Editor

Kevin Kavanagh

Medical Mycology

Cellular and Molecular Techniques

WILEY

Medical Mycology

Medical Mycology

Cellular and Molecular Techniques

Edited by

Kevin Kavanagh

Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland

John Wiley & Sons, Ltd

Telephone (+44) 1243 779777

Email (for orders and customer service enquiries): cs-books@wiley.co.uk
Visit our Home Page on www.wileyeurope.com or www.wiley.com

Other Wiley Editorial Offices

John Wiley & Sons Inc., 111 River Street, Hoboken, NJ 07030, USA
Jossey-Bass, 989 Market Street, San Francisco, CA 94103-1741, USA
Wiley-VCH Verlag GmbH, Boschstr. 12, D-69469 Weinheim, Germany
John Wiley & Sons Australia Ltd, 42 McDougall Street, Milton, Queensland 4064, Australia
John Wiley & Sons (Asia) Pte Ltd, 2 Clementi Loop #02-01, Jin Xing Distripark, Singapore 129809
John Wiley & Sons Canada Ltd, 6045 Freemont Blvd, Mississauga, ONT, L5R 4J3

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Library of Congress Cataloging-in-Publication Data

Medical mycology: cellular and molecular techniques / edited by Kevin Kavanagh.
p. ; cm.
Includes bibliographical references and index.
ISBN-13: 978-0-470-01923-8 (alk. paper)
ISBN-10: 0-470-01923-9 (alk. paper)
1. Medical mycology–Laboratory manuals. 2. Mycoses–Molecular aspects–Laboratory manuals. I. Kavanagh, Kevin.
[DNLM: 1. Candidiasis–diagnosis–Laboratory Manuals. 2. Candidiasis–immunology–Laboratory Manuals. 3. Antibodies, Monoclonal–diagnostic use–Laboratory Manuals. 4. Clinical Protocols–Laboratory Manuals. 5. Immunohistochemistry–methods–Laboratory Manuals. 6. Immunologic Factors–diagnostic use–Laboratory Manuals. 7. Mycology–methods–Laboratory Manuals. WC 470 M489 2006]
QR245.M434 2006
616.9'6901–dc22 2006028045

British Library Cataloguing in Publication Data

A catalogue record for this book is available from the British Library

ISBN-13 978-0-470-01923-8 ISBN-10 0-470-01923-9

Typeset in 10.5/12.5pt Times by Thomson Digital
Printed and bound in Great Britain by Antony Rowe Ltd., Chippenham, Wiltshire
This book is printed on acid-free paper responsibly manufactured from sustainable forestry in which at least two trees are planted for each one used for paper production.

Contents

Preface

Pathogenic fungi represent a serious threat to the life and health of immunocompromised patients and are responsible for the deaths of up to 5% of all those who die in hospitals in the developed world. In addition to such life-threatening infections, fungi are also responsible for a wide range of superficial infections (e.g. 'ringworm', 'thrush') which can affect apparently healthy individuals. As the numbers of patients immunocompromised due to underlying diseases or medical therapies continue to rise, the incidence of fungal infections will increase. Despite their impact on human health, our understanding of the factors that allow fungi to colonize the body and surmount the defences of the immune system is poorly developed. In addition, our armamentarium for treating fungal infections, until recently, has been stocked with drugs (e.g. amphotericin B) which induce severe side effects in the host and which target, in the case of the azoles and polyenes, the same fungal biosynthetic pathway. Another problem hampering the treatment of fungal infections is the difficulty in diagnosing systemic infections promptly so that chemotherapy can be initiated with maximum benefit to the patient.

The aim of this book was to assemble a range of cellular and molecular techniques in order to facilitate an enhanced understanding of fungal virulence to aid in the development of improved diagnostic and chemotherapeutic regimens. Each chapter is written by internationally recognized experts who have direct experience of the relevant techniques and in a format that should allow those new to the field of research to master procedures with the minimum of delay. Each protocol is a self-contained unit so the reader is not required to search through an entire chapter to understand the requirements of a specific protocol.

