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ORIGINAL ARTICLE

Specificity of the monoclonal antibody 3H8 in the immunohistochemical identification of *Candida* species

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Candida albicans **has been shown to be involved in the pathogenesis of adult periodontitis (AP). The diagnosis of *Candida*-associated AP depends largely on the identification of yeast and pseudomycelial forms in gingival tissue samples by using periodic acid-Schiff and Gomori methenamine silver stains. However, these stains are non-specific and also reveal confusing artifacts seemingly rather difficult to distinguish from yeasts. With the recent development and availability of monoclonal antibodies (Mabs) to various epitopes of *C. albicans*, for example Mab 3H8 which recognizes a mannoprotein, it is now possible to identify *Candida* in human tissue biopsies. To explore further the usefulness of this Mab in detecting *Candida* in periodontal disease the antibody was tested against a wide range of yeast species and strains and various morphological forms, grown in agar blocks at various temperatures and for various time periods. Furthermore, considering the location of the 3H8 epitope on the external cell wall of certain *C. albicans* strains, it seemed reasonable to determine whether the epitope could be expressed into the surrounding environment, further aiding the recognition of the organism in tissue. The 3H8 epitope appeared to be located at the external surface and on the septum between the mother cell and germ tube of some *C. albicans* strains but it was partially cryptic in the cell wall of other strains. Both yeast blastoconidia and pseudohyphae were labeled by the 3H8 antibody. *Candida lusitaniae*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* did not posses the epitope. The epitope was expressed extracellularly by both blastoconidia and pseudohyphae of *C. albicans*. This Mab appears to be suitable for the identification of *C. albicans* in periodontal tissue and may provide further insight into the role of *C. albicans* in the pathogenesis and diagnosis of periodontal diseases.**
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Introduction

Yeasts are opportunistic pathogens and common members of the normal oral flora in humans. *Candida albicans* is the most common yeast species in the human oral cavity. The transition from commensal to a harmful pathogenic state eventually depends on a decrease in host resistance, changes in the local microbiologic ecology, or changes in intrinsic fungal virulence (Samaranayake, 1990). Histopathologic changes related to the infection include inflammatory cell infiltration of the epithelium and connective tissue, intra-epithelial microabscesses, epithelial intercellular edema, and epithelial atrophy, hyperplasia and dysplasia (Budtz-Jørgensen, 1990). In the oral cavity yeasts can be found on mucosal surfaces and in saliva, and in the inflamed periodontal pockets. The sensitivity of immunohistochemistry using a monoclonal antibody was superior to periodic acid-Schiff stain or plaque culture in detection of Candida in tissues. (Järvensivu *et al*, 2004). Occasionally, other species of *Candida*, such as *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *C. krusei* have been implicated in periodontal diseases (Hannula *et al*, 1997; Ito *et al*, 2004). Even though the role of yeast in periodontitis is largely unknown, there is some evidence to suggest that yeasts may be part of the disease process (Järvensivu *et al*, 2004).

The monoclonal antibody 3H8 has been shown to recognize mannoproteins of high molecular mass present in the *C. albicans* cell wall. By using enzyme-linked immunosorbent assay, it has been shown that the presence of the epitope recognized by the monoclonal antibody 3H8 was similar in both yeast and mycelial cell

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walls of *C. albicans* (Marcilla *et al*, 1999). Immunohistochemical studies using this antibody have demonstrated its usefulness in specifically recognizing *C. albicans* in the kidney, lung, thyroid, esophagus, small bowel and gingival tissues (Marcilla *et al*, 1999; Järvensivu *et al*, 2004). To explore further the usefulness of this antibody in detecting *Candida* in periodontal diseases we wished to test the antibody against a wide range of yeast strains and species and various morphological forms. Furthermore, considering the location of the 3H8 epitope on the external cell wall of some *C. albicans* strains (Marcilla *et al*, 1999) it seemed appropriate to determine whether the epitope could be expressed into the surrounding environment, further aiding the recognition of the organism in tissues. Because of the limited number of tissue biopsies from patients with *Candida*-associated periodontal diseases (Järvensivu *et al*, 2004) we instead used an agar block technique where *Candida* species were grown under varying environmental conditions in agar which was then processed in a manner similar to that used for pathology tissue specimens. The 3H8 epitope appears to be located on the external surface of some *C. albicans* strains but was partially cryptic in the cell wall of other strains. It is likely that the epitope is expressed extracellularly. The use of agar blocks makes it easier to detect these epitopes because there are no interfering tissue structures in the background.

