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Production and Characterization of Yeast Killer Toxin Monoclonal Antibodies

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Monoclonal antibodies were obtained after fusion of mouse myeloma cells with spleen cells isolated from mice primed with a crude extract of yeast killer toxin produced by a strain of *Hansenula anomala*. Hybridomas were selected by specific immunoassay reaction of their fluid with crude yeast killer toxin extract. Among the monoclonal antibodies, which were characterized by the Western blot technique, one (designated KT4) proved to have precipitating properties, thus permitting the neutralization of the killer activity of the toxin. Experiments in double immunodiffusion showed that monoclonal antibody KT4 produced homologous precipitin bands by reacting with either the crude toxin used as immunogen or a toxic extract of *Hansenula mrakii*. It is suggested that these monoclonal antibodies will be useful for the purification, characterization, and understanding of the bioactions of yeast killer toxins.

Previous studies have shown that killer yeasts and their toxins might be effectively used to differentiate strains of opportunistic yeasts for epidemiological purposes (3, 4, 6). Later, we observed that the killer phenomenon, which was previously considered to be restricted to yeasts, was also displayed by killer yeasts against unrelated microorganisms (bacteria, hyphomycetes, lipophilic yeasts, aerobic actinomycetes, and achlorophyllous microorganisms) (9). The diverse biological activity of killer yeasts and the potential therapeutic effect shown by a crude toxic extract of *Hansenula anomala* topically applied to laboratory animals experimentally infected with *Malassezia furfur* and *Malassezia pachydermatis* (L. Polonelli, R. Lorenzini, F. De Bernardis, and G. Morace, Mycopathologia, Myco 172) prompted us to try to isolate and purify yeast killer toxins. Since conventional chemical procedures (gel-exclusion chromatography, chromatofocusing, and anion-exchange chromatography) proved to be unsuitable for the large-scale production of purified killer toxin, monoclonal antibodies were produced for the immunochemical purification (affinity chromatography) of yeast killer toxins.

In this study, we report the production and characterization of two monoclonal antibodies against a killer toxin produced by *H. anomala*.

Cultures. The yeast isolate used for the production of killer toxin, *H. anomala* UCSC 25F, was from our collection. The selection was made on the basis of its broad range of activity against unrelated microorganisms (9) and the demonstration of a potential therapeutic effect (Polonelli et al., Myco 172). *Hansenula mrakii* UCSC 255 and *Candida albicans* UCSC 10 were also from our culture collection.

Production of crude killer toxin (reference antigen). The production of crude killer toxin for mouse immunization was carried out as described elsewhere (6). Flasks (1-liter volume) with 500 ml of Sabouraud broth, buffered at pH 3 with 0.1 M citric acid and 0.2 M K₂HPO₄, were seeded with a loopful of a 48-h-old culture of *H. anomala* UCSC 25F and incubated at 22°C for 18 h at 70 rpm on a gyratory shaker. After this period, the cells were removed by centrifugation at 1,500 × g at 4°C, and the supernatant was filtered and

concentrated with a PM-10 membrane in a TCF10A ultrafiltration cell under N₂ pressure (Amicon Corp., Danvers, Mass.). The protein and polysaccharide concentrations in the concentrated (×50) product were 6 and 2 mg/ml, respectively, as determined by the Lowry method and anthrone test. Accordingly, a concentrated (×50) extract of the growth medium (Sabouraud broth, modified; Difco Laboratories, Detroit, Mich.) was prepared.

Mouse immunization. *H. anomala* UCSC 25F concentrated (×50) crude toxic extract was the antigen used for mouse immunization. Four BALB/c mice were injected according to the schedule previously used for mouse immunization with *C. albicans* soluble antigen (8). Briefly, 0.1 ml of the reference antigen was mixed with 0.1 ml of incomplete Freund adjuvant and injected into each mouse intraperitoneally once a week for 1 month. Three days before the fusion, a booster injection was given intraperitoneally with 0.1 ml of the reference antigen alone.