The book starts with a description of techniques for diagnosing *Candida* infections in tissue using a variety of immunochemical methods. Chapter 2 details procedures for examining pathogenic fungi by transmission electron microscopy and shows how analysis of this type gives an understanding of the interaction of fungal cells with host tissue. Chapter 3 describes protocols for studying the interaction of phagocytes with fungi and offers an insight into how the immune system deals with pathogenic fungi. A number of methods for studying the virulence of *Candida albicans* and related species are described in Chapter 4, while protocols for assessing the response of yeast to antifungal drugs are highlighted in Chapter 5. Chapter 6 continues this theme and describes the use of animal models for evaluating the efficacy of antifungal drugs. In recent years, proteomic analysis has revolutionized our study of the structure and function of cellular proteins, and the application of this technique to medical

mycology is described in Chapter 7. Chapter 8 is dedicated to detailing protocols for extracting and detecting nucleic acids from fungi. Chapter 9 describes the application of microarrays to the study of the pathogenicity of *C. albicans*. Chapter 10 details transformation protocols for use with the pulmonary pathogen *Aspergillus fumigatus*. This chapter is followed by another dedicated to *A. fumigatus*, where protocols for the generation of knock-out mutants are described, while Chapter 12 describes the use of microarray technology for studying the virulence of *A. fumigatus*. The penultimate chapter details techniques for studying the virulence of *Cryptococcus neoformans*, while Chapter 14 is a detailed description of the procedures for genetically manipulating the zygomycetes.

In a book of this size and scope it is inevitable that some techniques will be described in more than one place. A decision was taken not to remove multiple descriptions of techniques, so that the reader would be able to follow the specific guidelines of each chapter without having to refer to a standardized protocol that would undoubtedly miss some of the nuances necessary for a specific strain, species or experimental condition.

It is the hope of all the contributors to this book that the use of the protocols described here will increase our understanding of fungal virulence and ultimately enable the development of new and improved methods to diagnose and treat fungal infections.

Kevin Kavanagh

List of Contributors

Khaled H. Abu-Elteen
Department of Biological Science
Hashemite University
Zarqa 13133, Jordan

Darius Armstrong-James
Department of Infectious Diseases
Imperial College, London W12 0NN
England

Jean-Philippe Bouchara
Groupe d'Etude des Interactions
Hôte-Parasite, UPRES-EA 3142
Faculté de Pharmacie
16, boulevard Daviers 49045
Angers cedex, France

Axel A. Brakhage
Leibniz Institut für Naturstoff-Forschung
und Infektionsbilogie-Hans-Knöll-
Institut-Molekulare und angewandte
Mikrobiologie Beutenbergstr.11a
07745 Jena, Germany

Mathias Brock
Leibniz Institut für Naturstoff-Forschung
und Infektionsbilogie -Hans-Knöll-Institut-
Molekulare und angewandte
Mikrobiologie Beutenbergstr.11a
07745 Jena, Germany

David C. Coleman
Microbiology Division
Dublin Dental School and Hospital
Trinity College Dublin
Dublin 2, Ireland

Eric Dannaoui
Institut Pasteur, 25 rue du Dr Roux
75015 Paris, France

Alexander Gehrke
Leibniz Institut für Naturstoff-Forschung und
Infektionsbilogie-Hans-Knöll-Institut-
Molekulare und angewandte Mikrobiologie
Beutenbergstr.11a
07745 Jena, Germany

Pat Geraghty
Research Laboratory, RCSI Building
Beaumont Hospital, Beaumont, Dublin 9
Ireland

Mawieh Hamad
Department of Biological Science
Hashemite University, Zarqa 13133
Jordan

Jaako Hietanen
Department of Bacteriology & Immunology
University of Helsinki, Haartmaninkatu 3
00014 Helsinki, Finland

Hervé Hogues
Biotechnology Research Institute
National Research Council of Canada
6100 Royalmount Avenue, Montreal, QC
Canada H4P 2R2

Ashraf S. Ibrahim
David Geffen School of Medicine at UCLA
Division of Infectious Diseases (RB-2)
Los Angeles Biomedical Research Institute at
Harbor-UCLA Medical Center, 1124 W
Carson Street, Torrance, CA 90502, USA

Guilhem Janbon
Unité de Mycologie Moléculaire
CNRS FRE2849, Institut Pasteur
25 rue du Dr Roux
75015 Paris, France

