Oral Diseases (2004) 10, 106–112

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Periodontology/Oral Microbiology

Candida yeasts in chronic periodontitis tissues and subgingival microbial biofilms *in vivo*

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The frequency of *Candida* infection in periodontal tissues of chronic periodontitis (CP) patients and the extent of candidal penetration into gingival tissues was studied. Tissue specimens collected from 25 CP patients during periodontal flap operations of initial periodontal therapy were examined by immunohistochemistry using *Candida albicans*-specific antibodies. Sections were also stained with periodic acid-Schiff (PAS) and subgingival plaque samples from 17 patients were cultured. Immunoreactivity for *Candida* was present in four of the 25 CP specimens (16%). Only one yeast-positive specimen was found when PAS-staining was used (4%) and two yeast-positive specimens were found with plaque culture (8%). Hyphal formation seemed essential and candidal hyphae were found to extend into the periodontal connective tissue. The degree and type of inflammation adjacent to the hyphae varied from a site and a patient to another. The sensitivity of specific antibodies was superior to PAS-stain or plaque culture in detection of *Candida* in tissues.
Oral Diseases (2004) **10**, 106–112

Keywords: *Candida albicans*; monoclonal antibody 3H8; polyclonal antibody 158; chronic periodontitis; immunohistochemistry

Introduction

Periodontitis is an infection-induced inflammatory disease characterized by irreversible destruction of the tooth-supporting tissues (pocket epithelium, gingival connective tissue, periodontal ligament and alveolar bone) (Hamada, Holt and McGhee, 1991). Untreated periodontitis eventually may lead to tooth loss. Human periodontitis is associated with a widely diverse and complex subgingival microbiota encompassing both Gram-positive and Gram-negative bacteria, facultative and anaerobic organisms, and possibly yeasts. At least nearly 500 bacterial strains have been recovered from the subgingival crevice, a particularly well-studied microbial niche (Van Winkelhoff, Rams and Slots, 1996; Kroes, Lepp and Relman, 1999). Most of these strains are thought to be commensals, and a smaller number, potential opportunistic pathogens. The ability of one microbe to cause disease is greatly affected by the composition of the microbiota of the site. Chronic periodontitis (CP) results from a complex interplay of the mixed polymicrobial infection and host response. The adherent microbes evoke release of a number of inflammatory mediators in the underlying soft tissues. In fact, these activation products ultimately result in the destruction of host tissue (Chen *et al*, 2000).

Candida yeasts are opportunistic pathogens that cause disease in hosts who are compromised by underlying local or systemic pathological processes (Richardson, 1992). Candidosis is the most common fungal infection in humans after dermatophytosis. *Candida albicans* is an aerobic commensal, which can be cultured from the oral cavity of nearly every other adult (Arendorf and Walker, 1979; Odds, 1988). In the oral cavity, yeasts commonly colonize the tongue, palate, and buccal mucosa (Arendorf and Walker, 1980) and may occur in subgingival plaque of adults with severe periodontitis (Slots, Rams and Listgarten, 1988). Yeasts, especially *C. albicans*, have been recovered from periodontal pockets in a large number (7.1–19.6%) of patients with CP (Slots *et al*, 1988; Dahlén and Wikström, 1995; Rams, Flynn and Slots, 1997; Reynaud *et al*, 2001). In a recent survey by Reynaud *et al* (2001)) the prevalence of subjects with yeasts in the examined periodontal pockets was overall 15.6%. Using the electron microscope, yeasts were found to be invading in the gingival connective tissue of 26 of 60 samples from 12 patients

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Received 9 April 2003; revised 22 August 2003; accepted 25 September 2003

with juvenile periodontitis (JP) (González *et al*, 1987). Similar findings have not been reported of CP until now. It has been suggested that *Candida albicans* may contribute to the development of necrotizing periodontal diseases in HIV-infected patients (Odden *et al*, 1994). In addition to periodontal diseases (Rams and Slots, 1991), oral yeasts have been related with enamel (Hodson and Craig, 1972) and root caries (Lynch and Beighton, 1994).

