Reaction of *Candida albicans* cells of different morphology index with monoclonal antibodies specific for the hyphal form

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Summary. Two monoclonal antibodies (MAbs), 3D9 with reported specificity for *Candida albicans* hyphae, and 3B7 with reported specificity for morphological forms of *C. albicans* found *in vivo*, were tested by indirect immunofluorescence with *C. albicans* cells that were grown in 12 different environments (four different culture media incubated at various temperatures) and whose cellular morphology was estimated in terms of morphology index (Mi). Both MAbs reacted strongly with cells with $Mi > 3.0$, i.e., with pseudohyphal and hyphal forms, but in Eagle's medium at 26°C and in a modified Sabouraud's broth medium at 30°C, some reactivity was also found with cells of lower Mi (i.e., yeast forms). Therefore, it was concluded that the hyphal phenotype and the epitopes reactive with the MAbs were co-expressed but that the epitopes could also be expressed independently of the hyphal phenotype. The results confirm the propensity of *C. albicans* for variation of its surface antigenic composition.

Introduction

The existence of antigenic differences between the yeast and hyphal forms of *Candida albicans* has been known for many years.^{1,2} Several investigators have now prepared polyclonal antibodies^{3–8} and monoclonal antibodies (MAbs)^{9,10} that react specifically or predominantly with *C. albicans* hyphae in indirect immunofluorescence tests. Such antibodies usually show no reaction with *C. albicans* yeast cells grown *in vitro*, but reactions with yeast cells in tissues of experimentally infected animals have sometimes been described.¹⁰

Brawner *et al.*¹¹ have shown that expression of surface antigens by *C. albicans* in immunofluorescence tests can vary temporally and with the strain of candida used, and they recommended that experiments with apparent morphology-specific surface epitopes should ideally involve more than one *C. albicans* strain and several different growth media. The advent of an objective, quantitative method for measurement of morphological form in *C. albicans* (morphology index, Mi^{12}) provides a better situation than before for assessment of surface antigen expression, since reactions in immunofluorescence tests can be related to morphology on a cell by cell basis: each

cell can be individually measured to determine its Mi and that individual cell's fluorescence can be scored. The present investigation was undertaken to study the morphological specificity of two MAbs that appear to react predominantly with hyphal forms of *C. albicans*. Mi was used as the basis for morphology determination and the yeast was grown in various different growth environments selected to provide a wide range of cell morphologies.

Materials and methods

Fungus

C. albicans strain 73/055 was used in all experiments. It was maintained by subculture on Sabouraud's glucose agar and inocula were prepared by suspension of overnight growth of yeasts on this medium in water. Cell concentrations were estimated by haemocytometer counting and the volume of cell suspension used to inoculate broth media was calculated to give an initial yeast concentration of 10^6 /ml.

To generate cultures with various *C. albicans* morphologies, 13 different growth environments were used, and the cultures were incubated for 3 h. The growth conditions used were: Eagle's Minimal Essential Medium (EMEM; Gibco) without L-glutamine and buffered to pH 7.2 with 0.02 M HEPES, incubated

at 26°C, 30°C, 37°C and 40°C; Yeast Nitrogen Base (Difco) with glucose 10 g/L (YNBG), incubated at 26°C, 30°C, 37°C and 40°C; modified Sabouraud's broth (MSAB¹³) incubated at 30°C, 35°C and 40°C; Sabouraud's glucose broth (SGB; Oxoid) incubated at 30°C; and horse serum (Difco) incubated at 37°C.

Cells were harvested by centrifugation at 3000g for 10 min, washed twice with water and resuspended in water. The concentration of cells was adjusted to 10⁷/ml and 20-µl samples were placed as spots on glass microscope slides, dried in air and heat-fixed. Fixed slides were individually wrapped and stored at -20°C until used.¹⁴

Indirect immunofluorescence assay (IFA)

Two murine MAbs, both of which originated in France, were used in the experiments. MAb 3D9 was prepared in Angers and MAb 3B7 was prepared in Lille. The preparations of both MAbs used in these experiments were from murine ascitic fluid. Preliminary experiments to determine optimal reactivity in the IFA test led to routine use of MAb 3D9 undiluted and MAb 3B7 diluted 20-fold in phosphate-buffered saline (PBS). To test reactivity of the MAbs against *C. albicans* cells, an IFA procedure based on the one described by Page and Odds¹⁵ was used. To each heat-fixed specimen of cells was added 20 µl of MAb and the slides were incubated in a moist chamber at 37°C for 30 min. The slides were washed three times with fresh lots of PBS for periods of 3, 5 and 7 min then 20 µl of fluorescein-conjugated goat anti-mouse immunoglobulin (Sera-Lab), diluted 20-fold in PBS, was added. The slides were re-incubated at 37°C for 30 min and washed twice in PBS (30 min for each wash). The slides were dried in air and viewed on the same day whenever this was possible. Non-specific reactivity was assessed by means of control slides without cells, without the MAb addition and without addition of the fluorescent conjugate. Slides were viewed by a single operator, blinded to the source of the slides, with a Leitz ultraviolet microscope and a ×50 water-immersion objective. Fluorescence intensity was scored subjectively on a scale of 0 to 3+. Individual cells or cell units within hyphae almost always gave a uniform fluorescence so that no attempt was made to assess variations in fluorescence between different parts of a cell or hyphal unit.

Morphology index (Mi)

The morphology of *C. albicans* cells as viewed microscopically was expressed in terms of the Mi, a dimensionless ratio which was calculated from measurements obtained with the aid of a computerised image analysis system (Imagan; Kompira, Strathclyde). The length, maximum diameter and septal diameter of each separate cell or cell unit within conjoined cells was determined and used for calculation of Mi as described by Merson-Davies and Odds.¹²

Mi up to 1.5 usually indicates a spherical or spheroidal cell; Mi of ≥3.4 usually indicates a parallel-sided cylindrical cell, i.e., a true hyphal unit.

