A monoclonal antibody (MAb; MAb 6B3) which reacts specifically with a cell wall antigen found in all strains or isolates of Candida krusei was developed. MAb 6B3 was extensively tested by immunofluorescence assay for cross-reaction with many Candida, Cryptococcus, Saccharomyces, Trichosporon, and Rhodotorula species and was found to react only with the species C. krusei. The specific epitope is expressed on the surface of fungal cells and appears to reside on a protein moiety. Taking into account the increasing importance of fluconazole-resistant strains in nosocomial fungal infections, the very high degree of specificity of this MAb for C. krusei could be useful for the routine detection of C. krusei in culture or in tissue samples.

Among the members of the genus Candida, Candida albicans, Candida tropicalis, Candida glabrata, and Candida parapsilosis formerly represented more than 80% of the usual clinical Candida isolates, while Candida krusei was only sporadically isolated (11) and was regarded as a commensal organism (13, 24). During the past decade, C. krusei has been recognized as a true pathogen, particularly in immunocompromised patients. The resistance of this organism to fluconazole and the systematic use of this drug may explain the significant increase in the numbers of C. krusei infections, (9, 20, 31). In contrast to C. albicans, only a few articles concerning the potential virulence of C. krusei have been published (10, 12, 14, 15, 21, 22, 25, 26). Likewise, relatively few studies have been conducted to characterize antigens of C. krusei, and no monoclonal antibody (MAb) specific to this yeast has yet been developed. The present study concerns the description of a MAb (MAb 6B3) specific for the species C. krusei.

C. krusei ATCC 44507 (American Type Culture Collection) was used throughout this work unless otherwise indicated. Clinical isolates of C. krusei, Candida spp., and other fungi including Rhodotorula, Saccharomyces, and Cryptococcus were obtained from the Mycology Laboratory of the Medical Schools in the French cities of Angers, Grenoble, and Lyon. Each isolate was identified by using the ID 32C system (bioMérieux, Marcy l’Etoile-France). Among the isolates of C. krusei tested, 43 were typed by restriction endonuclease analysis and hybridization with the CKF1,2 DNA probe as described previously (1, 2).

Cultures were maintained on a Sabouraud glucose agar (SGA) slant (bioMérieux) at 22°C, and blastoconidia were prepared by growing the cells on this medium for 48 h at 37°C. In some experiments, the influences of growth in different media and at different temperatures (22 and 37°C) on the surface expression of the antigen reacting with MAb 6B3 were investigated. Seven isolates of C. krusei and one isolate each of C. albicans, C. tropicalis, or C. glabrata were cultured for 48 h in the following five media: SGA, SGA with chloramphenicol, SGA with gentamicin, 5% sheep blood Columbia agar, and chocolate agar (bioMérieux).

Cell antigens were extracted by the following four methods: (i) 10⁹ blastoconidia were incubated at 37°C with shaking in 1.5 ml of 50 mM EDTA-0.35 M 2-mercaptoethanol (2ME; pH 9; Sigma Chemical Co., St. Louis, Mo.) for 30 min; (ii) 10⁹ blastoconidia were digested with 1 ml of lyticase (1,000 U/ml; Arthrobacter luteus; ICN Pharmaceuticals France, Orsay, France) containing 1 mM phenylmethylsulfonyl fluoride (Sigma) for 1 h and 30 min at 37°C with shaking; (iii) 10⁶ cells were incubated at 37°C in 1.5 ml of 2% (wt/vol) sodium dodecyl sulfate (SDS; Sigma) (5, 18) for 30 min; and (iv) extraction by dithiothreitol (DTT) (Sigma) was performed as described by Smail and Jones (27). Solubilized antigenic components were recovered by centrifugation at 12,000 × g for 15 min, and they were then dialyzed against distilled water and lyophilized.

Enzyme-linked immunosorbent assays (ELISAs) were performed in triplicate in a microtitration plate (Falcon; Becton Dickinson, Lincoln Park, N.J.). Each well was coated with 100 μl of C. krusei extract at 10 or 100 μg of protein/ml in phosphate-buffered saline (PBS), and the plates were incubated for 2 h at 37°C or overnight at 4°C. After washing with PBS, the plates were blocked by adding 200 μl of PBS containing 1% bovine serum albumin (fraction V; Sigma). After washing with PBS with 0.05% Tween 20 (PBST), assays were performed by successively incubating the wells with the MAb for 1 h at 37°C, with a 1/2,000 dilution of a commercially available goat anti-mouse immunoglobulin G (IgG) peroxidase conjugate in PBST (Caltag Laboratories, South San Francisco, Calif.) for another hour and then the substrate solution containing ortho-phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide in phosphate citrate buffer (0.15 M; pH 5.0) for 30 min. All working volumes were 100 μl. The enzyme reaction was
stopped by the addition of 50 μl of 1 N H2SO4, and optical densities were read with a TiterTek Multiscan instrument at 492 nm (Flow Laboratories, Inc., McLean, Va.). The absorbance values given are the averages of three absorbance values.

SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli (16). Briefly, the extracts being analyzed were dissolved in buffer containing 62.5 mM Tris-HCl and 2% (wt/vol) SDS. After boiling for 2 min or incubation at 56°C for 30 or 60 min, 40 μl of each extract containing 20 or 60 μg of protein was placed on a 5 to 15% gradient polyacrylamide slab gel. Electrophoresis was performed in a gel electrophoresis apparatus (GE-2/4; Pharmacia, Uppsala, Sweden). Subsequently, the gels either were stained with Coomassie blue or were electrophoretically transferred to polyvinylidene difluoride sheets (Immobilon; Millipore Corp., Bedford, Mass.) as described previously (28). After the transfers, the membranes were blocked in 10% nonfat dry milk in PBS, washed in PBS, incubated with 1:20 or 1:100 dilutions of the MAbs, washed, and incubated with peroxidase-labelled, affinity-purified goat anti-mouse IgG (Caltag Laboratories). After the washings the sheets were placed in substrate solution (3,3’-diaminobenzidine [Sigma] and hydrogen peroxide) for 30 min.

Indirect immunofluorescence assays (IFAs) were carried out as described previously (19). A suspension of 10^6 blastoconidia/ml in PBS containing NaN3 (1‰) was prepared. Ten microliters of this suspension was placed on the wells in microplate slides (Polylabo, Strasbourg, France), and the suspension was allowed to dry at 30°C for 1 h. Twenty microliters of hybridoma culture supernatant undiluted or diluted in PBS was dropped over the cells. The slides were placed in a moist chamber at 37°C for 1 h and were then washed twice with PBS. Twenty microliters of fluorescein-conjugated goat anti-mouse immunoglobulin (Caltag Laboratories) was added to the slides at a dilution of 1:100 in PBS, and the slides were then reincubated at 37°C for 30 min and then washed twice with PBS. The preparations were in PBS containing 90% glycerol when they were mounted onto the slides. The preparations were examined with a Nikon microscope equipped with a reflected-light source of fluorescence. The same procedure was used for immuno-fluorescence studies of yeast cells grown under various conditions and for blastoconidia that were treated with different reagents.

The antigens were characterized by heat, enzymatic, and chemical agent treatments. The heat stabilities of the C. krusei antigens recognized by the MAbs were tested by heating 10^7 blastoconidia of C. krusei in 1 ml of PBS at 56°C for 30 or 60 min and at 100°C for 2 or 5 min. The effects of lyticase (2,000 U/ml; Sigma) and four proteases, pronase E (2.5 mg/ml; Merck, Darmstadt, Germany), proteinase K (16 μg/ml; Merck), trypsin (2.5 mg/ml; Sigma), and α-chymotrypsin (25 μg/ml; Merck), were tested by incubating 10^7 blastoconidia for 30 min at 37°C with shaking in 1 ml of enzymatic reagent in PBS. Control cells were incubated with PBS alone. Periodate oxidation was performed for 1 h at room temperature in the dark with 10^7 blastoconidia and 1 ml of 20 mM sodium periodate in 20 mM acetate-acetate buffer (pH 5). After washing in this buffer, the blastoconidia were incubated for 30 min with 1 ml of 1% (wt/vol) glucose to block the aldehyde groups generated by the periodate treatment and to prevent nonspecific reactions of the antibodies. The cells were then washed in PBS. Control cells were incubated with acetate buffer alone. Treatment with EDTA-2ME, DTT, or SDS was carried out as described above. After incubation with enzymes or chemical agents, the blastoconidia were washed in PBS and were fixed to the wells of a microscope slide. The antigenic activity of the treated blastoconidia was measured by IFA as described above. All experiments were performed in triplicate.

