Virchows Archiv A Pathol Anat (1991) 419:199–202

Virchows Archiv A
Pathological Anatomy and Histopathology

Candidiasis visualised by proteinase-directed immunofluorescence

R. Rüchel[1], F. Zimmermann[1], Birgitt Böning-Stutzer[1], and U. Helmchen[2,*]

Departments of [1] Medical Microbiology and [2] Pathology, University of Göttingen, Kreuzbergring 57, W-3400 Göttingen, Federal Republic of Germany

Received February 4, 1991 / Received after revision May 4, 1991 / Accepted May 7, 1991

Summary. The secretory aspartic proteinases of *Candida albicans* and *C. tropicalis* are potential factors for virulence produced during infection. By indirect immunofluorescence, we have demonstrated proteinase antigen on elements of both species in deparaffinized tissue sections derived from clinical cases of mucosal and deep-seated candidiasis. Occasionally, we observed a halo of fluorescence in the close vicinity of candidal cells, which may reflect secretion of the enzyme. In kidneys, a ring of amorphous fluorescent material surrounding candidal colonies may illustrate alkaline denaturation of secreted enzyme within a pH gradient, which is generated by the fungus. Our findings support the view that candidal proteinase may be a diagnostically relevant antigen.

Key words: Candidiasis – Secretory proteinase – Immunofluorescence

Introduction

Among the opportunistic fungi causing mucosal and deep-seated mycoses, *Candida albicans* and *C. tropicalis* play a dominant role (Odds 1988). Potential factors for virulence of these yeast-like fungi comprise dimorphism, fast phenotypic switching, and secretion of hydrolytic enzymes (for review, see Ghannoum and Abu-Elteen 1990). Among the candidal hydrolases, an aspartic proteinase (candidal proteinase) has attracted the most attention (Ross et al. 1990; Rüchel 1990).

Candidal proteinase is induced in experimental infection of human oral mucosa (Borg and Rüchel 1988) and phagocytic cells (Borg and Rüchel 1990). Previously, we have demonstrated the proteinase antigen in sera of certain patients suffering from candidiasis (Rüchel et al. 1988). A role for candidal proteinase as a diagnostic antigen can, however, only be assumed if the antigen can reliably be detected in situ during infection. For this purpose, we have investigated the expression of proteinase antigen in mycotic human tissue.

Patients and methods

The present study comprised 20 immuno-compromised patients who died between 1984 and 1986 in the Göttingen University Hospital (Zimmermann 1986). Postmortem examination revealed lesions suggesting invasive candidiasis, which were confirmed histopathologically, using the periodic acid-Schiff stain (PAS), Gomori methenamine silver stain (GMS) or Calcofluor white according to Hageage and Harrington (1984).

Tissues from the following patients were used in this study:

1. A 62-year-old male patient suffering from peritoneal carcinomatosis causing perforation of the sigmoid colon. The patient died of peritonitis, and a postmortem examination revealed disseminated candidiasis (lungs, kidneys) and thrush of the urinary bladder.
2. A 23-year-old male patient suffering from acute myeloblastic leukaemia. He underwent antineoplastic chemotherapy including cytosine arabinoside. During the following granulocytopenia, he developed partial ileus and pneumonia and died of respiratory failure. Autopsy revealed disseminated candidiasis involving the lungs, liver and gut.
3. A 42-year-old male homosexual suffering from AIDS. He developed Kaposi's sarcoma of the lungs and died of pulmonary obstruction without signs of serious opportunistic infection. Autopsy revealed typical candidal oesophagitis and fungal infiltrates of the lung, suggesting bronchopneumonia.
4. An 87-year-old male patient who was hospitalized for chronic diarrhoea and pneumonia. He developed fever and died of septic shock. *C. albicans* had been cultured premortem from an intravenous device, and necropsy revealed disseminated candidiasis (myocardium, lungs, and kidneys).
5. A 20-year-old male patient who was hospitalized for allergic pancytopenia. During subsequent corticosteroid therapy he developed fever and died of septic shock. Necropsy revealed disseminated candidiasis involving lungs, kidneys, heart and brain.

If fungal elements were disclosed by PAS or GMS staining, immunofluorescence was performed on adjacent tissue sections. The following procedure was adapted from Hed and Enestrom (1981) and was performed at room temperature. Thin sections were cut from formaldehyde-fixed, paraffin-embedded tissue, which were deparaffinized by xylene and graded ethanol as usual and exposed to bovine trypsin (Serva, Heidelberg, FRG, no. 37257).