Analysis of results

For each sample, the fluorescence intensity and Mi of 100 cells or cell units was determined. Cells scored as 0 or 1+ by IFA were counted as "negative" by IFA; cells scored as 2+ or 3+ were counted as "positive". Cells in each sample were grouped according to their Mi in intervals of 0.5, and the percentage of IFA-positive cells in each interval was determined. For each set of growth conditions, two or three separate samples were prepared.

Results

For each of the samples it was apparent that MAbs 3D9 and 3B7 often gave stronger fluorescence reactions with cells that fell into the higher Mi groups, indicating a preferential expression of the 3D9 and 3B7 epitopes by hyphal cells. However, there was considerable variation in patterns of reactivity, which appeared to be related to both the composition of the medium and the temperature of incubation. The results were very similar with both MAbs.

Cells grown in serum (mean Mi 3.5) showed no reactivity in the IFA tests. This was considered to be the result of blocking of reactive sites by serum proteins, many of which bind avidly to the surface of *C. albicans* hyphae;^{15,16} results with cells grown in serum were, therefore, ignored. For cells grown in YNBG, the overall level of IFA reactivity with the MAbs was quite low; at MI < 3.5, fewer than half of the cells were scored as positive by IFA. By contrast, in EMEM, most cells fluoresced strongly at temperatures higher than 26°C. Fig. 1 illustrates the distributions of IFA positivity with 3D9 among cells grown at three different temperatures in these two media.

Fewer than 25% of all the cells grown in MSAB at 30°C (mean Mi 2) were IFA-positive, and none of the cells grown in SGB at 30°C (mean Mi 1.75) was IFA-positive. At higher temperatures in MSAB, IFA-reactivity was confined almost entirely to the cells with Mi > 2.5, i.e., the pattern with MSAB resembled those for YNBG in fig. 1.

To gain an impression of the overall relation between Mi and expression of the two MAb epitopes, data from all the experiments were pooled to allow determination of IFA positivity for each increment of Mi independent of growth conditions. The result (fig. 2) indicates the distribution of 3D9 and 3B7 expression as a function of Mi calculated from measurement of more than 3000 cells grown in 12 different environments for each MAb. It is evident from fig. 2 that, for both MAbs, the epitope was expressed predominantly by cells with Mi > 3.0, and that fewer than 20% of cells with Mi < 2.5 expressed either epitope. The IFA

Fig. 1. IFA responses to MAb 3D9 of *C. albicans* 73/055 grown in EMEM and YNBG at (a) 26°C, (b) 30°C and (c) 40°C. The curves indicate the percentage distribution of IFA-positive cells in relation to Mi. Numbers adjacent to each point show the actual number of cells in each Mi interval; ○—○ EMEM, □—□ YNBG.

reactivity of MAb 3B7 was slightly higher than that of MAb 3D9 for cells with $Mi < 2.5$. Expression of the epitopes by more than 90% of the cells was found only for cells with $Mi > 4.0$.

Discussion

Brawner *et al.*¹¹ mentioned three aspects of experimentation on surface antigen expression, detected in *C. albicans* by IFA, in which failure to take account of possible variables might lead to invalid interpretation of findings in relation to cell morphology. They considered that all such experiments should take account of possible strain variations, temporal variations and variations due to growth environment. In the present study, constraints of resources restricted our work to a single strain of *C. albicans* grown for a single time period; however, the use of a wide variety of growth conditions for the candida confirmed that expression of the 3D9 and 3B7 epitopes was influenced by the choice of growth conditions. We believe our inability to explore strain and temporal variations was partly offset by our use of Mi as an objective marker of cell morphology that facilitated the interpretation of morphological observations made with cells from different environments.

For the *C. albicans* strain studied, and for cells

Fig. 2. IFA responses to MABs 3D9 and 3B7 as a function of Mi. The percentage of IFA-positive cells in each Mi interval was determined from data for 12 different growth environments. The curves were, therefore, derived from measurements involving more than 3000 individual *C. albicans* cells; ○—○ MAb 3D9, □—□ MAb 3B7.

grown for 3 h, it was clear that the overall reaction of MABs 3D9 and 3B7 was primarily with cells that had an $Mi > 3.0$ (fig. 2). This means the epitopes were expressed mainly by long pseudohyphal cell units and by true hyphal cell units, as was claimed by their

originators¹⁰ (A. Marot-Leblond, unpublished). However, in some growth environments, particularly EMEM (fig. 1), some *C. albicans* blastospores also expressed the epitope; a finding that confirms previous accounts of the extreme variability of expression of surface antigens by *C. albicans*.^{3,9,11,17-20} There are evidently particular growth environments that favour both the hyphal phenotype and the expression of the 3D9 and 3B7 epitopes, but the latter can also be expressed independently of hypha formation. The significance of expression of these epitopes to the pathogenesis of candida infections remains a matter of conjecture, but it is notable that Fortier *et al.*¹⁰ found strong reactions between MAb 3B7 and nearly

all the *C. albicans* cells found in infected tissues *in vivo*, regardless of their morphology.

Fruit *et al.*²¹ demonstrated clonal variation in expression of a *C. albicans* surface epitope named 5B2. Therefore, it is possible that clonal selection pressures within tissues may determine the pathological outcome of encounters between *C. albicans* and mammalian hosts; alternatively, the micro-environment within host tissues may favour expression of certain surface epitopes and these epitopes may be associated with virulence. Elucidation of these pathogenic processes at the molecular level and their relation to morphological phenotype in *C. albicans* will be the subject of future research.

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