helsinki, Finland;* [2]*Department of Pediatric Dentistry, Faculty of Dentistry, University of Garyounis, Benghazi, Libya;* [3]*Microbiology Unit/HUSLAB, Helsinki University Central Hospital, and Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Helsinki, Finland;* [4]*Department of Oral Pathology/Institute of Dentistry, University of Helsinki, Helsinki, Finland;* [5]*HUSLAB, Helsinki University Central Hospital, and Institute of Dentistry, University of Helsinki, Helsinki, Finland;* [6]*Medico-social, Dental Clinic, Bogazici University, Istanbul, Turkey;* [7]*Department of Medicine/invärtes medicin, Helsinki University Central Hospital, Helsinki, Finland;* [8]*ORTON Orthopaedic Hospital of the Invalid Foundation, Helsinki, Finland;* [9]*COXA Hospital for Joint Replacement, Tampere, Finland*

AIMS: T lymphocyte–antigen-presenting cell (APC) interaction plays a central role in T lymphocyte activation and APC maturation. We therefore studied the CD1a-positive Langerhans cells with respect to receptor activator of nuclear factor kappa B ligand (RANKL)-positive cells in chronic hyperplastic candidosis (CHC).

MATERIALS AND METHODS: Tissue sections of CHC were compared with leukoplakia and healthy oral mucosa using RANKL and CD1a monoclonal antibodies in an avidin–biotin peroxidase complex protocol. Two different antigen-retrieval protocols, pepsin preincubation and Tris–EDTA heat treatment, were used.

RESULTS: CD1a-positive Langerhans cells were in healthy and leukoplakia epithelium found in the middle layer, but in CHC in all layers of the epithelium, at the basement membrane and as mononuclear round cells in the lamina propria. Use of pepsin digestion enabled studies of mast cells and their activation in the form of degranulation of RANKL.

CONCLUSIONS: The numerical, morphological and topographical versatility of the CD1a-positive Langerhans cells in CHC can be clarified by dendritic cell (DC) recruitment into the epithelium. RANK-positive and RANKL-sensitive DCs have ample opportunity to interact with local T lymphocytes. Use of an optimized antigen-retrieval protocol enabled demonstration of an active engagement (degranulation) of mast cells, which represent a rapidly available source of soluble RANKL.

J Oral Pathol Med (2007) **36**: 329–36

Keywords: *Candida albicans*; receptor activator of nuclear factor kappa B ligand; Langerhans cell; mast cell; leukoplakia

Correspondence: Yrjö T. Konttinen, MD, PhD Biomedicum, PO Box 700, FIN-00029 HUS, Finland. Tel: +358 9 19125210, Fax: +358 9 19125218, E-mail: yrjo.konttinen@helsinki.fi
Accepted for publication December 21, 2006

Introduction

Candida species are fungi which normally inhabit mucosa-lined organs in the human body, including the mouth and the gastrointestinal and genitourinary tracts (1). *Candida* spp. are harmless commensals as long as the innate and acquired immune systems of the host remain intact. Once the immune status of the host is impaired, as in the case of compromising diseases, such as diabetes mellitus, HIV infection or due to the use of immunosuppressive drugs, *Candida* spp. exploit this situation and unmask their pathogenic potential. Therefore, they are known as opportunistic pathogens. Oral and cutaneous candidosis represent the most common fungal infection of the mouth and skin, respectively, characterized by an overgrowth of the fungus *Candida* (2, 3). The frequency of candidosis has increased in the last years with an accompanying rise in morbidity and mortality (4). Among all candidal species, *Candida albicans* is the most common one isolated from the oral cavity in health and disease (5). *Candida albicans* may encroach on the blood compartment of the host and disseminate hematogenously causing microabscesses in certain immunocompromised patients (6). Traditionally, *Candida* spp. have been considered nosocomial pathogens as candidaemia accounts for 10–15% of all nosocomial bloodstream infections (7). Chronic hyperplastic candidosis (CHC) is a form of oral candidosis characterized by hyphal invasion of the oral epithelium and association with squamous cell carcinoma (8, 9). CHC is manifested clinically as a speckled or homogeneous white patch which, unlike the pseudomembranous form of candidosis, cannot be scraped off by a gentle rubbing of the mucosa (10).

In oral candidosis, both specific and non-specific immune reactions are involved. Although cell-mediated immunity is of paramount importance in defending the host against oral candidosis, innate immunity remains the pioneer authority that appears to operate as soon as

the infectious process ensues. One of the cells which comprise an essential link between the innate and adaptive immune system is the dendritic cell (DC).