* *Present address:* Institut für Pathologie, Universitätsklinikum Eppendorf, W-2000 Hamburg 20, Federal Republic of Germany

Offprint requests to: R. Rüchel

After 15 min exposure to a freshly prepared solution of trypsin [1 mg/ml in phosphate buffered saline (PBS) at pH 7.4], the specimens were exposed twice for 10 min to the trypsin inhibitor phenylmethane-sulphonyl fluoride (PMSF, Sigma, St. Louis Mo. no. P 7626), which was made up at 10^{-3} M in PBS immediately before use.

After a series of rinses in PBS (5 × 2 min), non-specific protein binding sites were saturated with bovine serum albumin (BSA, Sigma no. A 7906) at 10 mg/ml in PBS for 30 min.

The first antibody (anti-proteinase, see below) was then added (10 µg/ml in PBS + 1% BSA). After 2 h and another series of rinses with PBS, the specimens were saturated with non-specific serum (20-fold diluted in PBS, 30 min), which corresponded to the species from which the following second antibody originated. After another series of rinses in PBS, the specimens were exposed to the fluorescein-labelled second antibody (diluted 30-fold in PBS-BSA). The fluorescein-antibody conjugates (anti-rabbit immunoglobulins, anti-guinea-pig immunoglobulins) were obtained from Dakopatts (Copenhagen, Denmark).

After a final series of rinses in PBS, the specimens were embedded in PBS-buffered glycerol with 1 mg/ml *p*-phenylenediamine (Sigma, P 1519) according to Johnson et al. (1982), and were covered with a coverslip. The specimens were viewed with a Zeiss microscope (Zeiss, Oberkochen, FRG), equipped with epifluorescent illumination and appropriate filters. Recordings were made on Kodak Ektachrome 400 ASA 35 mm film.

The anti-proteinase antibodies were derived from immune sera, which were elicited in rabbit or guinea-pig in this laboratory, using affinity-purified candidal proteinases. The specificity of these antibodies has been previously established (Borg and Rüchel 1988). The antisera have also been shown to be virtually free of anti-*Aspergillus* activity (Berger et al. 1988).

Results

Evidence for the presence of candidal proteinase antigen was found in every organ which was positive with PAS, GMS or Calcofluor staining, such as mucosa, central nervous system, lung, heart, liver, pancreas and kidney.

The proteinase antigen was always affiliated with the cell wall of blastoconidia and filamentous fungal cells.

Strong proteinase-dependent fluorescence was observed with superficially growing fungi exemplified by thrush of the urinary bladder (Fig. 1). Comparable fluorescence was observed in invasive intestinal candidiasis (Fig. 2a). The specificity of this immune reaction can be judged by the image of a control without specific antibody (Fig. 2b).

Likewise, a strong specific fluorescence was observed in mycotic lung (Fig. 3) and myocardium (Fig. 4a). The image after GMS staining of a corresponding section from the latter case is shown in Fig. 4b.

A halo of granular fluorescent material in the vicinity of fungal cells was observed in the brain (Fig. 5), and in another case of myocardial candidiasis (data not shown).

Likewise, fungal elements in tissue sections from cases of oral thrush, candidal oesophagitis, gastric mucosal candidiasis, candidal bronchopneumonia and interstitial pneumonia, pancreatic candidiasis, candidal endocarditis, and meningitis were tested for proteinase antigen. In all these samples, proteinase antigen was detected (Zimmermann 1986). The intensity of immune fluorescence was lower than usual in tissue from candidal endocarditis and meningitis, where the fungal cells were buried in fibrin clots (results not shown).

In mycotic kidney, amorphous deposits of proteinase antigen were found to form a ring around fungal colonies (Fig. 6). This type of deposition was found in all the three examined cases of candidal nephritis and may reflect the secretion and subsequent denaturation of the fungal enzyme.

Discussion

Deep-seated candidiasis, like other opportunistic mycoses, can be diagnosed histologically. However, fungal elements in tissue are often not distinguishable by standard staining procedures (Luna and Tortoledo 1985). Differentiation of fungal elements, even in formaldehyde-fixed, paraffin-embedded tissue, can be attempted immunologically (Kobayashi et al. 1988; Reijula 1989). In these studies, either whole cell antigens or a non-defined mixture of cytoplasmic antigens of the fungi were used as targets.