The diagnosis of cutaneous and mucosal candidosis depends largely on the identification of yeast pseudomycelial forms in tissue samples by using periodic acid-Schiff (PAS) and Gomori methenamine silver (GMS) stains. However, these stains are non-specific and also reveal confusing artefacts seemingly rather difficult to distinguish from yeasts. The structural similarities between different fungi are a further source of diagnostic difficulties. Histopathological techniques evidently do not identify pathogenic fungi to the species level. To enhance the *in situ* identification of fungi in clinical specimens, a number of both direct and indirect immunohistochemical techniques have been developed, notably immunoperoxidase-based methods (Jensen *et al*, 1996). With the recent development and availability of monoclonal antibodies (mAb) to various epitopes of *C. albicans* and *C. dubliniensis* (Marcilla *et al*, 1999) it is now possible to identify *Candida* in human tissue biopsies to species level. Although the role of yeasts in CP is largely unclear there is evidence to suggest that yeasts can be implicated in the pathogenesis of the tissue destructive periodontal disease process. With this background we studied the frequency of *Candida* infection in periodontal tissues of CP patients and the extent of Candidal penetration to the gingival tissue by using immunohistochemical techniques.

Materials and methods

Patient samples

Samples of diseased gingiva were collected from 25 patients (15 women and 10 men; 53.4 ± 11.0 years) with moderate to severe generalized adult type CP as judged by clinical measurements (Table 1). The CP patients chosen had radiographic evidence for bone loss of 20–50% on many teeth and a mean loss of attachment ranging from 4 to 6 mm. Samples were obtained from premolar–molar regions during flap surgery of the initial periodontal therapy and consisted of oral and sulcular epithelium, subgingival plaque, and underlying connective tissue. The clinical periodontal examination was undertaken by using a pressure-sensitive periodontal probe set to give a probing force of 20 g. All teeth present were examined at six sites and periodontal pockets over 3.5 mm were recorded. The Community Periodontal Index of Treatment Needs (CPITN) (Ainamo *et al*, 1982), presence of suppuration and bleeding on probing were also recorded before the periodontal treatment. After careful dissection gingival samples were rinsed with 0.9% NaCl before fixation in 10% formaldehyde. All specimens were immediately formalin-fixed and subsequently paraffin-embedded at the Department of Oral Pathology, Institute of Dentistry, University of Helsinki. Subgingival plaque samples taken by curettage from deep suppurating periodontal pockets from 17 of the 25 patients were cultured as part of routine diagnostics (Oral Microbiology, Helsinki University Central Hospital Laboratory Diagnostics, Helsinki, Finland). The study was approved by the ethical committee of the Institutes of Dentistry, University of Helsinki and the subjects were enrolled into the study and treated in compliance with the Helsinki Agreement as revised in 1983.

Table 1 Age, gender and periodontal characteristics of study subjects

Subjects	*Candida albicans*-positive ($n = 4$)	*Candida albicans*-negative ($n = 21$)
Age (years)		
Mean ± s.d.	54.8 ± 13.2	53.0 ± 11.1
Range	36–67	36–76
Gender ratio		
Female/male	2/2	8/13
CPITN index (before treatment)		
Mean[a] ± s.d.	3.0 ± 0.3	3.0 ± 0.5
Range	2–4	2–4
CPITN index at sample site		
Mean[b] ± s.d.	3.5 ± 0.6	3.7 ± 0.5
Range	3–4	3–4

[a]The average of all values of each subject.
[b]The average of the values at the sample sites.
CPITN, Community Periodental Index of Treatment Needs.