Because of their rarity and lack of specific surface markers, DCs have long been ignored and have just recently succeeded in catching researchers' attention (11). DCs are leukocytes which originate in the bone marrow, and their role in the induction of primary immune response has been well established. DCs belong to a family of antigen-presenting cells (APC) which infiltrate most of the tissues of the body. In the epithelium, DCs were first identified in 1980 in experimental inflammatory reactions, where DCs were found closely located near lymphocyte-like cells within 4–6 h of a topical application of an allergen (12). Being very versatile, DCs have different morphologies and functions depending on their degree of maturation, e.g. mucosal DCs (called Langerhans cells), upon which we conducted this study, tend to have numerous cellular dendritic projections which conform to their roles in capturing antigens and presenting them to T cells. Existence of Langerhans cells in oral epithelia was confirmed by Daniels *et al*. where they identified, using modified ATPase histochemistry and monoclonal antibodies, such cells in mucosal regions of postmortem mouths (13).

In CHC (also called candidal leukoplakia), detection of Langerhans cells was reported (14).

Lately, the role of DCs in the host defense against candidosis has become more evident by showing their phagocytic capacity of candidal yeasts and hyphae as well as processing their antigens (15).

Mast cells, on the other hand, are known best for their roles in mediation of allergy through their expression of high-affinity receptors (FcεRI) for immunoglobulin E. So far, no study has been conducted to clarify whether mast cells are involved in the immune system against oral candidosis.

In the present study, we sought to evaluate the immunological role of DCs and formulate a hypothesis about their interactions with T lymphocytes in response to receptor activator of nuclear factor kappa B ligand (RANKL) which according to recent studies is also found in mast cells (16).

Materials and methods

Samples

The local ethical committee approved the study protocol. Biopsy samples of oral mucosal lesions were obtained from patients with CHC undergoing examination of mucosal lesions, while biopsies of candida-negative leukoplakia were used as candida-negative hyperplastic mucosal controls. CHC was defined as an invasive candidosis characterized by hyphal in-growth into the oral epithelium leading to the formation of well-demarcated, palpable and raised homogenous and/or nodular lesions of the mucosal membrane varying from small translucent whitish areas to large opaque plaques. They, in contrast to the pseudomembranous candidosis, cannot be rubbed off. Histologically, the homogenous lesions were characterized by hyperorthokeratinization or hyperparakeratinization, whereas the nodular lesions displayed varying thickness of the hyperplastic surface epithelium, which was always characterized by parakeratosis and occasionally by hyperkeratosis and/or slight dysplasia. Chronic inflammatory cell infiltrates were found in lamina propria. These lesions are described in some detail in Table 1.

Patients included in this study represent 10 consecutive patients, in whom the clinical suspicion of CHC was confirmed in the biopsy specimen using hematoxylin–eosin and in whom the presence of candida was confirmed using Periodic Acid Schiff (PAS) staining and/or Dentocult CA® culture test (Orion Diagnostica, Espoo, Finland). Thus, 10 samples of CHC, 10 of leukoplakia and three of healthy controls were analyzed in the present study. For routine diagnostic purposes, at least 5–10 hematoxylin–eosin and PAS-stained sections were analyzed by an experienced MD and PhD specialist in oral pathology (JH), but more were always asked if in doubt. Similar strategy was followed in the immunohistochemical evaluation of the samples using CD1a and RANKL staining, which were, if only possible, performed from consecutive sections, usually so that at least two slides were stained for both, although naturally more were produced if for some reason necessary. All sections were analyzed semiquantitatively using a light microscope and documented using a digital camera attached to the microscope. The main findings (positive cells) are graded in numerical order.

Immunohistochemistry

Paraffin-embedded sections of CHC, leukoplakia and healthy controls were deparaffinized in xylene, dehydrated through a graded ethanol series and washed in distilled water. The experiment was done using two different antigen-retrieval protocols. In some experiments, antigen epitopes hidden in tissue sections by fixation process were disclosed by enzymatic digestion in which sections were immersed in a solution containing pepsin and distilled water (1:250) containing 0.1 1N HCl. All sections were kept in +37°C incubator for 30 min and profusely washed with running water. Some slides were instead pre-treated using heat-induced antigen retrieval performed by immersing the slides in Tris–EDTA buffer (10 mM Tris and 1 mM EDTA, pH ~9.0), followed by a period of 10 min of heating in a microwave at 600 W, with checking of the plastic box after the first 5 min to ascertain that it had enough fluid to evaporate and to avoid drying-up of the slides. Slides were kept 30 min at room temperature to cool them down.