We have used detector antibodies specific for a defined antigen, the extracellular aspartic proteinases of *C. albicans* and *C. tropicalis*. The reactivity of the proteinase antigen was enhanced by unmasking with trypsin. The additional pretreatment with pepsin (Hed and Eneström 1981) was avoided, since pepsin belongs to the aspartic proteinases like candidal proteinase.

Secretory aspartic candidal proteinases (E.C. 3.4.23.6) are potential factors for virulence which have been characterized from *C. albicans*, *C. tropicalis* and *C. parapsilosis* (Rüchel 1990), and which have recently attracted much attention (De Bernardis et al. 1989; Tsuboi et al. 1989; Ray and Payne 1990; Ross et al. 1990). The inhibition of candidal proteinase in vivo has a modulating effect on systemic murine candidosis (Rüchel et al. 1990). The gene of a secretory candidal proteinase has recently been cloned and sequenced (Hube et al., 1991).

Immunohistological evidence for secretion of the enzyme during murine candidiasis was first provided by Macdonald and Odds (1980), who observed a specific immunofluorescence of the fungal cells and a halo of fluorescence in their immediate vicinity. Subsequently, we demonstrated the antigen in mycotic human epidermis (Rüchel 1984). This study was extended to various types of mycotic human tissues to investigate the con-

Fig. 1. Pseudomembranous candidiasis of the urinary bladder (patient 1): demonstration of fungal elements by specific immunofluorescence of *Candida albicans* proteinase. *Bar* = 30 µm

Fig. 2. a Invasive intestinal candidiasis (patient 2): demonstration of candidal elements in gut mucosa by immunofluorescence of *Candida* proteinase. *C. albicans* was grown from surveillance cultures. *Bar* = 100 µm. **b** Adjacent mucosal section after treatment as in **a**. The specific antibodies, however, were replaced by non-adsorbed gammaglobulins of the rabbit preimmunization serum

Fig. 3. Pulmonary candidiasis (patient 3): demonstration of fungal elements by immunofluorescence of candidal proteinase. *C. albicans* was grown from respiratory secretion. *Bar* = 35 µm