Kevin Kavanagh
Department of Biology
National University of Ireland Maynooth
Co. Kildare, Ireland

Nancy Lee
Unité de Mycologie Moléculaire
CNRS FRE2849, Institut Pasteur
25 rue du Dr Roux
75015 Paris, France

Gary Moran
Microbiology Division
Dublin Dental School and Hospital
Trinity College Dublin
Dublin 2, Ireland

Alan Murphy
Department of Biology
National University of Ireland Maynooth
Co. Kildare, Ireland

André Nantel
Biotechnology Research Institute
National Research Council of Canada
6100 Royalmount Avenue, Montreal, QC
Canada H4P 2R2

Nir Osherov
Dept of Human Microbiology
Sackler School of Medicine
Tel Aviv University Ramat Aviv
Tel-Aviv 69978, Israel

Emmanuelle Pinjon
Microbiology Division
Dublin Dental School and Hospital
Trinity College Dublin
Dublin 2, Ireland

Riina Rautemaa
Department of Bacteriology & Immunology
University of Helsinki, Haartmaninkatu 3
00014 Helsinki, Finland

Malcolm Richardson
Department of Bacteriology & Immunology
University of Helsinki, Haartmaninkatu 3
00014 Helsinki, Finland

Tracey Rigby
Biotechnology Research Institute
National Research Council of Canada
6100 Royalmount Avenue, Montreal, QC
Canada H4P 2R2

Tom Rogers
Department of Clinical Microbiology
St James Hospital, Trinity College
Dublin, Ireland

Emmanuel Roilides
3rd Dept Pediatrics, Aristotle University
Hippokration Hospital
Konstantinoupoleos 49
GR-54642 Thessaloniki, Greece

Jacob Romano
Dept of Human Microbiology
Sackler School of Medicine
Tel Aviv University, Ramat Aviv
Tel-Aviv 69978, Israel

Maria Simitsopoulou
3rd Dept Pediatrics, Aristotle University
Hippokration Hospital
Konstantinoupoleos 49
GR-54642 Thessaloniki, Greece

Christopher D. Skory
National Center for Agricultural Utilization
Research, USDA
Agricultural Research Service
1815 North University Street
Peoria IL 61604, USA

Venelina Sugareva
Leibniz Institut für Naturstoff-Forschung und Infektionsbilogie-Hans-Knöll-Institut-Molekulare und angewandte Mikrobiologie
Beutenbergstr.11a
07745 Jena, Germany

Derek J. Sullivan
Microbiology Division
Dublin Dental School and Hospital
Trinity College Dublin
Dublin 2, Ireland

Guy Tronchin
Groupe d'Etude des Interactions Hôte-Parasite, UPRES-EA 3142
Faculté de Pharmacie, 16 boulevard Daviers
49045 Angers cedex, France

Malcolm Whiteway
Biotechnology Research Institute
National Research Council of Canada
6100 Royalmount Avenue, Montreal, QC
Canada H4P 2R2

1

Diagnosis of *Candida* infection in tissue by immunohistochemistry

Malcolm D. Richardson, Riina Rautemaa and **Jarkko Hietanen**

1.1 Introduction

As a result of their large size, polysaccharide content and morphological diversity, fungi can be detected readily and may also to a certain level be identified in histological sections by conventional light microscopy. Application of immunohistochemical techniques may, in several cases, be the only means of establishing an accurate aetiological diagnosis in fixed-tissue sections because of morphological similarities among the tissue forms of several fungal genera (e.g. aspergillosis, fusariosis and scedosporiosis), when atypical forms of the fungus are present or when fungal elements are sparse (Jensen *et al*., 1996; Jensen and Chandler, 2005).