Materials and methods

Strains and condition of growth
Candida species were maintained on Sabouraud dextrose agar and suspended in sterile distilled water at an appropriate concentration.

Antibodies and reagents
Mouse monoclonal antibody designated 3H8 was used as the primary antibody. 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, and available on request) was raised against a zymolyase-solubilized preparation from blastoconidial cell walls of *C. albicans* ATCC 26555 and recognizes high molecular mannoproteins (HMM) present in the cell wall (Marcilla *et al*, 1999). Biotinylated anti-mouse IgG from Vectastain® kit (Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody. Phosphate-buffered saline (PBS) was used as a buffer. In some instances, bovine serum albumin (BSA; Behringverke Gmbh, Marburg, Germany) was added to PBS (PBS–BSA) to reduce non-specific reactions. Control stainings were performed by omitting the primary antibody.

Agar block culture and histochemical processing
One milliliter of yeast suspension in either normal human serum or RPMI-1640 was mixed with 1 ml of 2% or 4% warm purified agarose (Pharmacia, Uppsala, Sweden) and poured into individual compartments of plastic repli dishes (Bibby Sterilin Ltd, Stone, Staffordshire, UK) and incubated for specified periods. After solidifying, the embedded agarose material was fixed in 10% formal saline for 24 h before processing through graded concentrations of alcohol and xylene and embedded in paraffin wax.

Immunohistochemical staining

Four micrometer-thick, formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol series and in water. Sections were incubated in pepsin (5 mg pepsin + 5 ml H_2O + 50 μl 1 N HCl) for 45 min in a humid chamber and washed three times for 5 min in PBS. Endogenous peroxidase activity was inhibited with 0.3% H_2O_2 in methanol for 30 min, then the sections were washed with PBS three times for 5 min. A modification of the Vectastain® Kit protocol was used. The sections were first incubated in normal horse blocking serum from the kit diluted 1:50 in 2% PBS–BSA. The sections were then incubated with the monoclonal primary antibody (3H8, 1:500, diluted 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 the specimens had been washed three times, the sections were incubated for 30 min at 37°C with biotinylated anti-mouse IgG secondary antibody solution from the kit diluted 1:200 in 0.1% PBS–BSA. After three washes the sections were incubated with the kit reagent for 30 min at 37°C and then washed three times with buffer. Peroxidase binding sites were revealed with 3-amino-9-ethylcarbazole with 0.03% hydrogen peroxidase. Finally, the slides were washed with tap water and then counterstained with Mayerś hematoxylin for 4 min and again rinsed with tap water before mounting in glysergel (DAKO Corporation, Carpinteria, CA, USA). The specimens were examined with an Olympus BX light microscope (Olympus Corporation, Tokyo, Japan) and a Hamamatsu $ORCA_{IIIm}$ digital color camera (Hamamatsu Photonics, Hamamatsu City, Japan) together with an Openlab 2.2.5 imaging application (Improvision, Coventry, UK).

Expression of antigens by Candida species
To test the specificity of the monoclonal antibody 3H8 to detect HMM expressed by different *Candida* species, 12 isolates of *C. albicans*, six isolates of *C. lusitaniae*, five isolates of *C. glabrata*, four isolates of *C. parapsilosis*, three isolates of *C. krusei* and two isolates of *C. tropicalis* were examined. All strains were initially identified by using standard morphological and biochemical methods (Evans and Richardson, 1989). The isolates were cultured in RPMI-1640 medium (Sigma-Aldrich, Helsinki, Finland) supplemented with 2% glucose. One milliliter volumes of the yeast suspensions were then added to 1 ml of 2% agarose. After solidifying, the embedded agarose material was fixed, embedded and stained as described before.