Production and screening of hybridomas. Hybridization was performed according to the procedures previously described (7). Three days after the booster injection, the mice were sacrificed, and their spleens were removed. The spleen cells were fused with an equal number of NS1 myeloma cells by using polyethylene glycol 1000. The cell culture was subjected to a 1-month-long selection regime which included adding hypoxanthine, aminopterin, and thymidine to the culture medium. To screen for the hybrids producing the desired antibody, the hybrid fluids were comparatively tested by enzyme-linked immunosorbent assay (10) in either Nunc microtitration plates (GIBCO Laboratories, Grand Island, N.Y.) coated with the reference antigen (concentrated [×50] crude toxic extract of *H. anomala* UCSC 25F diluted 1:1,000 in carbonate buffer [pH 9.6]) or microtitration plates coated with equally diluted growth medium extract. Hybridoma cultures secreting antibodies reacting only with the reference antigen were propagated and cloned twice by limiting dilution in the presence of mouse thymocytes. Clones continuing to produce the antibody were expanded. The monoclonality and immunoglobulin class of the antibodies produced were ascertained by testing them by immunodiffusion against rabbit anti-mouse immunoglobulins (Miles Laboratories, Inc., Elkhart, Ind.).

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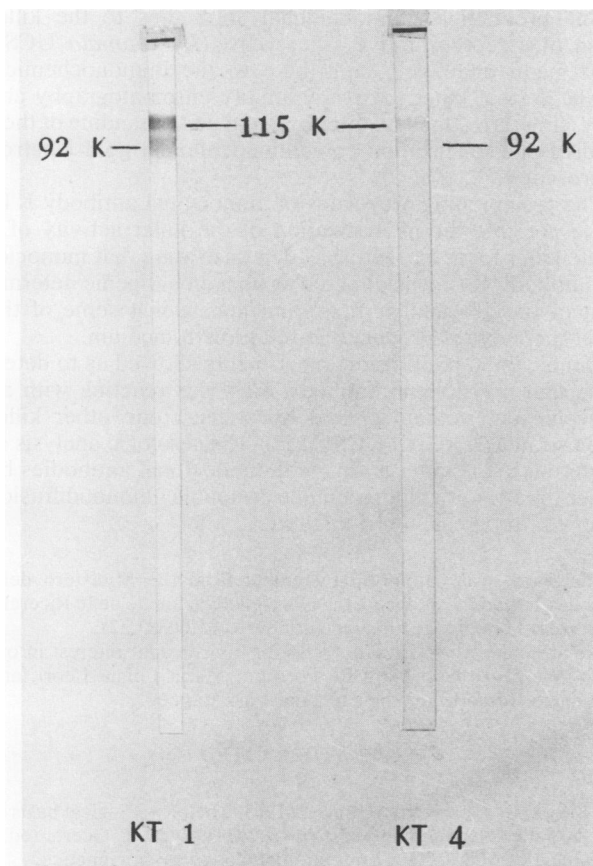


FIG. 1. Western blot analysis of killer toxin monoclonal antibodies. Their molecular masses are expressed in kilodaltons.

Western blot analysis. Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis of the crude killer toxin and its subsequent transfer for detecting monoclonal antibodies against polypeptides transferred to nitrocellulose were carried out as previously reported (5). Electrophoresis was performed at 15 mA for 18 h, and then the proteins were electrically transferred to nitrocellulose sheets at 60 V for 3 h. The nitrocellulose sheets were cut into strips and incubated at room temperature with 5% horse serum for 30 min in phosphate-buffered saline (pH 7.6). Subsequently, the strips were incubated at 37°C with 2 ml of a 1:10 dilution of hybridoma culture fluid in a gyratory shaker for 30 min. They were successively washed for 10 min with phosphate-buffered saline, blocked with 5% horse serum in phosphate-buffered saline, and incubated for 30 min at 37°C in a shaker with 3 ml of horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin G (IgG). After being washed for 30 min with two changes of phosphate-buffered saline, the strips were stained with 4-chloro-1-naphthol and hydrogen peroxide.