Tissue sections were stained using the avidin–biotin peroxidase complex (ABC) staining. All sections were washed in 10 mM phosphate buffered, 0.1 mM saline, pH 7.4 (PBS), 3×5 min. 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. One set of sections was always

Table 1 Clinical and demographic data of the patients with chronic hyperplastic candidosis (CHC) and leukoplakia (LP)

Number	*Gender*	*Age*	*Location of the lesion*	*Clinical presentation*	*Additional information*
1 CHC	M	59	Tongue	Diffuse keratinization	Heavy smoker
2 CHC	F	45	Tongue	Red/white lesion	Painful
3 CHC	F	59	Tongue	Homogenous	
4 CHC	F	67	Palate	Verrucous	Prosthesis
5 CHC	F	55	Cheek/commissures	Hyperplastic	Heavy smoker
6 CHC	F	53	Tongue	Nodular	Sharp tooth edge
7 CHC	M	44	Tongue	Ulcerative	
8 CHC	M	53	Palate	Verrucous	
9 CHC	F	81	Cheek	Papular leukoplakia	Carcinoma of tongue
10 CHC	F	85	Tongue	Hyperplastic	
1 LP	F	51	Alveolar ridge	White batch	
2 LP	F	55	Tongue	White batch	
3 LP	F	52	Cheek	Striated batch	
4 LP	F	73	Floor of the mouth	Exophytic	Prosthesis, heavy smoker
5 LP	F	66	Palate	Striated	
6 LP	F	64	Floor of the mouth	Homogenous	
7 LP	F	50	Alveolar ridge	Exophytic	
8 LP	F	48	Cheek	Striated	
9 LP	F	84	Alveolar ridge	Exophytic, pigmented	Prosthesis
10 LP	M	49	Alveolar ridge	White batch	

Number	*Gender*	*Age*	*Location of the sample*	*Reason presenting patient to the clinic (no inflammation)*
1 CR	F	56	Upper left sulcus mucosa	Resection of left upper incisor
2 CR	F	57	Upper left sulcus mucosa	Wisdom tooth operation
3 CR	F	44	Lower right sulcus mucosa	Orthodontic treatment

incubated in 5 μg/ml monoclonal mouse anti-human RANKL IgG_{2b} (TRANCE/TNFSF11; R&D Systems Inc., Minneapolis MN, USA), while a consecutive set of sections were incubated in 0.012 μg/ml monoclonal mouse anti-human CD1a IgG_1 (DAKO Corporation, 6392 Via Real, Carpinteria, CA, USA). All sections were left over night in a humid box at +4°C. Next day, sections were incubated in biotinylated horse anti-mouse IgG antibody followed by ABC according to the manufacturer's instructions (Vector Laboratory). Finally, the sites of peroxidase binding were revealed with a combination of 300 μl of 3% H_2O_2 and 0.023% 3,3′-diaminobenzidine tetra-hydrochloride solution (35 mg of DAB in 150 ml PBS, Sigma Chemical Co., St Louis, MO, USA). Control sections of both sets were treated with mouse 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 CHC 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 CHC, candidal hyphae were usually growing in a perpendicular direction into the epithelium and were randomly distributed along the epithelial surface colonizing some areas while absent in others. Numerical grading was used to estimate the number of yeast cells and hyphae in individual cases (Table 2).

Staining of CHC sections with hematoxylin and 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 CHC samples, whereas inflammatory cell infiltrates, composed of lymphocytes and plasma cells, were seen in lamina propria in all sections.

Immunohistochemical staining

CD1a-positive DCs were found in the epithelia of CHC, leukoplakia, and healthy controls. CD1a-positive cells formed a network which varied in location from the very basal layers of the epithelium to suprabasal layers, which, however, were mostly found in the middle layer of the healthy and leukoplakia epithelium. The overall content of Langerhans cells was variable so that in CHC the quantity of CD1a-positive cells ranged from high (+++) to low (+) with a very obvious tendency to match the estimated quantity of candidal hyphae in each case (Table 2). In CHC Langerhans cells were seen throughout the whole epithelium, in the upper, middle and lower third, while, as mentioned, in leukoplakia and healthy control sections, Langerhans cells were preferentially located in the middle third of the epithelium (Fig. 1a and b) and their number tended to be in the medium range (++) (Table 2). In all three sample groups, Langerhans cells were found either isolated or as

Table 2 Grading of CD1a and RANKL staining in chronic hyperplastic candidosis (CHC), leukoplakia (LP) and healthy control (H)