Antibodies and reagents

Mouse mAb designated 3H8 and rabbit polyclonal antibody (pAb) designated 158 were used as primary antibodies to detect *C. albicans*. The 3H8 mAb (IgG1; 2.5 mg ml^{-1}; a generous gift from Prof. R. Robert at Société de Recherche et de Réalisations Biotechnologiques, Paris, France) is raised against a Zymolyase-solubilized preparation from blastoconidia cell walls of *C. albicans* ATCC26555 and recognizes high molecular mannoproteins present in the cell wall (Marcilla *et al*, 1999). The 158 pAb (4.5 mg ml^{-1}; Biodesign International, Saco, ME, USA) recognizes numerous proteins in a soluble *C. albicans* extract. It has minimal cross-reactivity with human serum, urine and spinal fluid but does cross-react with other yeasts as it has not been absorbed. Biotinylated anti-mouse or anti-rabbit IgG from Vectastain® kit (Vector Laboratories, Burlingame, CA, USA) were used as secondary antibodies. Phosphate-buffered saline (PBS) was used as a buffer. In some instances, bovine serum albumin (BSA, Behringverke GmbH, Germany) was added to PBS (PBS-BSA) to reduce non-specific reactions.

Periodic acid-Schiff stain

Four micrometre-thick, formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol series and in water. Before staining with Schiffs' leuco-fuchsin reagents the sections were first exposed to periodic acid.

Immunohistochemical staining

Four micrometer-thick, formalin-fixed, paraffin-embedded sections were deparaffinized as described above. The sections were then incubated with pepsin for 45 min in a humid chamber and washed three times for 5 min with PBS. To inhibit endogenous peroxidase activity the sections were then incubated with 0.3% H_2O_2 in methanol for 30 min followed by three washes with PBS. A modification of the Vectastain® kit protocol was used. To inhibit non-specific staining the sections were first incubated with normal horse serum from the kit (1:50 in 2% PBS-BSA). Then the sections were incubated with the primary antibody (3H8 or 158) against *C. albicans* (1:500 and 1:5000 accordingly in 1% PBS-BSA) for 30 min at 37°C and then kept overnight at 4°C in a humid chamber. Control stainings were performed by omitting the primary antibody. The next day after three washes the sections were incubated for 30 min at 37°C with the corresponding biotinylated anti-mouse or anti-rabbit secondary antibody from the kit (1:200 in 0.1% PBS-BSA). The sections were then washed and incubated with the kit reagent for 30 min at 37°C and then washed again. Peroxidase-binding sites were revealed with 3-amino-9-ethylcarbazole (AEC) with 0.03% hydrogen peroxidase. Finally, the slides were washed with tap water and counterstained with Mayer's haematoxylin for 4 min and then rinsed with tap water before mounting with glycerol (Dako Corporation, Carpinteria, CA, USA). The specimens were examined with a Olympus BX light microscope and a Hamamatsu $ORCA_{IIIm}$ digital colour camera (Hamamatsu Photonics, Hamamatsu city, Japan) together with an OPENLAB 2.2.5 imaging application (Improvision, Coventry, UK).

Plaque culture

Subgingival plaque samples were collected from deep suppurating periodontal pockets (> 5 mm) prior to flap surgery operations and antimicrobial therapy. The samples were transported in viability-maintaining Gothenburg anaerobe medium (VMGA III medium) and 10-fold dilutions (10^0–10^{-5}) were cultured routinely aerobically on horse blood and trypsin soy supplemented with bacitracin and vancomycin (TSBV) agars and on Sabouraud's agar, and anaerobically on enriched brucella blood agar at the Oral Microbiology Unit of the Helsinki University Central Hospital Laboratory Diagnostics. After 3 days of aerobic incubation yeasts and *Actinobacillus actinomycetemcomitans* were identified using standard morphological and biochemical methods, and the proportion of *C. albicans* of all culturable yeasts was calculated. After 7 days of anaerobic incubation *Porphyromonas gingivalis, Prevotella intermedia/nigrescens, Bacteroides forsythus, Peptostreptococcus micros* and *Campylobacter rectus*-bacteria were isolated. Bacterial identification was based on the colony morphology and pigmentation, staining and biochemical reactions.

Results

Gingival tissue specimens were collected from 25 CP patients (15 women and 10 men; 53.4 ± 11.0 years). Four CP samples (16%; two women and two men; 54.8 ± 13.2 years) showed positive staining for *C. albicans* either with the pAb or mAb. Predominantly, pseudomycelial forms were immunoreactive. Only one yeast-positive specimen was found when PAS-staining was used (4%; patient 4) and two yeast-positive specimens were found with plaque culture (8%; patients 1 and 2). In general, the pAb gave a stronger signal than the monoclonal and as it had not been absorbed it could cross-react with other yeasts. The pAb also appeared to detect *Candida*-derived antigens in the periodontal tissues in addition to the specific staining of hyphal elements and candidal cells. With the mAb staining of the surrounding tissues could not be detected.