Expression of antigens by distinct morphological forms of C. albicans
To investigate the expression of extracellular antigen HMM by blastoconidia, pseudohyphae or hyphae of

C. albicans, normal human serum was inoculated with blastoconidia of strain ATCC 28366 and incubated at either 30°C or 37°C for up to 30 h. At the following time points an aliquot of serum was added to molten 2% agarose which was allowed to set as described previously: 3, 6, 24 and 30 h. The preparations were then fixed, embedded and stained as before.

Kinetics of antigen production

To study the expression of *Candida* antigen during hyphal growth as a function of time, one isolate of *C. albicans* (ATCC 28366) was cultured in normal human serum. One milliliter of the suspension was then added to 1 ml of 2% warm agarose. Nine tubes of the above-described type of sample were produced. These tubes were incubated at 37°C for 0, 1, 2, 3, 4, 5, 6, 24 and 30 h. After incubation, the samples were fixed, embedded and stained as before.

Results

Expression of 3H8 epitope by Candida species

To determine the specificity of the monoclonal antibody 3H8, 32 isolates of *Candida* incorporating strains of *C. albicans*, *C. lusitaniae*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis* species were cultured in agar and stained with the MAb. Of these isolates, positive staining was evident only with those isolates previously identified as *C. albicans* (Figure 1a–d).

With *C. albicans*, very intensive staining of the blastoconidial cell wall was seen (Figure 1a). In contrast, lateral and polar buds were negative (Figure 1a). Furthermore, staining of mother cell–daughter cell junction was not seen. The entire cell wall of both pseudohyphae and hyphae was intensely stained, but at the apex area staining was light.

The yeast cell morphology of *C. glabrata*, and the dimorphic growth forms of *C. krusei* and *C. parapsilosis* were negatively stained, indicating further that the Mab was specific for the mannoprotein of *C. albicans* (Figure 1b–d). *Candida lusitaniae* and *C. tropicalis* were also negatively stained.

Expression of 3H8 epitope by distinct morphological forms of C. albicans

To investigate the expression of the mannoprotein antigen by the morphological forms of *C. albicans*, normal human serum was inoculated with blastoconidia of strain ATCC 28366 and incubated at either 30°C or 37°C for up to 30 h.

After incubation for 3 h at 30°C the yeast cells were intensely stained, indicating that the mannoprotein epitope had accumulated homogenously over the cell wall surface (Figure 2a). There was a positive signal in the parent yeast cells and particularly at the bud scar region. After 30 h at 30°C there appeared to be antigen expression extracellularly in the region immediately around the cell (Figure 2b).

