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Antigen-Antibody Crossed Electrophoresis (Laurell) Applied to the Study of the Antigenic Structure of *Candida albicans*

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By means of the antigen-antibody crossed electrophoresis procedure of Laurell, 68 antigens were demonstrated in *Candida albicans*. This is about four times the number of antigens described earlier by means of classical immunoelectrophoresis. The procedures for obtaining this result are described, including the preparation of antigen, the immunization of rabbits, and the method of N. M. G. Harboe for the production of purified and concentrated rabbit antibodies suitable for quantitative immunoelectrophoresis. The immunoplates were stained by means of the sensitive Coomassie brilliant blue R. The various quantitative immunoelectrophoretic methods offer considerable possibilities for qualitative and quantitative characterization of antigens, even in complex mixtures, and are therefore well suited for the investigation of microbial antigens.

The antigenic structure of *Candida albicans* has been investigated extensively. Agglutination methods have revealed a total number of 7 to 10 antigens (10, 12), the Ouchterlony technique has revealed 5 to 7 antigens (1, 11), and Grabar immunoelectrophoresis has revealed 15 to 16 antigens in this microorganism (2, 3). In the present study, it was possible to demonstrate 68 antigens in *C. albicans* by use of the antigen-antibody crossed electrophoresis procedure of Laurell (9) and of purified concentrated rabbit antibodies.

MATERIALS AND METHODS

Antigens. A strain of *C. albicans* A (B 311) was obtained by courtesy of H. F. Hasenclever, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Antigen no. 1. Antigen no. 1 (for the initial immunization of rabbits) was prepared as follows. A 48-hr culture on Sabouraud medium (37 C) was scraped off the plates, washed three times in 0.154 M NaCl, killed with 2% phenol, partly disintegrated in a Potter-Elvehjem homogenizer, and finally lyophilized.

Antigen no. 2. Antigen no. 2 (for immunoelectrophoresis and final immunization of rabbits) was prepared in the following way. A 10-g amount of a washed 48-hr culture on Sabouraud medium (37 C) suspended in 10 ml of 0.154 M NaCl was homogenized in a Braun cell-homogenizer (MSK) at 2,800 rev/min for 120 sec with 50 g of glass beads (0.45 to 0.50 mm) by using liquid CO_2 as a coolant. The homogenate was centrifuged for 60 min at 105,000 × *g* at 4 C in a Beckman L2-65 B ultracentrifuge (rotor 30), and the resulting supernatant fluid was stored at −20 C. By refractometry, the colloid concentration was determined to be 25 g per liter with human serum as the standard.

Immunization of rabbits. The rabbits were injected and bled by the following schedule. The first four injections were given with intervals of 14 days, and the following injections were given with intervals of 45 days. Bleeding of the rabbits (45 ml of blood from an ear vein) was carried out 8 days after an injection of antigen, the first bleeding following the fourth injection and subsequently following each injection.

During 1 year, the rabbits were injected intracutaneously with doses of 1 mg of antigen no. 1 suspended in Freund's incomplete adjuvant. After 1 year, antigen no. 1 was replaced by antigen no. 2. Each dose consisted of 50 μliters of the antigen suspended in 50 μliters of Freund's incomplete adjuvant.

The antibodies used for the immunoelectrophoresis shown in Fig. 1 were purified from a pool of antisera from 13 rabbits (bleeding no. 5 after the antigen for immunization was changed).

Purification and concentration of the rabbit antibodies. Purification of the immunoglobulin G and immunoglobulin A fractions from the pool of rabbit antisera was performed by the method of N. M. G. Harboe (*personal communication*). The fractions were salted out at room temperature with ammonium sulfate (250 g per liter of serum) overnight. After washing with 1.75 M ammonium sulfate, the precipitate was transferred to a dialysis bag and dialyzed

against distilled water and subsequently against acetate buffer (*p*H 5.0; ionic strength, 0.05). This procedure was performed twice. During the dialysis, the ammonium sulfate was removed and a large precipitate of lipoproteins appeared. The precipitated lipoproteins were removed by centrifugation. Subsequently, diethylaminoethyl A 50 Sephadex chromatography was performed, and the antibodies were eluted with acetate buffer (*p*H 5.0; ionic strength, 0.05). The antibodies were concentrated by repeated salting out with ammonium sulfate (300 g per liter). After dialysis (against distilled water and finally 0.154 M NaCl), NaN_3 was added to a concentration of 15 mM, and storage was at 4 C. In the antibody pool used for the immunoelectrophoresis shown in Fig. 1, the total protein concentration was determined by refractometry to be 27 g per liter, and by agarose electrophoresis it was shown that 96% of the proteins were gamma globulins.

Antigen-antibody crossed electrophoresis. The antigen-antibody crossed electrophoresis (9) was carried out largely according to Clarke and Freeman (5) with 1% agarose gel (Litex, Glostrup, Denmark) in barbital buffer (*p*H 8.6; ionic strength, 0.02). The first-dimension electrophoresis of the *Candida* antigen was performed at 12 C, applying 10 v per cm for 50 min. The second-dimension electrophoresis was run at 12 C, applying 3 v per cm for 18 hr through a gel containing 10 μliters of rabbit antibody preparation per cm^2. In some experiments, the Sabouraud medium was used as antigen. Dimensions of the plates were 10 by 20 cm, and the thickness of the gel was 1 mm. The nonprecipitated proteins were removed by washing the gel with 0.1 M NaCl (24 hr) and distilled water (1 hr). After drying, the plates were stained for 10 min in a solution of 0.5% (w/v) Coomassie brilliant blue R (Michrome) in ethanol-glacial acetic acid-water (45:10:45). Excess dye was removed by repeated washings in dye-free solvent.