Subgingival plaque was included in most patients samples. Three of four yeast-positive samples included plaque in all of which a large number of candidal hyphae could be demonstrated (Figure 3a,b). In none of the yeast-positive samples could hyphal penetration into the epithelium be seen. However, in one case *Candida* structures could be detected in an intraepithelial tear (Figure 4d). The epithelium was often detached from the underlying connective tissue (Figures 1a, 3 and 4d). The *Candida* hyphae were typically found at the border of the epithelium and the underlying connective tissue (Figures 1a and 2d). Hyphal elements also appeared to be attached to the periodontal connective tissue (Figures 1a, 2b and 4c).

In three cases strong, chronic and compact inflammatory cell infiltrates could be seen in the vicinity of the candidal hyphae (Figures 1–3). In one case the inflammation was mixed and moderate (Figure 3c). In general, the density of inflammatory cells adjacent to the candidal structures was higher in the deeper parts of the specimens. In two patient samples the degree of the local inflammatory reaction varied greatly from one infected site to another. Thus, areas of very little inflammatory cell infiltrate adjacent to hyphae were also detected (Figures 1b and 4b). The inflammatory cell infiltrate typically composed of chronic mononuclear inflammatory cells (Figures 1d, 2b and 4c). However, occasional neutrophilic leucocytes could also be detected (Figure 3c).

Subgingival plaque samples from deep suppurating periodontal pockets from 17 of the 25 patients were cultured as part of routine diagnostics. An average of three bacterial pathogens, namely, *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Prevotella intermedia/nigrescens, Bacteroides forsythus, Peptostreptococcus micros* and *Campylobacter rectus* were isolated. In addition, two patient samples showed fungal growth on bacteriological agar. Both these patients were found to be *C. albicans*-positive also by immunohistochemistry (patients 1 and 2).

Discussion

Candida albicans is regarded as the potentially most pathogenic fungus normally found in the oral cavity. In tissues it appears mostly in pseudohyphal form and can tentatively be distinguished from other fungi by its