The prerequisite for all immunohistochemical staining systems is a primary antibody properly characterized, especially in terms of specificity under optimal conditions. The technique used for obtaining an immunohistochemical diagnosis of mycoses may be either direct (conjugated primary antibodies) or indirect (conjugated secondary antibodies or tertiary enzyme complexes). In accordance with other immunohistochemical staining systems, the reaction complexes may be visualized with the help of fluorchromes (e.g. fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate R (TRITC), gold-silver complexes or complexes of enzymes (e.g. peroxidase-antiperoxidase (PAP) techniques, and alkaline phosphatase-antialkaline phosphatase (APAAP) techniques. In addition, avidin-biotin enzyme complex (ABC) methods may be used with horseradish peroxidase (HRP) and galactosidase. The major advantage of using enzyme immunohistochemistry compared with immunofluorescence techniques is that permanent sections are

Medical Mycology: Cellular and Molecular Techniques. Edited by Kevin Kavanagh

provided, specialized microscopes are not needed and pathological reactions may be assessed simultaneously during the evaluation of the immunoreactivity. A range of different fungal forms or fungal elements are frequently observed within lesions, especially when more than one organ is studied, and it must be ascertained whether these elements belong to a single or more taxa. In such cases, dual immunostaining techniques are useful tools for obtaining a reliable diagnosis.

Apart from tissue sections, immunohistochemical techniques can also be used to identify fungi in smears of lesional exudates, bronchial washings, bronchoalveolar lavage fluids, blood, bone marrow, cerebral-spinal fluid and in sputum specimens that have been enzymatically or chemically digested. An important limitation to the widespread application of immunochemical techniques and their use in the routine diagnosis of mycoses lies in the fact that sensitive and specific reagents are usually derived from multiple heterologously adsorbed polyclonal antisera which are not commercially available. However, in recent years more specific monoclonal antibodies have been developed, some of which are available commercially.

Before immunostaining, localization and presumptive identification of fungal elements in Gomori-methenamine-silver (GMS)-stained tissue sections enable the pathologist to narrow the aetiologic possibilities so that the most appropriate panel of immunoreagents can be employed. A number of appropriate control procedures are essential in the evaluation of immunohistochemical diagnostic assays (e.g. replacement of antifungal antibodies by antibodies raised against irrelevant antigens and including both positive and negative control sections with each batch of slides). As the somatic and cell-wall antigens of most pathogenic fungi survive formalin fixation and processing into paraffin quite well, immunohistochemical techniques can be performed retrospectively on archival material. Newly acquired antibodies can be evaluated using known fungal species cultured in agar and fixed and sectioned, as for tissue material.

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 a commensal to a harmful pathogenic state eventually depends on a decrease in host resistance, changes in the local ecology or changes in intrinsic fungal virulence. The histopathologic changes related to the infection include inflammatory cell infiltration of the epithelium and connective tissue, intraepithelial microabscesses, epithelial intercellular oedema and epithelial atrophy, hyperplasia and dysplasia. In the oral cavity, yeasts can be found on mucosal surfaces and in saliva but also in the inflamed periodontal pockets (Järvensivu *et al.*, 2004). Even though the role of yeast in periodontitis is largely unknown, there is some evidence to suggest that yeasts may be implicated in the disease process.

The monoclonal antibody 3H8 has been shown to recognize mannoproteins of high molecular mass present in the cell walls of *C. albicans*. By ELISA (enzyme-linked immunosorbent assay), it has been shown that the presence of the epitope recognized

by monoclonal 3H8 was similar in both the yeast and mycelial cell walls of *C. albicans* (Marcilla *et al*., 1999). Immunohistochemical studies using this antibody have demonstrated its usefulness in specifically recognizing *C. albicans* in kidney, lung, thyroid, oesophagus, small bowel and gingival tissues (Marcilla *et al*., 1999; Järvensivu *et al*., 2004). To explore further the usefulness of this monoclonal antibody in detecting *Candida* in periodontal diseases, we wished to test the antibody against a wide range of yeasts 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 reasonable to determine whether the epitope could be expressed into the surrounding environment, further aiding the recognition of the organism in the tissues.

C. albicans has been shown to be involved in the pathogenesis of adult periodontitis (AP). The potential candidal diagnosis of AP depends largely on the identification of yeast and pseudomycelial forms in gingival tissue samples by using periodic acid-Schiff and GMS stains. However, these stains are non-specific and also reveal confusing artefacts 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.

The following protocols describe the evaluation of a relatively new monoclonal antibody for immunohistochemistry and its use in the detection of *C. albicans* morphological forms in tissue, using adult periodontitis as a model.