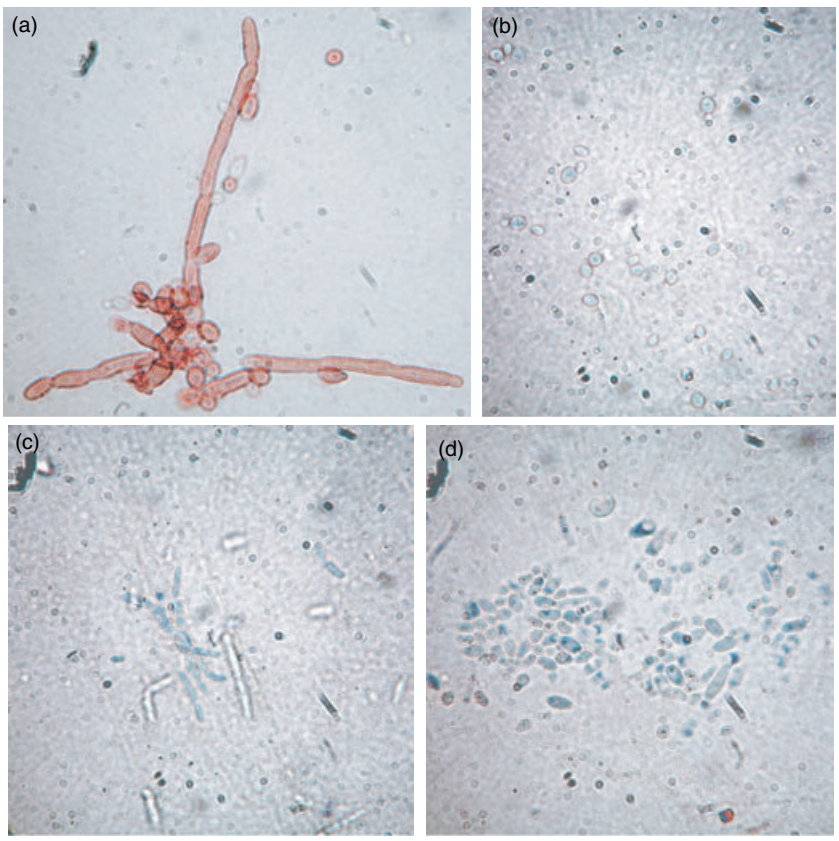

Figure 1 Expression of 3H8 epitope by *Candida* species (immunohistochemical staining with mAb 3H8): (**a**) With *C. albicans*, very intensive staining of the blastospore cell wall was seen whereas 'immature-small buds' and cell–daughter junctions were negatively stained. Other *Candida* species were negatively stained: (**b**) *C. glabrata*, (**c**) *C. krusei* and (**d**) *C. parapsilosis*. However, it is still possible to see their typical morphology