Preparation of the MAb was carried out by immunization of BALB/c mice (Iffa Credo, l’Arbresle, France) with three subcutaneous injections, at 2-week intervals, of 10^6 formalin-killed blastoconidia emulsified in 100 μl of Freund’s complete adjuvant (Sigma) for the first injection and in Freund’s incomplete adjuvant (Sigma) for the following two booster injections. A final booster injection of 10^6 formalin-killed blastoconidia in 100 μl of 0.15 M NaCl was given intravenously 3 days before the mouse was killed. Hybridoma cells were produced by the method described by Dippolo et al. (3). Splenocytes were fused with mouse plasmacytoma X63/Ag.63 in the presence of 0.4% polyethylene glycol 1500 (Merck). The fusion products were diluted in 200 ml of selective medium containing 100 μg of hypoxanthine, 15 μM thymidine, and 0.4 μM aminopterin (HAT medium; Sigma), and the mixture was plated out at 100 μl per well in 20 96-well trays. Ten days after fusion, aliquots of medium from wells with growing hybridomas were screened by IFA for the production of antibodies directed to blastoconidia of C. krusei. Positive hybrids were immediately subcloned twice by limiting dilution in 96-well plates and were stored in liquid nitrogen. MAbs were obtained from confluent hybridoma cultures in a nonselective medium.

Two fusions were carried out, and 593 hybridoma cell lines were produced. Supernatants from 10 of these cell lines produced antibodies that recognized C. krusei by IFA. Among them, only one of the cell lines (6B3), preliminarily tested by IFA against blastoconidia from other Candida species, indicated that it was specific for C. krusei. This hybridoma secreted an IgG and was selected for further studies. To investigate the strain and species specificities of MAB 6B3, 73 isolates or strains of C. krusei, 138 isolates of other Candida species, and 27 isolates of other fungal genera were cultured on SGA for 48 h at 37°C and were examined by IFA for surface antigen expression. The antigen recognized by MAB 6B3 was not detectable in genera other than Candida (Table 1). In the genus Candida, no binding to the other species of yeast tested other than C. krusei was

| Organisms            | No. of strains tested | No. of strains that reacted with MAb 6B3 |
|----------------------|-----------------------|-----------------------------------------|
| Candida krusei       | 73                    | 73                                      |
| Candida albicans     | 45                    | 0                                       |
| Candida glabrata     | 37                    | 0                                       |
| Candida tropicalis   | 18                    | 0                                       |
| Candida kefr         | 8                     | 0                                       |
| Candida parapsilosis | 10                    | 0                                       |
| Candida guilliermondii | 4                  | 0                                       |
| Candida norvegensis  | 6                     | 0                                       |
| Candida lactaniæ     | 2                     | 0                                       |
| Candida lipolytica   | 2                     | 0                                       |
| Candida famata       | 2                     | 0                                       |
| Candida lambica      | 2                     | 0                                       |
| Candida inconspicua  | 3                     | 0                                       |
| Rhodotorula spp.     | 3                     | 0                                       |
| Cryptococcus neoformans | 6                     | 0                                       |
| Saccharomyces cerevisiae | 10                  | 0                                       |
| Trichosporon spp.    | 8                     | 0                                       |
noticed under the conditions supporting cell development. All isolates and reference strains of *C. krusei* were positive by IFA with MAb 6B3. Both blastoconidia and pseudohyphae (when present) reacted with the MAb. The results of control experiments performed without MAb 6B3 were found to be negative. According to these results, MAb 6B3 recognizes a specific *C. krusei* epitope that is expressed on the surfaces of the blastoconidia of *C. krusei*.

IFA values were not affected by heating the blastoconidia of *C. krusei* for 30 or 60 min at 56°C. However, no immunofluorescent reaction was obtained after heating at 100°C. Pronase E and trypsin treatment of *C. krusei* reduced strongly the level of fixation of MAb 6B3. Preparations treated with other enzymes such as proteinase K, α-chymotrypsin, or lyticase retained the same activities as those treated with buffer. Exposure of *C. krusei* blastoconidia to sodium periodate, EDTA-2ME, DTT, or SDS did not affect or weakly reduce the level of fixation of MAb 6B3 (Table 2).

Soluble extracts made with DTT, EDTA-2ME, SDS, or lyticase and tested with MAb 6B3 by ELISA showed no positive signal. These extracts were separated by SDS-PAGE and detected immunologically by using MAb 6B3 to identify the apparent molecular mass of the antigenic component. None of these extracts gave a positive reaction, whatever the temperature (100, 56, or 37°C) or the time (2, 30, or 60 min) of sample treatment before electrophoresis. Although the antigen was expressed on the surface of *C. krusei*, it was not identified in the soluble extracts tested by ELISA or Western blotting. These results suggest two hypotheses: (i) MAb 6B3 recognizes a conformational or a native protein epitope which is present on intact cells and which could be denatured following chemical or enzymatic extraction or (ii) the antigen recognized by MAb 6B3 may not be released in the extracts made with EDTA-2ME, lyticase, DTT, or SDS. The hypothesis that antigen is resistant to solubilization is strengthened by the results obtained by IFA: MAb 6B3 still stains the blastoconidial surface after treatments with the various chemicals or lyticase.