			CD1a-positive DCs		*RANKL-positive cells*	
Cases	*Yeasts and hyphae*	*Keratin*	*DCs in Epithelium*	*DCs in CT*	*Epithelial cells*	*CT cells, e.g. mast cells, other cells*
CHC-1	+ + +	–	+ +	+ +	–	+ +
CHC-2	+	0	+	+ +	+ +	+ + +
CHC-3	+ + +	–	+ + +	+ +	±	+
CHC-4	+	–	+	+ +	±	+
CHC-5	+	–	+ +	+	±	+ +
CHC-6	+ + +	–	+ + +	+ +	±	+ +
CHC-7	+	–	+	+	+ +	+ +
CHC-8	+ +	–	+ + +	+	+	+ +
CHC-9	+ + +	–	+ + +	+ +	+	+ + +
CHC10	+ + +	–	+ + +	+ +	+ +	+ + +
Neg.	–	–	–	–	–	–
LP-1	–	–	+ +	+ +	±	+ +
LP-2	–	–	+ +	+	±	+ +
LP-3	–	–	+/–	+	±	+
LP-4	–	–	+	+ +	±	+ +
LP-5	–	–	+ +	+ +	±	+
LP-6	–	–	+	+ +	±	+ +
LP-7	–	–	+	+ +	+	+ +
LP-8	–	–	+ +	+ +	±	+ +
LP-9	–	–	+ +	–	±	+ +
LP-10	–	–	+ +	+	±	–
H-1	–	0	+ +	+ +	–	–
H-2	–	–	+ +	+ +	–	+
H-3	–	–	+	+ +	–	+ +

(0) The structure itself is not present; (–) negative; (±) only occasional; (+) some; (+ +) moderate; and (+ + +) high numbers of positive cell. DC = dendritic cell; CT = connective tissue.

clusters (Fig. 1c and d). In the epithelium, CD1a-positive cells had a typical dendritic morphology, whereas in the lamina propria, they had a more regular shape of inflammatory mononuclear cells. In some sections of CHC, the Langerhans cells were found as a front at the lower segment of the epithelium, or even lying in a very close proximity to the basement membrane (Fig. 1e and f respectively). Occasionally, Langerhans cells looked as if they were actually traversing the epithelium–lamina propria interface, which was a feature unique to CHC samples (Fig. 1g). Infiltration of the epithelium with CD1a Langerhans cells was particularly intense in heavily candida-infected sections and the localization of the CD1a-positive Langerhans cells was quite variable in CHC between different patients and different areas of the same biopsy, possibly reflecting the dynamic involvement of Langerhans cells in tissue pathology in this condition.

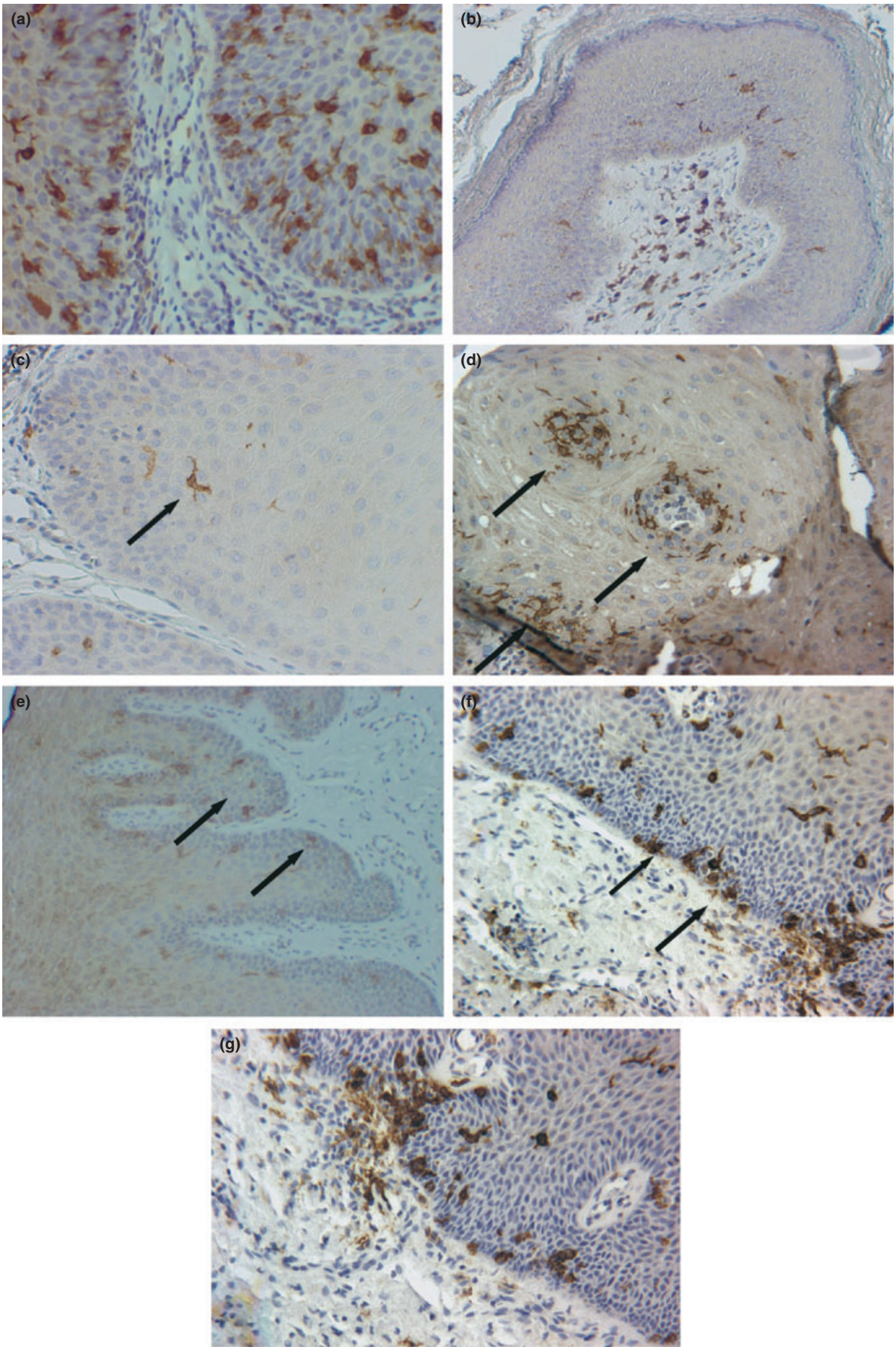
In the lamina propria, Langerhans cells were seen scattered near inflammatory cell infiltrates. Hematoxylin–eosin staining showed heavy chronic inflammatory cell infiltrates in the lamina propria in CHC, less in leukoplakia, whereas only occasional tissue monocyte/macrophages and lymphocytes were seen in healthy control sections.