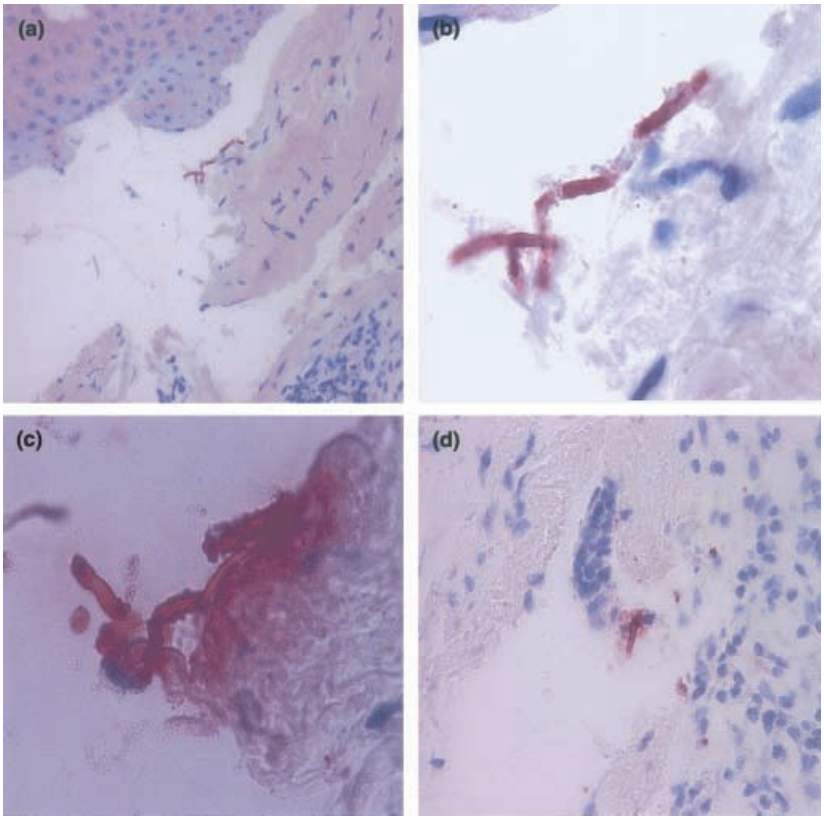

Figure 1 Adherence of *Candida albicans* hyphae to the periodontal connective tissue of patient 1. With the monoclonal antibody three to four *Candida* hyphae can be seen on the surface of connective tissue which has become detached from the overlaying epithelium. This group of *Candida* hyphae is surrounded by a mild inflammatory reaction (a, ×200; b, ×1000). Staining with the polyclonal antibody demonstrates six to eight *Candida* hyphae in almost the same localization also without a remarkable inflammatory reaction (c, ×1000). In a deeper part of the specimen one hypha surrounded by a rather strong compact inflammatory cell infiltrate can be seen (d, ×400). This infiltrate seems to consist mainly of mononuclear inflammatory cells

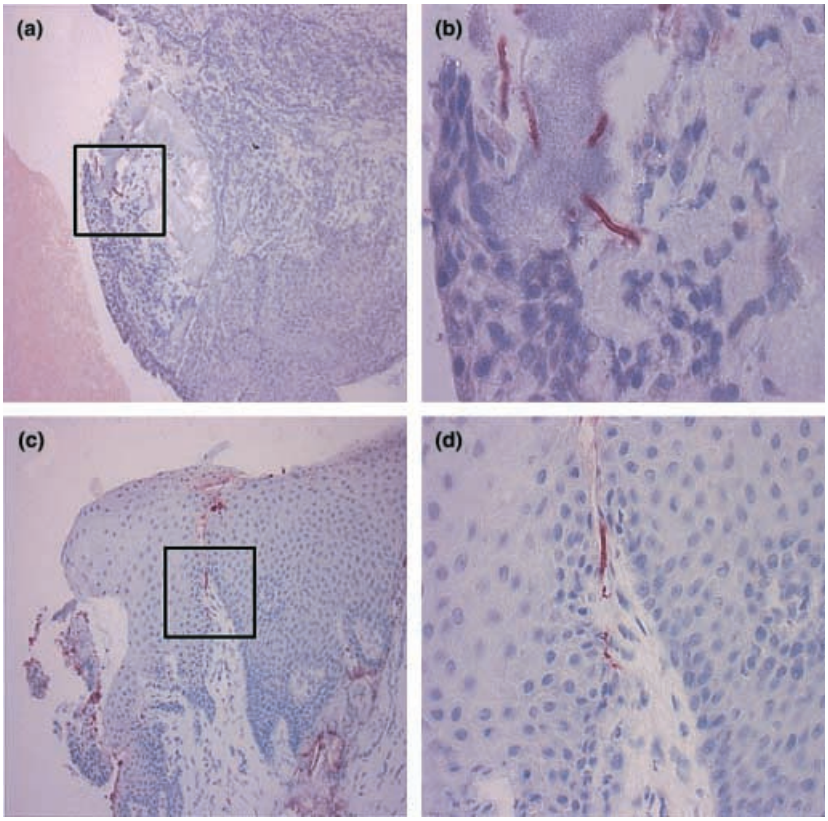

Figure 2 Immunolocalization of *Candida* hyphae in gingival pockets of patient 2. With the monoclonal antibody approximately four to five *Candida* hyphae can be seen in the vicinity of the periodontal pocket (a, ×200 and enlargement of selected area; b, ×1000). They are located in a bacterial plaque near the pocket epithelium and the underlying connective tissue. At least one hyphal element appears to be penetrating into the connective tissue in which a strong, chronic inflammatory reaction is visible (b). With the polyclonal antibody one single *Candida* hypha can be detected in another region of the section. This hypha is aligned along the border between the periodontal connective tissue and the epithelium without causing a remarkable inflammatory reaction (c, ×200 and enlargement of selected area; d, ×600)