1.2 Specificity of monoclonal antibody 3H8 for *C. albicans*

Because of the limited number of tissue biopsies from patients with *Candida*-associated periodontal diseases (Järvensivu *et al*., 2004), it is very convenient to use an agar block technique where *Candida* species are grown under varying environmental conditions in agar, which is then processed in a similar manner to that used for pathology tissue specimens. The 3H8 epitope appears to be located at the external surface of some *C. albicans* strains but is partially cryptic in the cell wall of other strains. It is highly probable that the epitope is expressed extracellularly. The use of agar blocks makes it easier to detect these epitopes because there are not any interfering tissue structures as background.

To evaluate the usefulness of mabs in detecting *Candida* in periodontal disease, the antibody should be 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 epitopes on the external cell wall of certain *C. albicans* strains, it is useful to determine whether the epitope can be expressed into the surrounding environment, further aiding the recognition of the organism in tissue.

Protocol 1.1
Testing of specificity of monoclonal antibody 3H8

Equipment, materials and reagents

Candida isolates maintained on glucose-peptone agar

RPMI-1640 medium (Sigma-Aldrich, Poole, Dorset, UK) supplemented with 2% glucose

Agarose (Sigma-Aldrich)

Repli-dishes

Pepsin

Phosphate-buffered saline

Antibodies. A mouse monoclonal antibody, for example 3H8, is used as the primary antibody (IgG1; Société de Recherche et de Réalisations Biotechnologiques, Paris, France). It is raised against a zymolyase-solubilized preparation from the blastoconidial cell walls of *C. albicans* ATCC26555 and recognizes high-molecular-weight mannoproteins present in the cell wall. Biotinylated anti-mouse IgG as a component of the Vectastain® kit (Vector Laboratories, Burlingame, CA, USA) is used as a secondary antibody. Phosphate-buffered saline (PBS) is used as a buffer. In some instances, bovine serum albumin (BSA; Behringverke Gmbh, Germany) can be added to PBS (PBS-BSA) to reduce non-specific reactions.

Method

1. **Agar block culture and histochemical processing.** To test the specificity of the monoclonal antibody 3H8 to detect high-molecular-weight mannoproteins expressed by different *Candida* species, use, for example, strains of *C. albicans*, *C. lusitaniae*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis*. Culture the isolates in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2% glucose. Add 1 ml of the suspensions to 1 ml of 2% molten agarose (held at 50 °C) and pour into individual compartments of plastic replica dishes. After solidifying, the embedded agarose material is fixed in 10% formal saline for 24 h before processing through graded concentrations of alcohol and xylene and embedded in paraffin wax. Cut 4-μm-thick sections.

2. **Immunohistochemical staining.** De-paraffinize sections in xylene and rehydrate in graded alcohol series and in water. Incubate sections in pepsin (5 mg pepsin

+ 5 ml H_2O + 50 µl 1 N HCl) for 45 min in a humid chamber and wash 3 times for 5 min in PBS. Inhibit endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 30 min, and then wash the sections with PBS 3 times for 5 min. First incubate the sections in normal horse blocking serum from the kit diluted 1:50 in 2% PBS-BSA. Then incubate the sections with the monoclonal primary antibody (3H8, 1:500, diluted in 1% PBS-BSA) for 30 min at 37 °C and keep overnight at 4 °C in a humid chamber. Controls are performed by omitting the primary antibody. The following day, after washing 3 times, incubate the sections for 30 min at 37 °C with biotinylated anti-mouse IgG secondary antibody solution from the kit diluted 1:200 into 0.1% PBS-BSA. After three washes, the sections are then incubated with the kit reagent for 30 min at 37 °C and then washed 3 times with buffer. Peroxidase binding sites are revealed with 3-amino-9-ethylcarbazole (AEC) with 0.03% hydrogen peroxidase. Finally, the slides are washed with tap water and then counter-stained with Mayer's haematoxylin for 4 min and again rinsed with tap water before mounting with glysergel (DAKO Corporation, CA, USA).

C. albicans is positively stained by the monoclonal antibody, whereas the yeast cell morphology of *C. glabrata*, and the dimorphic growth forms of *C. krusei*, *C. parapsilosis*, *C. lusitaniae* and *C. tropicalis* are negatively stained indicating further that the mab is specific for the mannoprotein of *C. albicans* (Figure 1.1).