RESULTS

During the first year of immunization with antigen no. 1 (the phenol antigen), it was possible to demonstrate a maximal number of 46 precipitates in the immunoplates by using concentrated rabbit antibodies. However, after the antigen for immunization was changed to antigen no. 2, a considerable increase in the number of precipitates occurred. In the immunoplate shown in Fig. 1, it was possible to see 67 individual precipitates. With 1 μliter of antigen, instead of 10 μliters, an additional precipitate delimiting a large area could be seen. Thus, 68 antigens were demonstrated in *C. albicans* in the present study.

Cathodic migrating antigens were studied in other experiments. However, the precipitates were broad and poorly defined and showed reactions of identity with some of the anodic precipitates.

About 15 of the 68 precipitates were faint, and some of these did not appear in every immunoelectrophoretic run, probably because of small changes in the degree of background staining. No antigens could be demonstrated in the Sabouraud medium.

DISCUSSION

The 68 antigens demonstrated in *C. albicans* constitute about four times the number of antigens reported in earlier immunoelectrophoretic studies of this species (2, 3). However, the anti-

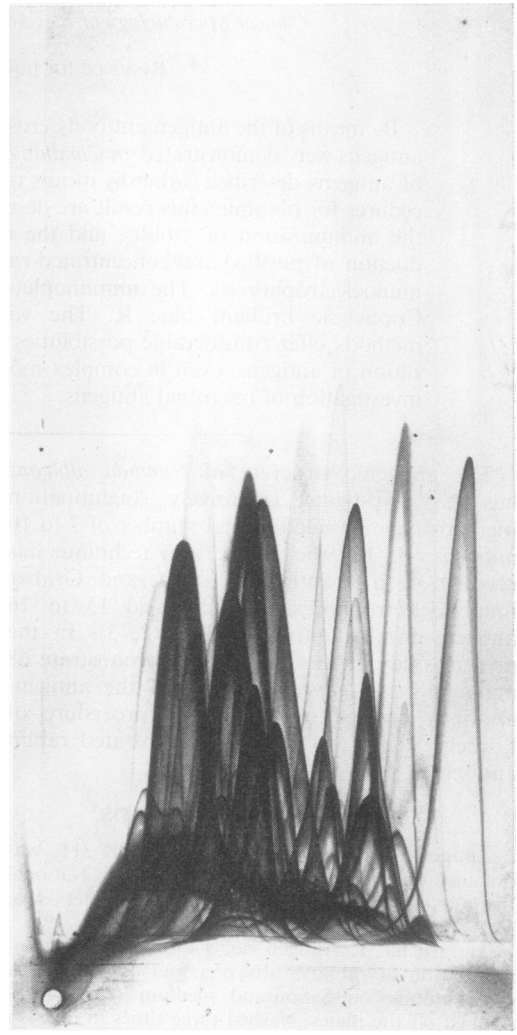

FIG. 1. *Sixty-seven precipitates obtained by antigen-antibody crossed electrophoresis (Laurell) of water-soluble Candida albicans antigens. The first-dimension electrophoresis of 10 μliters of antigen was performed at 12 C, applying 10 v per cm for 50 min (anode to the right). The second-dimension electrophoresis, through rabbit antibody-containing gel, was carried out at 12 C, applying 3 v per cm for 18 hr (anode at the top). Dimensions of the plate: 10 by 20 cm. Staining: Coomassie brilliant blue R.*

gen preparation procedure, the immunization of rabbits, and the immunochemical techniques are not comparable.

The extraction of water-soluble *C. albicans* antigens was carried out by using an effective cell homogenizer. In all other respects, the antigen was handled as carefully as possible, i.e., avoiding heating and denaturing agents. The final ultracentrifugation was performed to remove cell debris which disturbed the exact application of the antigen in the wells of the gel.

For lack of a better antigen, the rabbits were initially injected with phenol-killed partially disintegrated cells. However, when it was revealed that disintegration by means of glass beads was well suited for extraction of the antigens, the immunization of the rabbits was changed. The result was an almost immediate increase in the number of precipitates from 46 to 68. Obviously, phenol treatment should be avoided when the purpose of immunization is to reveal as many antigens as possible.

The purification of the rabbit antibodies by the method used in this study gives a high yield of IgG and IgA (N. M. G. Harboe, *personal communication*). The lipoproteins were removed by the method of N. M. G. Harboe (*personal communication*) to diminish the background staining of the immunoplates. The final concentration procedure was performed to detect precipitins of low titers.

The antigen-antibody crossed electrophoresis (Fig. 1) was carried out by means of 10 μliters of antigen and 2 ml of the concentrated antibody pool (corresponding to about 10 ml of unconcentrated rabbit serum). Thus, the consumption of antibodies was very high compared to the amounts usually employed for immunoelectrophoresis.

The Coomassie staining was used since it is more sensitive than other stains, such as, for example, amido black (4).

The methods used in this study for demonstrating the great complexity of *C. albicans* may be applied to other microorganisms, the purpose being to reveal a multitude of antigens. Furthermore, the quantitative nature of the antigen-antibody crossed electrophoresis (5, 9) allows quantitation without purification of the individual antigens. A prerequisite for quantitation is a safe identification of the antigens. This might seem to be an impossible task in a complicated precipitate pattern as that demonstrated in this study. However, several recently developed quantitative immunoelectrophoretic methods are very effective tools in this respect (6–8). The application of quantitative immunoelectrophoretic methods to the study of microbial antigens may therefore offer considerable possibilities for qualitative and quantitative characterization of these antigens which are often present in very complex mixtures.

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