Production of ascites fluids and immunodiffusion procedures. The production of ascites fluids for obtaining concentrated immunoglobulins and the immunodiffusion procedure for detecting the precipitating properties of ascites fluids with killer toxins were performed according to procedures reported elsewhere (G. Morace, G. Amalfitano, and L. Polonelli, *Mycopathologia*, in press). Hybridoma cells (10^7) were injected intraperitoneally into pristane-treated syngeneic mice. The precipitating properties of the ascites fluid

were ascertained by an agar-gel double immunodiffusion technique with the reference antigen.

Neutralization of killer toxin. Neutralization of the toxic activity of the killer toxin was carried out by adding graduated amounts of precipitating monoclonal antibody ascites fluid to 1 ml of crude killer toxin. The neutralizing properties of the precipitating monoclonal antibody were ascertained after 24 h at 4°C by discarding the sediment and testing the supernatants (restored to the initial volume with a Minicon B15 concentrator [Amicon Corp.]) for toxic activity against a recognized sensitive strain of *C. albicans*, UCSC 10. Control tests were carried out by using ascites fluid containing a nonspecific monoclonal antibody.

Three thousand hybridomas were obtained from the fusion of NS1 myeloma cells with spleen cells from the four mice immunized with the crude toxic extract of *H. anomala* UCSC 25F. Comparative enzyme-linked immunosorbent assay, used for selecting the antibody-producing hybrids, was used to determine the reactivity of the antibodies in the culture fluid. The hybrids that produced antibodies which reacted with the antigen constituted by the growth medium were discarded. The hybrids that produced antibodies which reacted only with the reference antigen were expanded and cloned. Ten clones were obtained, and monoclonal antibodies of the IgG class were produced. When the *H. anomala* UCSC 25F reference antigen was electrophoretically separated in denaturing gels and then immobilized on nitrocellulose strips, we detected only slight diversity among the monoclonal antibodies to the killer toxin proteins. One type of monoclonal antibody (designated KT1) showed a greater avidity than the other (designated KT4), although both of them reacted with the antigenic determinants that had molecular masses of 92 and 115 kilodaltons (Fig. 1).

Antibody-rich ascitic fluids produced from both of the expanded clones reacted differently in immunodiffusion tests with the homologous reference antigen. Monoclonal antibody KT4 produced a clear precipitin band, whereas monoclonal antibody KT1 failed to precipitate with the antigen.

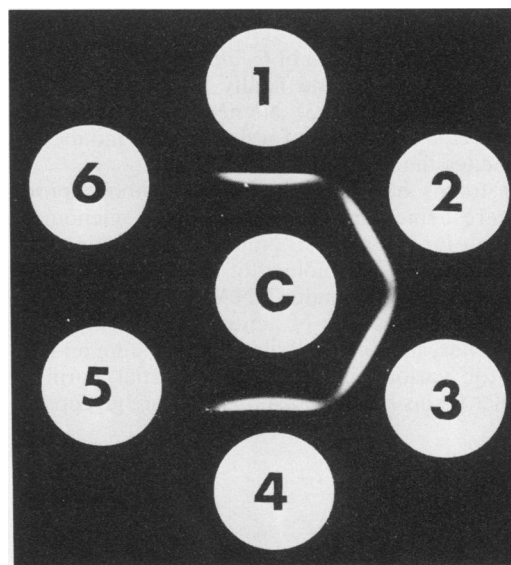


FIG. 2. Double immunodiffusion of killer toxin monoclonal antibody KT4. Well contents: C, KT4 monoclonal antibody; 1 and 4, *H. anomala* UCSC 25F crude killer toxin; 2 and 3, *H. mrakii* UCSC 255 crude killer toxin; 5 and 6, concentrated ($\times 50$) medium extract.