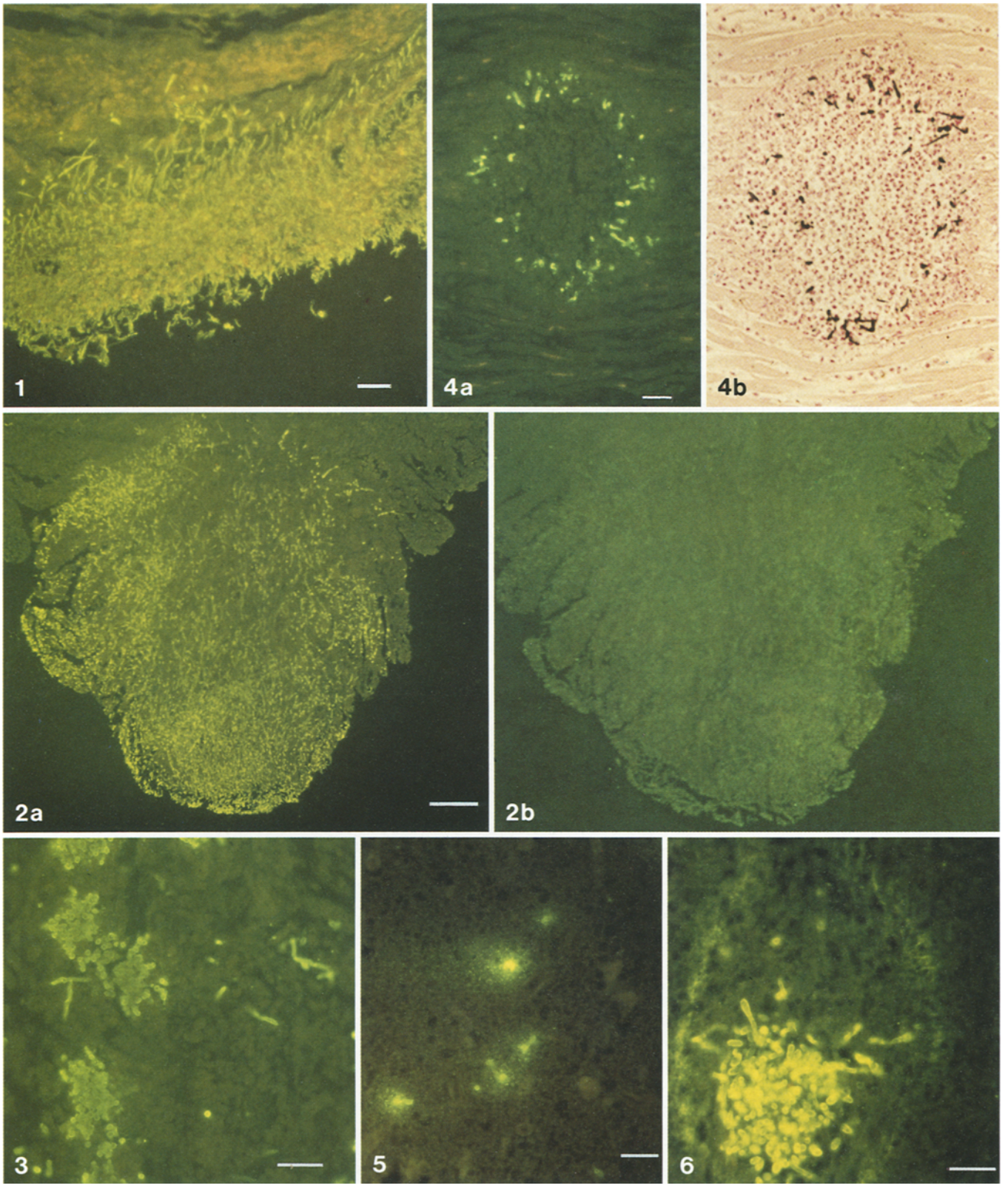

Fig. 4. a Myocardial candidiasis (patient 4): demonstration of fungal elements by immunofluorescence of candidal proteinase in the periphery of a haematogenous microabscess. Differential reactivity with antibodies against proteinases of *C. albicans* and *C. tropicalis* suggested presence of the latter. *Bar* = 30 μm. **b** Adjacent myocardial section: Grocott-methenamine staining

Fig. 5. Candida encephalitis (patient 5): demonstration of fungal elements by immunofluorescence of candidal proteinase. Note the halo of amorphous fluorescent matter surrounding fungal cells. *C. albicans* was grown post mortem from various organs. *Bar* = 20 μm

Fig. 6. *Candida* nephritis (patient 4): demonstration of fungal cells by immunofluorescence of fungal proteinase, using antibodies from guinea-pig, which cross-reacted with all candidal proteinases. Note the ring of amorphous fluorescent matter surrounding the candidal colony. *Bar* = 10 μm

sistency of expression of the proteinase antigen during infection.

The specificity of the anti-proteinase antibodies used has been previously established (Borg and Rüchel 1988). Various murine monoclonal antibodies against candidal proteinases were not suited for immunofluorescence under the conditions of this study (M. Borg, personal communication). Proteinase-related fluorescence was found consistently on the surface of candidal cells, except in a case of candidal nephritis, which was possibly due to the non-proteolytic yeast *C. glabrata*. In this specimen blastoconidia were exclusively seen, which reacted only with antibodies against fungal cell wall antigens (unpublished data). Filamentous fungal cells were always found to be antigen-positive, suggesting that only the common serotype A was present if *C. albicans* was involved (Borg and Rüchel 1990). Lowered intensity of fluorescence must be expected, if the fungal cells are embedded in fibrin clots, for example, in endocarditis. A halo of fluorescence, as observed by Macdonald and Odds (1980), was occasionally seen in candidal myocarditis and encephalitis. However, in renal candidiasis, proteinase was mostly deposited around fungal colonies in a unique ring-like fashion. The ring pattern not only illustrates secretion of proteinase, but it is also indicative of denaturation, which appears to involve dimerization of the enzyme (Rüchel et al. 1982), and which occurs at approximately neutral pH at some distance from the fungal colonies. In the close vicinity of fungal colonies, the pH is likely to be as low as pH 5 due to secretion of organic acids (Stewart et al. 1989). Here the enzyme is likely to act rather indiscriminately on most proteinaceous substrates. Close to neutral pH, specific effects such as activation of blood coagulation or the kallikrein-kinin system may be effected by those candidal proteinases which are relatively resistant to denaturation (Rüchel et al. 1982; Kaminishi et al. 1990). Such enzymes can enter the blood, where they can be detected immunologically (Rüchel et al. 1988); they can reach distant sites of the body and act as factors of virulence. Likewise, circulating candidal proteinases may act as serological markers of invasive candidiasis, and they may even serve as targets for anti-mycotic therapy.

Acknowledgements. We are indebted to Mrs. E. Bothmann (Department of Pathology) for generously providing the tissue sections used in this study, which was supported by the Deutsche Forschungsgemeinschaft (grant Ru 306).