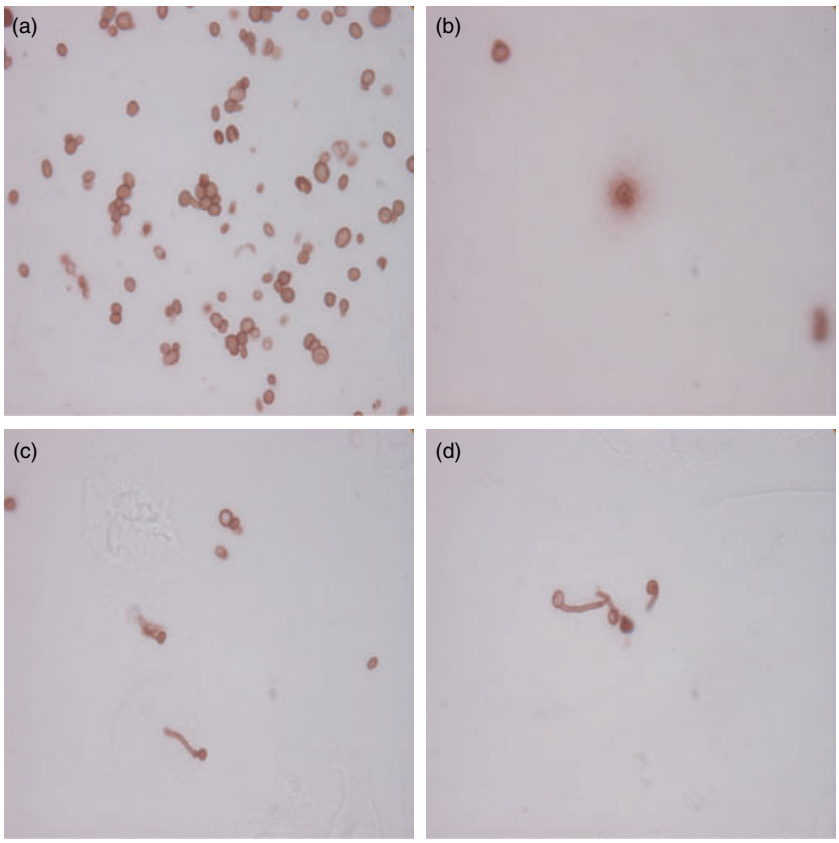

Figure 2 Expression of antigens by distinct morphological forms of *C. albicans*. (**a**) When *C. albicans* cells were incubated for 3 h at 30°C, the blastoconidial cell wall was intensely stained. (**b**) Blastoconidia cultured for 30 h at 30°C appeared to express antigen extracellularly as evidenced by positive staining of the agar section adjacent to individual cells. Yeast cells incubated at 37°C for 24 h (**c**) and 30 h (**d**) produced hyphae whose cell walls were positively stained

Yeast cells incubated at 37°C for 24 and 30 h had formed hyphae (Figure 2c,d). The intensity of staining indicated that the mannoprotein epitope was expressed uniformly on the cell wall of both yeast and hyphal growth forms.

Kinetics of antigen production

To determine the kinetics of mannoprotein expression during hyphal elongation, *C. albicans* (ATCC 28366) was cultured in normal human serum. One milliliter of the suspension was then added to 1 ml of 2% warm electrophoresis-grade agarose. These preparations were then incubated at 37°C for 0, 1, 2, 3, 4, 5, 6, 24 and 30 h. After 1 h incubation budding yeast cells were seen. These were positively stained with the MAb (Figure 3a). By 4 h germination had commenced (Figure 3b). During early germ tube growth the mannoprotein was highlighted along the length of the elongating cell wall (Figure 3b). The antigen was also seen in the region of the septum between the parent cell and the early germ tube. Older germ tubes were similarly stained (Figure 3c,d). After hyphal elongation for 24 h the mannoprotein appeared to be located extracellularly (Figure 3d).

Discussion

The results of this study provide evidence that the MAb 3H8 is a powerful and useful tool in the identification of *C. albicans*; and could be useful in the detection of *C. albicans* in tissues by immunohistochemistry. In this study the use of agar blocks made it easier to detect cell wall epitopes and yeast structures as there are no interfering tissue structures in the background.

The use of specific reagents helped to distinguish *C. albicans* from other *Candida* species such as *C. lusitaniae*, *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. tropicalis*. The absence of immunoreactivity of normal human tissues (Marcilla *et al*, 1999; Järvensivu *et al*, 2004), together with the fact that no immunostaining of other yeasts had been found, supports the value of this antibody in the specific diagnosis of candidosis caused by *C. albicans*. The ability to identify *Candida* in tissue lesions in cases of oral candidosis, (for example in *Candida*-associated periodontitis, mucosal candidosis or chronic hyperplastic candidosis) will help to clarify the role of individual *Candida* spp. in the pathogenesis of these infections. *Candida* species are frequently associated with superficial infection of mucosal membranes and fatal systemic infection in debilitated individuals may also occur. Chronic hyperplastic candidosis is a particularly important form of oral candidosis as it is associated with the development of epithelial dysplasia and intraoral squamous cell carcinoma.

Candida albicans is dimorphic, implying that vegetative growth of the fungus can occur in either a yeast form, reproducing by budding or by binary fission, or alternatively in a filamentous form in which elongated cells are elaborated and cells fail to separate after