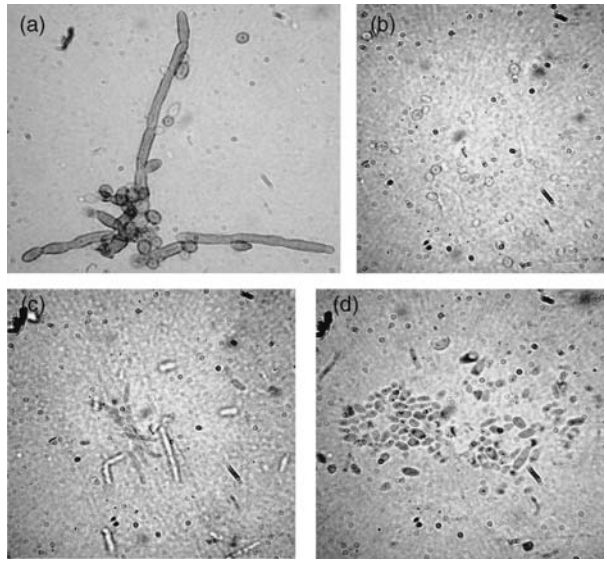

Figure 1.1 Expression of 3H8 epitope by *Candida* species (immunohistochemical staining with mab 3H8): (a) For *C. albicans*, very intensive staining of blastospore cell wall is seen. Small buds and cell-daughter junctions are negatively stained. Other *Candida* species are negatively stained: (b) *C. glabrata*, (c) *C. krusei*, (d) *C. parapsilosis*. However, it is still possible to see their typical morphology

1.3 Evaluation of monoclonal antibody 3H8 for the detection of *C. albicans* morphological forms

A range of different fungal morphological forms are frequently observed within lesions and it must be ascertained whether these elements belong to a single or more taxa. All species of *Candida* produce oval yeast-like cells, 3–6 μm, and mycelial elements composed of pseudohyphae and true hyphae. Pseudohyphae are composed of elongated yeast-like cells that remain attached end-to-end in chains. They are distinguished from true hyphae by the presence of prominent constrictions at points of attachment between adjacent cells, whereas the septate hyphae, 3 to 5 μm in width, are tubular and have parallel contours. Since the distinction between pseudohyphae and true hyphae is rarely of practical significance in histopathological diagnosis, they can be referred to collectively as mycelial elements.

The combination of yeast-like cells, pseudohyphae and true hyphae distinguishes *Candida* species from most other yeast-like pathogens in histopathological sections. *Trichosporon* species, which produce both yeast-like cells and mycelial elements, may provide a vexing problem in differential diagnosis. The yeast-like cells of *Trichosporon* are somewhat larger and more pleomorphic than those of *Candida*, and their hyphae produce rectangular arthroconidia. Weakly pigmented agents of phaeohyphomycosis can also be mistaken for *Candida* in histopathological sections. In difficult cases, a provisional morphological diagnosis of candidosis can be confirmed by culture or immunohistochemistry.

Protocol 1.2
Evaluation of monoclonal antibody 3H8 for the detection of *C. albicans* morphological forms

Equipment, materials and reagents

Candida albicans ATCC 28366

Normal human serum

Highly purified agarose (electrophoresis grade)

Method

Expression of antigens by morphological forms of *C. albicans*. To investigate the expression of extracellular antigenic mannoproteins by blastospores, pseudohyphae or hyphae of *C. albicans*, normal human serum is inoculated with blastospores of reference strain ATCC 28366 and incubated at either 30 °C or 37 °C for up to 30 h. After 3, 6, 24 and 30 h remove an aliquot of the inoculated serum and add this to molten 2% agarose and allow to set in compartmentalized replica dishes, or similar. Fix the samples, embed and stain as described in Protocol 1.1.