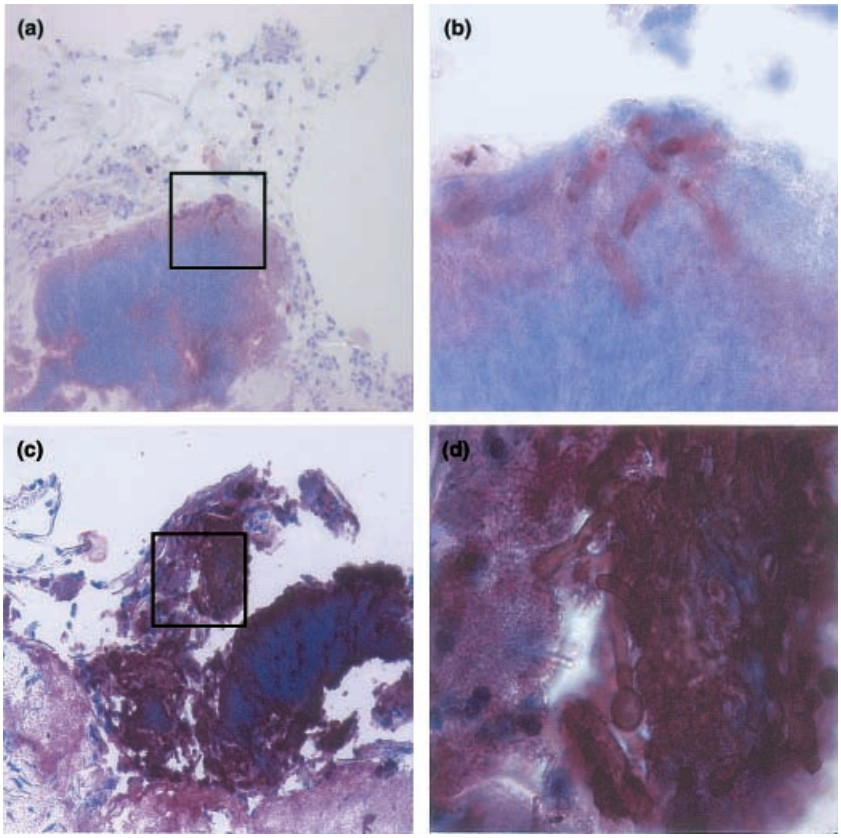

Figure 3 *Candida* hyphae in the plaque of an ulcerated gingival pocket of patient 3. A bacterial plaque deposit is surrounded by connective tissue and some remaining parakeratinized squames of the pocket epithelium. Staining with the monoclonal antibody reveals *Candida* hyphae in the periphery of the plaque (a, ×200 and enlargement of selected area; b, ×1000). A moderate inflammatory cell infiltrate composed of both acute and chronic cells can be seen in the surrounding tissues (a). The same plaque from a slightly different direction can also be seen with the polyclonal antibody (c, ×200 and enlargement of selected area; d, ×1000). The polyclonal antibody gives a much stronger signal than the monoclonal antibody and a large number of *Candida* hyphae can be demonstrated in the plaque. The surrounding inflammatory cell infiltrate mostly consists of mononuclear inflammatory cells although occasional neutrophilic leucocytes are present

morphology. *Candida albicans* can also appear as yeast cell forms, especially when colonizing epithelial surfaces. Therefore, more specific methods, like immunohistochemistry, are necessary for precise identification. Changes in the environmental conditions trigger germination. The gingival pocket and gingival crevicular fluid are favourable environments for germination and hyphal growth of *Candida*. *Candida* hyphae have the ability to penetrate host tissue, and hyphae adhere to host surfaces to a greater extent compared with yeast cells. Thus, hyphae are important in the attachment and disease process.