References

Berger U, Bertozzi-Süssmann B, Hässner K, Rüchel R, Zimmermann O (1988) Fatal pulmonary multiple infection with *Aspergillus sp.* and *Candida tropicalis*. Mycoses 31 [Suppl 1]:39–46

Borg M, Rüchel R (1988) Expression of extracellular acid proteinase by proteolytic *Candida spp.* during experimental infection of oral mucosa. Infect Immun 56:626–631

Borg M, Rüchel R (1990) Demonstration of fungal proteinase during phagocytosis of *Candida albicans* and *Candida tropicalis*. J Med Vet Mycol 28:3–14

DeBernardis F, Lorenzini R, Verticchio R, Agatensi L, Cassone A (1989) Isolation, acid proteinase secretion, and experimental pathogenicity of *Candida parapsilosis* from outpatients with vaginitis. J Clin Microbiol 27:2598–2603

Ghannoum MA, Abu-Elteen KH (1990) Pathogenicity determinants of *Candida*. Mycoses 33:265–282

Hageage GJ, Harrington BJ (1984) Use of Calcofluor white in clinical mycology. Lab Med 15:109–112

Hed J, Enestöm S (1981) Detection of immune deposits in glomeruli: the masking effect on antigenicity of formalin in the presence of proteins. J Immunol Methods 41:57–62

Hube B, Turver CJ, Odds FC, Eiffert H, Boulnois GJ, Köchel H, Rüchel R (1991) Sequence of the Candida albicans gene encoding the secretory aspartate proteinase. J Med Vet Mycol 29:129–132

Johnson GD, Davidson RS, McNamee KC, Russell G, Goodwin D, Holborow EJ (1982) Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. J Immunol Methods 55:231–242

Kaminishi H, Tanaka M, Cho T, Maeda H, Hagihara Y (1990) Activation of the plasma kallikrein-kinin system by *Candida albicans* proteinase. Infect Immun 58:2139–2143

Kobayashi K, Hayama M, Hotchi M (1988) The application of immunoperoxidase staining for the detection of causative fungi in tissue specimens of mycosis. Mycopathologia 102:107–113

Luna MA, Tortoledo ME (1985) Histologic identification and pathological patterns of disease due to Candida. In: Bodey GP, Fainstein V (eds) Candidiasis. Raven Press, New York, pp 13–27

Macdonald F, Odds FC (1980) Inducible proteinase of *Candida albicans* in diagnostic serology and in the pathogenesis of systemic candidosis. J Med Microbiol 13:423–435

Odds FC (1988) *Candida* and candidosis. Bailliere-Tindall, London

Ray TL, Payne CD (1990) Comparative production and rapid purification of *Candida* acid proteinase from protein-supplemented cultures. Infect Immun 58:508–514

Reijula KE (1989) Detection of *Candida albicans* in disseminated candidosis by immunofluorescence staining. Mycopathologia 106:1–3

Ross IK, De Bernardis F, Emerson GW, Cassone A, Sullivan PA (1990) The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase-deficient mutant. J Gen Microbiol 136:687–694

Rüchel R (1984) A variety of Candida proteinases and their possible targets of proteolytic attack in the host. Zentralbl Bakteriol Hyg A 257:266–274

Rüchel R (1990) Virulence factors of *Candida* species. In: Samaranayake LP, MacFarlane TW (eds) Oral candidosis. Wright, London, pp 47–65

Rüchel R, Tegeler R, Trost M (1982) A comparison of secretory proteinases from different strains of *Candida albicans*. Sabouraudia 20:233–244

Rüchel R, Böning-Stutzer B, Mari A (1988) A synoptical approach to the diagnosis of candidosis, relying on serological antigen and antibody tests, on culture, and on evaluation of clinical data. Mycoses 31:87–106

Stewart E, Hawser S, Gow NAR (1989) Changes in internal and external pH accompanying growth of *Candida albicans:* studies of non-dimorphic variants. Arch Microbiol 151:149–153

Tsuboi R, Matsuda K, Ko IJ, Ogawa H (1989) Correlation between culture medium pH, extracellular proteinase activity, and cell growth of *Candida albicans* in insoluble stratum corneum-supplemented media. Arch Dermatol Res 281:342–345

Zimmermann CF (1986) Immunhistologischer Nachweis von Candida Protease bei verschiedenen Manifestationen der Candida-Mykose. DDS Thesis, Faculty of Medicine, University of Göttingen, FRG