In CHC and leukoplakia, the epithelia showed some RANKL immunoreaction particularly at the region of the epithelial ridges without any apparent localization in discrete cells, except in cells which were infiltrating the epithelium. The epithelium of leukoplakia stained more weakly than CHC. Epithelium of healthy control sections was completely devoid of any immunoreaction to RANKL.

When pepsin pre-treatment was used, it was noticed that the morphological characteristics of the RANKL-positive cells in lamina propria were typical of mast cells. In CHC, RANKL-positive mast cells displayed granular cytoplasmic RANKL staining, which was faint and associated with pericellular RANKL-positive granules and matrix (Fig. 2a). In contrast, the number of mast cells was relatively low in leukoplakia and healthy control sections and RANKL staining was in these cells confined to the cytoplasm without any signs of extracellular release of RANKL (Fig. 2b).

If instead of pepsin, Tris–EDTA heat pre-treatment was used for antigen retrieval, also other cells, e.g. fibroblasts and lymphocytes, were found to be RANKL positive. These other RANKL-positive cells were more intensely stained than mast cells and they were distributed mainly in the subepithelial areas. Side-by-side comparison of pepsin pre-treated and Tris–EDTA heat pre-treated sections from the same location demonstrates nicely this difference (Fig. 2c and d).

Figure 1 Staining of oral epithelium and underlying connective tissue for CD1a in chronic hyperplastic candidosis (CHC) and leukoplakia of the oral mucosa. In CHC, CD1a-positive Langerhans cells can be seen anywhere throughout the whole epithelium (a) while in leukoplakia they are confined mainly to the middle third (b). In CHC, leukoplakia, or healthy controls, Langerhans cells can be found (arrow) lying individually (c) or organized (arrows) collectively (d). In CHC, Langerhans cells were in some samples in some areas found mainly (arrows) in the lower third of the epithelium (e) or just lying on (f) or even traversing (arrows) through the basement membrane towards or from the lamina propria (g). Original magnifications ×200 (b, c, d, e), ×400 (a, f, g).


(a)
(b)
(c)
(d)
(e)
(f)
(g)