Our findings suggest that *C. albicans* could have a role in the infrastructure of periodontal microbial plaque and in its adherence to the periodontal tissues. Unlike most microbes, *Candida* is rather tolerant to innate and cell-mediated immunity although it provokes inflammation in the surrounding tissues. In our study *Candida* was typically found in the outer layers of the plaque and seemed to act as a barrier between the host immunity and the inner layers of the mixed biofilm. *Candida albicans* may thus have a role in the immune evasion of the plaque in periodontal infections and in the provocation of destructive inflammation in the underlying tissues.

Our results also indicate that hyphal germination starts in the gingival pocket. *Candida* hyphae were typically found at the border of the sulcular epithelium and in the underlying connective tissue and the number of candidal hyphae seen was generally rather low. Interestingly, fungal elements could not be seen in the epithelium. Nor were obvious yeast forms seen in the samples. The predominance of hyphal forms in the samples support the visual finding of candidal tissue penetration and attachment in contrast to technical artefacts or smears. The patients participating into this study were immunocompetent and had not been under any local or systemic antimicrobial medication prior to the flap operations. Therefore, it is interesting that fungi were seen deep in the periodontal tissues. The presence of the hyphae in the connective tissue can be explained either by the pathogenicity of *C. albicans* or by the local immunesuppression caused by the severe periodontal disease. The lack of candidal structures in the epithelium may be coincidential and as a result of the small number of *C. albicans* seen in the tissues in general. Evidently the fungus must have passed the epithelium to invade the underlying connective tissue. *Candida albicans* can express both cell surface-bound and secreted proteinases capable of degrading major extracellular matrix and basement membrane components such as collagens and fibronectin (Kaminishi *et al*, 1986; El-Moudni *et al*, 1995; Rodier *et al*, 1999). These *C. albicans* proteinases can focally also induce the tissue destructive host cell proteinase network, similarly to proteases expressed by certain oral bacteria such as *P. gingivalis* (Uitto *et al*, 1989; Ding *et al*, 1997).

The mAb used was raised against a *C. albicans* mannoprotein (Marcilla *et al*, 1999). This work presented here suggests that the mannoprotein antigen is