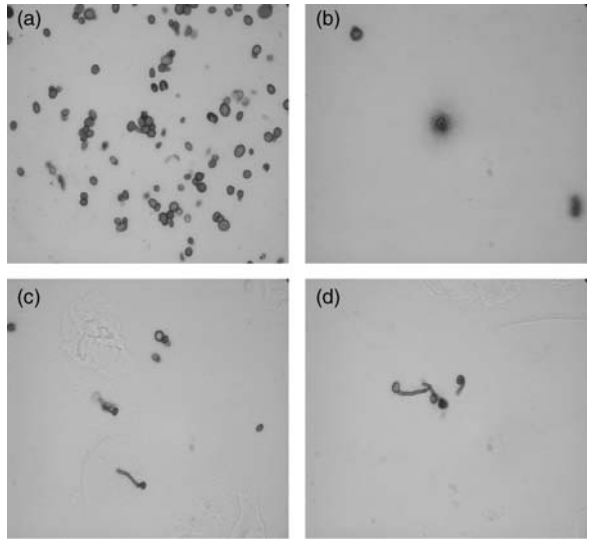

Figure 1.2 Expression of antigens by morphological forms of *C. albicans*. (a) When *C. albicans* cells are incubated for 3 h at 30 °C, cell-wall antigens are preferentially stained. (b) After incubation for 30 h at 30 °C, yeast cells can be seen to express antigen extracellularly. The cells incubated at 37 °C for 24 h (c) and 30 h (d) form hyphae which are positively stained

After incubation for 3 h at 30 °C, yeast cells are intensely stained indicating that the mannoprotein epitope has accumulated homogenously over the cell wall surface (Figure 1.2a). A positive signal in the parent yeast cells and particularly at the bud scar region will be seen. After 30 h incubation at 30 °C antigen expression will be observed extracellularly in the region immediately around the cell (Figure 1.2b).

Yeast cells incubated at 37 °C for 24 h and 30 h will form hyphae (Figure 1.2c, Figure 1.2d), and the staining reaction will indicate that the mannoprotein epitope is expressed uniformly on the cell wall of both yeast and hyphal growth forms.

1.4 Application of immunohistochemistry in the diagnosis of *Candida* periodontal disease

Periodontitis is an infection-induced inflammatory disease characterized by irreversible destruction of the tooth-supporting tissues (epithelium, gingival connective tissue and alveolar bone). 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 500 bacterial strains have been recovered from the subgingival crevice, a particularly well-studied microbial niche (Kroes *et al*., 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.

Adult periodontitis (AP) results from a complex interplay of the mixed microbial 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.

C. albicans is an aerobic commensal which can be cultured from the oral cavity of nearly every other adult (Arendorf and Walker, 1979). In the oral cavity, yeasts commonly colonize the tongue, palate and buccal mucosa (Arendorf and Walker, 1980) and may occur in the subgingival plaque of adults with severe periodontitis (Slots *et al*., 1988). Yeasts, especially *C. albicans*, have been recovered from periodontal pockets in a large number (7.1–19.6%) of patients with AP (Dahlén and Wikström, 1995; Rams *et al*., 1997; Reynaud *et al*., 2001; Slots *et al*., 1988). In a recent survey by Reynaud *et al.* (2001), the prevalence of subjects with yeasts in the periodontal pockets was 15.6%. Using the electron microscope, yeasts were found to be invading in the gingival connective tissue of 26 out of 60 samples from 12 patients with juvenile periodontitis (JP) (González *et al*., 1987). Similar findings have not been reported of AP until now. It has been suggested that *C. albicans* may contribute to the development of necrotizing periodontal diseases in HIV-infected patients (Odden *et al*., 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; Jensen and Chandler, 2005). With the recent development and availability of monoclonal antibodies to various epitopes of *C. albicans* and *C. dubliniensis* (Järvensivu *et al*., 2004; Marcilla *et al*., 1999), it is now possible to identify *Candida* in human tissue biopsies to the species level. Even though the role of yeasts in AP is largely unclear, there is evidence to suggest that yeasts can be implicated in the pathogenesis of the tissue-destructive periodontal disease process.

Protocol 1.3
Use of monoclonal antibody 3H8 in the detection of *C. albicans* in tissue

Equipment, materials and reagents

Patient tissue samples

Monoclonal antibody 3H8: IgG1; 2.5 mg/ml; Société de Recherche et de Réalisations Biotechnologiques, Paris, France

Rabbit polyclonal antibody 158: 4.5 mg/ml; Biodesign International, Saco, Maine, USA

Biotinylated anti-mouse or anti-rabbit IgG: Vectastain® kit (Vector Laboratories, Burlingame, CA, USA)

BSA

10% formalin

Method

1. Fix patient tissue samples in formalin and paraffin-embed.

2. For periodic acid-Schiff (PAS) staining de-paraffinize 4 μm-thick, formalin-fixed, paraffin-embedded sections in xylene and rehydrate in graded alcohol series and in water. Before staining with Schiff's leucofuchsin reagents, expose the sections to periodic acid.