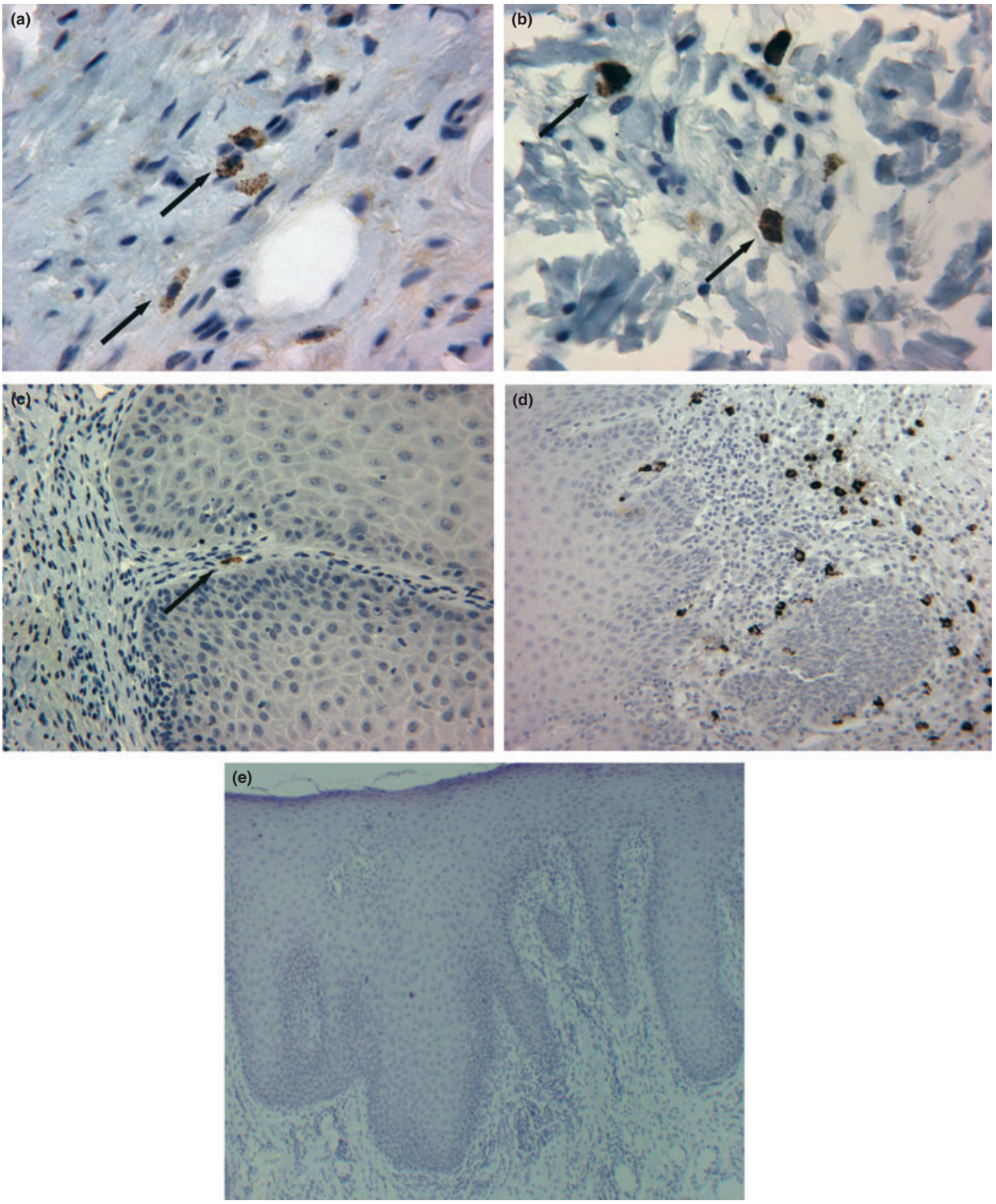


Figure 2 Staining of oral epithelium and underlying connective tissue for RANKL in chronic hyperplastic candidosis (CHC) and healthy tissue. Panel A demonstrates a magnified scene of RANKL-positive mast cells around an arteriole. The cells display a rather granular, weak staining (arrows), most likely due to the release of their granular contents extracellularly as RANKL is also seen in the pericellular area. In contrast, panel B shows that RANKL-positive mast cells in healthy samples tend to reveal a stronger, opaque staining pattern (arrows). In panel C, after use of pepsin in antigen-retrieval protocol before immunohistochemical staining, only mast cells retain their RANKL positivity, here trapped between the epithelial ridges (arrow), whereas in panel D when Tris–EDTA was used for antigen retrieval instead of pepsin, many RANKL-positive cells can be seen as the surface RANKL apparently retains its immunoreactivity. Negative control for either CD1a or RANKL did not show any staining (e). Original magnification ×200 (e), ×400 (c, d), ×600 (a, b).

Neither CD1a nor RANKL-positive cells were observed in negative staining controls (Fig. 2e), which confirmed the specificity of the cellular, pericellular, and epithelial staining.