The variability of antigen expression was examined by observing the surface reactivities of cells grown under various conditions. Incubation with MAb 6B3 revealed that antigen expression was detectable on the blastoconidia of *C. krusei* whatever medium or temperature (22 or 37°C) was used. However, the reactivity observed by IFA was low when the blastoconidia were obtained on blood agar (Table 3).

Tsuchiya et al. (29, 30) used polyclonal antibodies (PABs) to study the antigenic patterns of the more medically important yeasts by cross-absorption experiments. For *C. krusei*, by using PAB, Fukazawa et al. (7, 8) and Tsuchiya et al. (30) determined three main antigenic factors: factors 1, 5, and 11. Factor 11 was identified as *C. krusei* specific. The antigen recognized by MAb 6B3 is different from factor 11, which presents a mannose moiety. Several lines of evidence indicate that the epitope recognized by MAb 6B3 presents a protein moiety. First, it was susceptible to treatment with heat at 100°C. Second, treatment with some proteolytic enzymes (pronase, trypsin) resulted in a strong reduction of fluorescent staining. Finally, treatment of *C. krusei* blastoconidia by sodium periodate did not affect recognition by the MAb.

In conclusion, we provide here the first report of the development of a MAb which identifies a *C. krusei*-specific antigen. Its use in the latex test described by Freydiere et al. (6) is useful for the rapid and convenient identification of *C. krusei* colonies. A similar test, Bichrolatex Albicans (Fumouze Diagnostics, Asnières, France), which uses a MAb (MAb LIB 3H8) specific for *C. albicans*, was developed for the rapid identification of *C. albicans* colonies (4, 17, 23). In addition, MAb 6B3 could also be used for the identification of *C. krusei* in tissue samples or in normally sterile fluids.

### Table 2. Quantitative IFA titers of MAb 6B3 in culture supernatant tested with treated C. krusei ATCC 44507 blastoconidia

| Treatment | IFA titer |
|-----------|-----------|
| None      | 1:200     |
| 56°C, 30 min | 1:200     |
| 56°C, 60 min | 1:200     |
| 100°C, 2 min | 1:200     |
| 100°C, 5 min | —         |
| Lyticase  | 1:200     |
| Pronase E | 1:6       |
| Proteinase K | 1:200     |
| Trypsin   | 1:12      |
| α-Chymotrypsin | 1:200     |
| Lysozyme  | 1:200     |
| EDTA-2ME  | 1:100     |
| DTT       | 1:100     |
| SDS       | 1:100     |

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### Table 3. Quantitative IFA titers of MAb 6B3 against isolates of *C. krusei* and other Candida spp cultured in SGA, SGA with chloramphenicol, SGA with gentamicin, 5% sheep blood Columbia agar, or chocolate agar

| Organism     | IFA titer in the following medium at the indicated temp: |
|--------------|--------------------------------------------------------|
|              | SGA | SGA with chloramphenicol | SGA with gentamicin | SBA sheep blood Columbia agar | Chocolate agar |
|              | 22°C | 37°C | 22°C | 37°C | 22°C | 37°C | 22°C | 37°C | 22°C | 37°C |
| *C. krusei*  |     |     |     |     |     |     |     |     |     |     |
| 1            | 1:100 | 1:100 | 1:100 | 1:100 | 1:100 | 1:100 | 1:25 | 1:50 | 1:50 | 1:50 |
| 2            | 1:200 | 1:200 | 1:200 | 1:200 | 1:200 | 1:200 | 1:25 | 1:25 | 1:25 | 1:25 |
| 3            | 1:200 | 1:200 | 1:200 | 1:200 | 1:200 | 1:200 | 1:25 | 1:25 | 1:25 | 1:25 |
| 4            | 1:100 | 1:100 | 1:100 | 1:100 | 1:100 | 1:100 | 1:12 | 1:12 | 1:12 | 1:12 |
| 5            | 1:200 | 1:200 | 1:100 | 1:100 | 1:200 | 1:200 | 1:12 | 1:12 | 1:12 | 1:12 |
| 6            | 1:100 | 1:100 | 1:100 | 1:100 | 1:100 | 1:100 | 1:12 | 1:12 | 1:12 | 1:12 |
| 7            | 1:100 | 1:100 | 1:100 | 1:100 | 1:100 | 1:100 | 1:12 | 1:12 | 1:12 | 1:12 |
| *C. albicans*|     |     |     |     |     |     |     |     |     |     |
| *C. tropicalis*| —   | —   | —   | —   | —   | —   | —   | —   | —   | —   |
| *C. glabrata* |     |     |     |     |     |     |     |     |     | —   |

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* — negative reaction at a 1:3 dilution of culture supernatant.
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