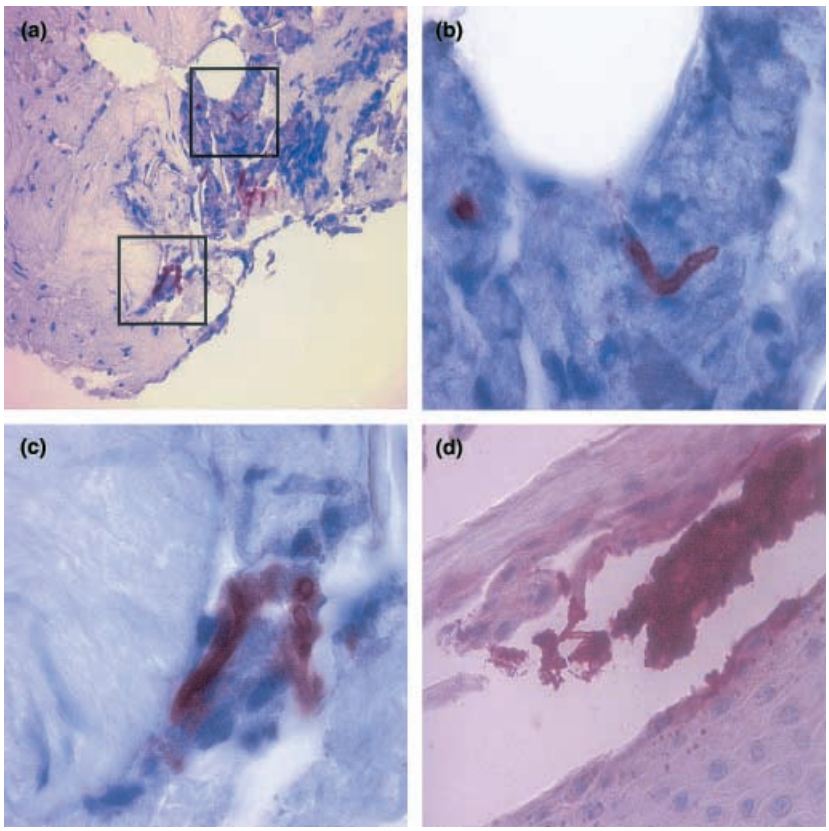

Figure 4 Adherence of *C. albicans* hyphae to the periodontal tissues of patient 4. In a cross-section of a gingival papilla two distinct areas showing *Candida* hypha can be demonstrated by using the monoclonal antibody (a, ×200, enlargement of the upper selected area; b, ×1000 and of the lower selected area; c, ×1000). In the upper selected area one *Candida* hypha attached by its tip to the connective tissue can be seen (b). In this area no remarkable inflammatory response is present. However, in the lower selected area the hyphal elements are surrounded by a rich mononuclear cell infiltrate (c). In another region of the section an intraepithelial deposit of *Candida* hyphae can be seen in a tear of the epithelium using the polyclonal antibody (d, ×400)

expressed in greater quantity on the surface of the hyphae than on the yeast cells and that the antigen can also be found intracellularly. Others have shown that mannoproteins are not expressed on the surface of *Candida* hyphal elements (Sanjuán *et al*, 1995). In periodontitis it may be that the expression of such an immunologically relevant mannoprotein does allow *C. albicans* to evade the local specific and non-specific immunoresponses, in contrast to other tissue types and organs. Although studies of antigenic variability in *C. albicans* are few it is apparent that *Candida* surface antigens are not expressed equally among different strains. In addition, surface antigens appear to be expressed dynamically as a function of their metabolic and morphological state during yeast and hyphal growth *in vitro*. The same differential expression may occur *in vivo*, as well. In the present work it is quite clear that the hyphal elements of *C. albicans* stained equally among the four patients with *Candida*-associated periodontitis. Although it has been shown that a single strain of *C. albicans* may undergo antigenic variation during pathogenesis it seemed that the hyphal elements of the *C. albicans* strains demonstrated here stained equally with either the polyclonal or mAb used. The pAb, which recognizes numerous proteins in soluble candidal extracts, revealed the extent of spreading of *Candida*-derived proteins in the plaque and periodontal tissues.

In general, the clinical, bacteriological and histological findings of the *C. albicans*-infected patients did not significantly differ from the other CP patients of this study. The histology of all the samples was a typical view of CP. The candidal structures had evoked a variable amount of inflammation in their surroundings. In three cases strong, chronic and compact inflammatory cell infiltrates could be seen in the vicinity of the candidal hyphae. In one case the inflammation was mixed and moderate. In general, the density of inflammatory cells adjacent to the candidal structures was higher in the deeper parts of the specimens. In two patient samples the degree of the local inflammatory reaction varied greatly from one infected site to another. Thus, areas of very little inflammatory cell infiltrate adjacent to hyphae were also detected. The inflammatory cell infiltrate typically composed of chronic mononuclear inflammatory cells. However, occasional neutrophilic leucocytes could also be detected.

Our results are in accordance with the results of previous studies that yeasts can be found in some 15% of patients with chronic periodontal diseases. The sensitivity of specific antibodies was superior to PAS-stain or plaque culture in detection of *Candida* in tissues. Only one yeast-positive specimen was found when PAS-staining was used (4%) and two yeast-positive specimens were found with plaque culture (8%). The role played by *C. albicans* in periodontal diseases is not clear and further studies are needed to demonstrate the clinical significance of the findings. However, only a few species of microorganisms normally found in diseased gingival pockets can penetrate the epithelia surface and provoke inflammation. To our knowledge there are only a few previous publications that demonstrate that yeasts are present in

gingival tissue (González *et al*, 1987; Odden *et al*, 1994). The current work supports the contention that *Candida* can be part of the periodontal disease process.

Acknowledgements

This study was supported by the Finnish Dental Society Apollonia, the Sigrid Juselius Foundation, the Wilhelm and Else Stockmann Foundation, the HUCH-EVOCT1020 Y0002 and TIH 4113J grants, the Academy of Finland and the University of Helsinki.

Authors gratefully acknowledge Marjatta Kivekäs for technical assistance with the biopsies and Kristina von Boguslawski for the generous gift of the polyclonal antibody.

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