Discussion

CD1a-positive Langerhans cells were found in mucosal sections from CHC, leukoplakia, and healthy control tissues well in accordance with their role as faithful sentinels throughout the mucosal regions of the host body. DCs and neutrophils play an essential role in extinguishing invasive *C. albicans*. Immature and mature DCs can produce chemotactic factors that activate and recruit neutrophils (17). We have examined samples from patients with CHC for the presence of CD1a-positive Langerhans cells and RANKL and compared them with leukoplakia and healthy control tissues. Leukoplakia is defined by the World Health Organization (WHO) as a white patch or plaque which cannot clinically or pathologically be characterized as any other disease (18). We also sought to see the difference in the immunological response between CHC and leukoplakia as CHC is considered as leukoplakia superimposed by *C. albicans* infection. Although CD1a Langerhans cells were found in all three study groups, their number and pattern of distribution were different, probably due to recruitment and migration.

The DC numbers are debatable in other oral infections and inflammations, e.g. chronic periodontitis. In three separate studies, it was found that the number of DCs increased (19), decreased (20), or did not change (21) in chronic periodontitis. Jotwani *et al.* has explained this discrepancy by the possibility of the difference in the stage of the disease (22). Inter- and intraindividual (intrasample) variation in the number and localization of CD1a-positive Langerhans cells was also found in the present study in CHC, which probably reflects the dynamic nature of their involvement in local disease mechanisms. We also found a close match between the Langerhans cell numbers in the epithelium of CHC and the intensity of candidal yeasts and hyphae. However, we still think that the number of Langerhans cells *per se* in such mucosal infectious lesions does not form a very important or reliable parameter to assess the severity of the infection. Otherwise, all CHC sections should have shown higher numbers of Langerhans cells than their *Candida*-negative counterparts and comparators, i.e. leukoplakia and healthy controls. To the contrary, some leukoplakia and healthy control sections did contain nearly equal or even higher Langerhans cell numbers than CHC. In CHC, however, Langerhans cells were seen in all epithelial regions including the basement membrane area at the epithelial-lamina propria junction. This disparity can be attributed to the role of Langerhans cells in capturing and engulfing the antigen, i.e. *C. albicans*, which could explain the presence of Langerhans cells in the upper part of the epithelium. Once the *C. albicans* is engulfed, Langerhans cells start to migrate away from the epithelium towards the lamina propria.

Receptor activator of nuclear factor kappa B ligand is a membrane-bound ligand which belongs to tumor necrosis factor superfamily. It is produced by osteoblasts, chondrocytes and other mesenchymal cells as well as by various immune cells, such as activated T cells (23). It binds to its receptor RANK on the surface of monocyte/macrophages, osteoclast progenitors and antigen-presenting cells leading to their differentiation (24, 25). T cells can modulate the function of DCs (26) through release of RANKL and augment the capacity of DCs to stimulate naïve T-cell proliferation in a mixed lymphocyte reaction (27). We have previously shown, using immunoblotting and immunohistochemistry methods, that naïve mast cells and mast cells generated from hematopoietic stem cells produce RANKL (16). It was found that RANKL was released peri- or extracellularly by mast cells in the atherosclerotic plaques, while it was confined within the cytoplasmic boundaries in non-sclerotic regions. Now, we proceeded with this observation to assess whether it holds true for mucosal infections involving antigen-presenting cell–T lymphocyte interactions. It was observed that when the samples are formalin fixed and paraffin embedded, followed by pepsin digestion, mast cells can be specifically studied as probably all cells containing cell surface RANKL become negative. In contrast, when Tris–EDTA heat treatment was used for antigen retrieval, also all the other RANKL-positive cells stain, including lymphocytes and fibroblasts, could be seen. The reason might lie in the different location of the antigen (RANKL) in different cells, as well as to the mechanism of action of the antigen-retrieving agent. Pepsin is a proteolytic enzyme so it entails the risk of cleaving some epitopes on the cell surface. Pepsin appears to destroy most of RANKL proteins which had been expressed on cell surfaces. In mast cells, however, RANKL is stored in cytoplasmic granules. As pepsin cannot penetrate plasma membranes (either cellular or granular) composed mostly of lipid, RANKL proteins produced by mast cells avoid the action of pepsin. The Tris–EDTA-based solution, on the other hand, is designed to break the protein cross-links, without endangering the antigen proper itself. Thus, all RANKL-positive cells were seen after this method of antigen retrieval. This type of 'fixation artifact' can be used if in particular the eventual role of mast cell RANKL is in focus of interest.

In CHC, RANKL-positive mast cells were weakly stained due to extracellular release of their RANKL-laden granules, while in leukoplakia and healthy controls RANKL staining in resting mast cells was homogeneous and completely intracellular. We suggest that this mast cell activation and RANKL release strengthens and speeds up the maturation of the RANK-positive Langerhans cells, when they communicate with the RANKL-positive T cells.

References

1. Appleton SS. Candidiasis: pathogenesis, clinical characteristics, and treatment. *J Calif Dent Assoc* 2000; **28**: 942–8.