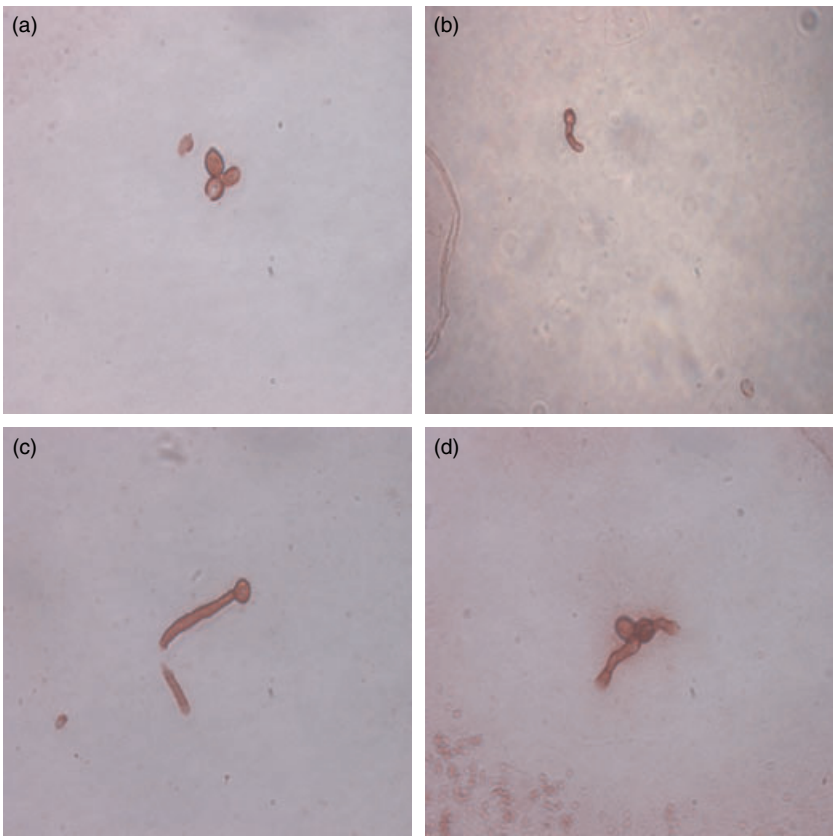

Figure 3 Kinetics of antigen production. (**a**) After 1 h incubation budding yeast cells were seen. These were positively stained with the Mab 3H8. (**b**) By 4 h germination had commenced. During early germ tube growth the mannoprotein was highlighted along the length of the elongating cell wall. The antigen was also seen in the region of the septum between the parent cell and the early germ tube. (**c**) Older germ tubes (incubated for 6 h) were similarly stained. (**d**) After hyphal elongation for 24 h there appeared to be extracellular expression of the mannoprotein

cytokinesis, thereby forming filaments of pseudohyphae or hyphae. Previous studies suggest that the host interacts differently against filamentous elements of *C. albicans* than with yeast (blastoconidia) forms. It has been shown that there are distinctive surface differences between blastoconidia and germ tubes (Sudström and Kenny, 1984). The present study demonstrated that the mannoprotein antigen was expressed on the surface of both blastoconidia and pseudohyphae. The antigen was also seen intracellularly and it is likely that the epitope is expressed extracellularly. Surface antigens appear to be expressed dynamically as a function of their metabolic and morphologic state during yeast and hyphal growth *in vitro*. Lateral buds were negatively stained. Mannoprotein antigens are not expressed until further cell expansion has occurred (Gow, 2002). In contrast, mannoproteins are not expressed on the surface of *Candida* hyphal elements (Sanjuán *et al*, 1995). Molinari *et al* (1993) showed by postembedding methods that both yeast and hyphal forms of *C. albicans* synthesized the relevant mannoproteins and incorporated them in a similar fashion into the inner layers of the cell wall, but 'export' of these mannoproteins to the outermost, capsular layer occurred in yeast, but not in hyphal, cells. The authors suggest that this differential expression could be a means by which the fungus evades or attenuates the host response as mannoproteins are a main immunogenic component of *Candida*.

In conclusion, this study has confirmed and has demonstrated that the MAb 3H8 recognizes a mannoproteinaceous epitope of the *C. albicans* cell wall which may account for its high specificity (Marcilla *et al*, 1999). This antibody also shows specific and strong cell surface immunostaining of *C. albicans* in paraffin-embedded tissues, being particularly effective in the detection of mycelial forms (Marcilla *et al*, 1999; Järvensivu *et al*, 2004). These results also suggest that this MAb could be a powerful and useful tool in distinguishing *C. albicans* from other species of *Candida*.

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