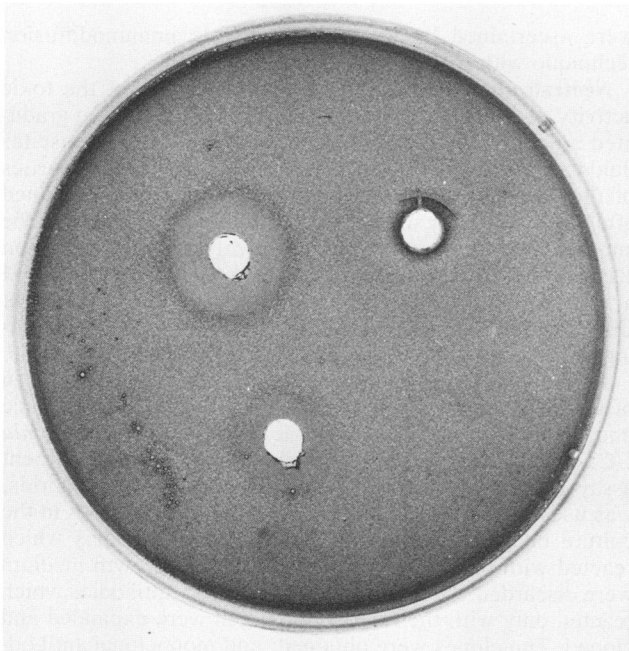


FIG. 3. Gradual decrease of activity of yeast killer toxin from *H. anomala* UCSC 25F against *C. albicans* UCSC 10 with the addition of graduated amounts of KT4 monoclonal antibody.

Monoclonal antibody KT4 produced homologous precipitin bands by reacting with the concentrated ($\times 50$) crude toxic extract of a different killer yeast (*H. mrakii* UCSC 255). However, it reacted negatively against the equally concentrated growth medium extract (Fig. 2).

By adding graduated amounts of KT4-precipitating monoclonal antibody (ascitic fluid) to vials containing 1 ml of *H. anomala* UCSC 25F crude killer toxin, it was possible to observe graduated amounts of sediment after 1 night at 4°C (as the antibody concentration increased, so did that of the immunoprecipitate). Tests on the toxic activity of the supernatants, which were restored to their original volume, against the sensitive strain of *C. albicans*, UCSC 10, showed a gradual decrease in and finally the neutralization of the killer toxin activity (Fig. 3). No neutralization was observed by adding equal amounts of a nonspecific monoclonal antibody (ascites fluid) used as a control.

Killer toxins have proven to be metabolic products of great interest since the time that the killer phenomenon was discovered in yeasts (1). Numerous studies have been done on the molecular biology of the yeast killer factor, the detection of double-stranded RNA viruslike particles (retroviruses), the mechanism of action, and the involvement of nuclear genes, making the killer toxin system an interesting system for various investigations. Partial purification of yeast killer toxins has revealed that they are glycoproteins (2).

The production of monoclonal antibodies to the killer toxin of a representative killer yeast (*H. anomala* UCSC 25F) might effectively contribute to the immunochemical purification of killer toxins by affinity chromatography and the characterization (gel filtration) and understanding of their binding and localization by immunocolloidal gold electron microscopy.

The precipitating properties of monoclonal antibody KT4 made possible the neutralization of the killer activity of a crude killer toxin extract, thus demonstrating that monoclonal antibody KT4 was directed against an antigenic determinant of the yeast killer toxin and not against some of the other metabolites produced in the growth medium.

Double immunodiffusion experiments allowed us to determine that monoclonal antibody KT4 was reacting with an antigenic determinant shared by at least one other killer toxin strain (*H. mrakii* UCSC 255). The serologic analysis of numerous yeast killer toxins with monoclonal antibodies by either the Western blot technique or double immunodiffusion is now in progress in our institute.

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