2. Rossie K, Guggenheimer J. Oral candidiasis: clinical manifestations, diagnosis, and treatment. *Pract Periodontics Aesthet Dent* 1997; **9**: 635–41; quiz 642.
3. Odds FC. Pathogenesis of candida infections. *J Am Acad Dermatol* 1994; **31**: S2–5.
4. Weig M, Gross U, Muhlschlegel F. Clinical aspects and pathogenesis of candida infection. *Trends Microbiol* 1998; **6**: 468–70.
5. Farah CS, Ashman RB, Challacombe SJ. Oral candidosis. *Clin Dermatol* 2000; **18**: 553–62.
6. Filler SG, Swerdloff JN, Hobbs C, Luckett PM. Penetration and damage of endothelial cells by *Candida albicans*. *Infect Immun* 1995; **63**: 976–83.
7. Pfaller M, Wenzel R. Impact of the changing epidemiology of fungal infections in the 1990s. *Eur J Clin Microbiol Infect Dis* 1992; **11**: 287–91.
8. Cawson RA, Lehner T. Chronic hyperplastic candidiasis – candidal leukoplakia. *Br J Dermatol* 1968; **80**: 9–16.
9. Bartie KL, Williams DW, Wilson MJ, Potts AJ, Lewis MA. Differential invasion of *Candida albicans* isolates in an in vitro model of oral candidosis. *Oral Microbiol Immunol* 2004; **19**: 293–6.
10. Bartie KL, Williams DW, Wilson MJ, Potts AJ, Lewis MA. PCR fingerprinting of *Candida albicans* associated with chronic hyperplastic candidosis and other oral conditions. *J Clin Microbiol* 2001; **39**: 4066–75.
11. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245–52.
12. Silberberg-Sinakin I, Thorbecke J. Contact hypersensitivity and Langerhans cells. *J Invest Dermatol* 1980; **75**: 61–7.
13. Daniels TE. Human mucosal Langerhans cells: postmortem identification of regional variations in oral mucosa. *J Invest Dermatol* 1984; **82**: 21–4.
14. Daniels TE, Schwartz O, Larsen V, Dabelsteen E, Pindborg J. Langerhans cells in candidal leukoplakia. *J Oral Pathol* 1985; **14**: 733–9.
15. Newman SL, Holly A. *Candida albicans* is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. *Infect Immun* 2001; **69**: 6813–22.
16. Ali A, Lax A, Liljeström M et al. Mast cells in atherosclerosis as a source of the cytokine RANKL. *Clin Chem Lab Med* 2006; **44**: 672–4.
17. Scimone ML, Lutzky VP, Zittermann SI et al. Migration of polymorphonuclear leucocytes is influenced by dendritic cells. *Immunology* 2005; **114**: 375–85.
18. Sciubba JJ. Oral leukoplakia. *Crit Rev Oral Biol Med* 1995; **6**: 147–60.
19. Saglie FR, Pertuiset JH, Smith CT et al. The presence of bacteria in the oral epithelium in periodontal disease. III. Correlation with Langerhans cells. *J Periodontol* 1987; **58**: 417–22.
20. Seguier S, Godeau G, Brousse N. Immunohistological and morphometric analysis of intra-epithelial lymphocytes and Langerhans cells in healthy and diseased human gingival tissues. *Arch Oral Biol* 2000; **45**: 441–52.
21. Gemmell E, Carter CL, Hart DN, Drysdale KE, Seymour GJ. Antigen-presenting cells in human periodontal disease tissues. *Oral Microbiol Immunol* 2002; **17**: 388–93.
22. Jotwani R, Cutler CW. Multiple dendritic cell (DC) subpopulations in human gingiva and association of mature DCs with CD4+ T-cells in situ. *J Dent Res* 2003; **82**: 736–41.
23. Hofbauer LC, Shui C, Riggs BL et al. Effects of immunosuppressants on receptor activator of NF-kappaB ligand and osteoprotegerin production by human osteoblastic and coronary artery smooth muscle cells. *Biochem Biophys Res Commun* 2001; **280**: 334–9.
24. Burgess TL, Qian Y, Kaufman S et al. The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol* 1999; **145**: 527–38.
25. Matsuzaki K, Udagawa N, Takahashi N et al. Osteoclast differentiation factor (ODF) induces osteoclast-like cell formation in human peripheral blood mononuclear cell cultures. *Biochem Biophys Res Commun* 1998; **246**: 199–204.
26. Josien R, Wong BR, Li HL, Steinman RM, Choi Y. TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J Immunol* 1999; **162**: 2562–8.
27. Anderson DM, Maraskovsky E, Billingsley WL et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 1997; **390**: 175–9.