3. For immunohistochemical staining, de-paraffinize 4 μm-thick, formalin-fixed, paraffin-embedded sections as described above. Incubate the sections with pepsin for 45 min in a humid chamber and wash 3 times for 5 min with PBS. To inhibit endogenous peroxidase activity, the sections are 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 can be used. To inhibit non-specific staining, incubate the sections with normal horse serum from the kit (1:50 in 2% PBS-BSA). The sections are 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 kept overnight at 4 °C in a humid chamber. Control stainings are performed by omitting the primary antibody. The next day, after three washes, incubate sections 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 are washed and incubated with the kit reagent for 30 min at 37 °C and then washed again. Peroxidase binding sites are revealed with 3-amino-9-ethylcarbazole (AEC) with 0.03% hydrogen peroxidase. Finally, wash the slides with tap water and counterstain with Mayer's haematoxylin for 4 min and then rinse with tap water before mounting with glycerol (DAKO Corporation, CA, USA).

Typically, positive staining for *C. albicans* either with the polyclonal or monoclonal antibody is seen (Figure 1.3). Predominantly, pseudomycelial forms are immunoreactive. In general, the polyclonal antibody gives a stronger signal than the monoclonal antibody. The polyclonal antibody used here will also detect *Candida*-derived antigens in the periodontal tissues in addition to the specific staining of hyphal elements and candidal cells.

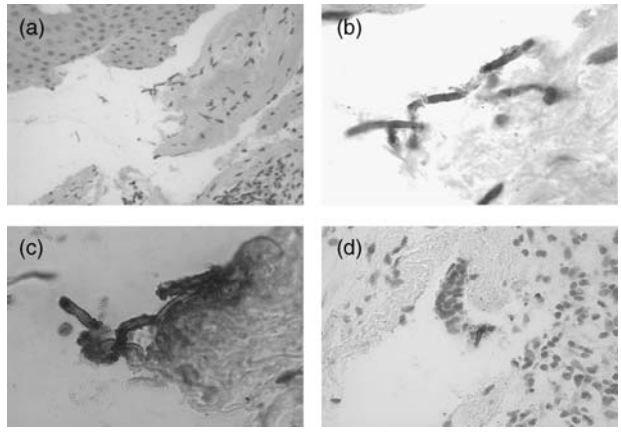

Figure 1.3 Adherence of *C. albicans* hyphae to periodontal connective tissue. With the mab 3H8, three to four *Candida* hyphae can be seen on the surface of connective tissue which has become detached from the overlying epithelium. This group of *Candida* hyphae is surrounded by a mild inflammatory reaction (Figure 3A, ×200 and Figure 3B, ×1000). Staining with the polyclonal antibody demonstrates six to eight *Candida* hyphae in almost the same localization also without a remarkable inflammatory reaction (Figure 3C, ×1000). In a deeper part of the specimen one hypha surrounded by a rather strong compact inflammatory cell infiltrate can be seen (Figure 3D, ×400). This infiltrate seems to consist mainly of mononuclear inflammatory cells

C. albicans is regarded as potentially the 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 morphology. *C. albicans* can also appear as a yeast cell form, 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 the 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 to yeast cells. Thus, hyphae are important in the attachment and disease process.

In one evaluation of the monoclonal antibody 3H8, *C. albicans* was detected by immunohistochemistry in the tissues of four (16%) adult periodontitis patients. Three of these tissue samples also contained subgingival plaque in which candidal structures could be detected in every case. Staining of hyphal elements with the specific monoclonal antibody verified that the organism seen was *C. albicans*. *C. albicans* was often seen in the outer layers of the plaque and some hyphae reached towards the more central parts. It is clear that hyphal germination had already started in the gingival pocket. These findings suggest that *C. albicans* could have a role in the infrastructure of periodontal microbial plaque and in its adherence to the periodontal tissues.