Journal of Chromatography, 306 (1984) 117—124
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1991

PURIFICATION OF SOLUBLE SPECIFIC ANTIGENS OF SYSTEMIC CANDIDIASIS BY ANTIBODY AFFINITY CHROMATOGRAPHY

A. BENBOUZID

Institut Pasteur de Lyon, Laboratoire de Mycologie et Microbiologie Industrielle, 77, Rue Pasteur, 69365 Lyon Cedex 2 (France)

R. GUINET*

*Institut Pasteur de Lyon, Laboratoire de Mycologie et Microbiologie Industrielle, 77, Rue Pasteur, 69365 Lyon Cedex 2 (France) and *Departement de Microbiologie et Immunologie Pharmaceutique, Faculté de Pharmacie, Université Claude Bernard — Lyon I, 8, Avenue Rockefeller, 69373 Lyon Cedex 2 (France)*

and

H. CLOPPET

Institut Pasteur de Lyon, Laboratoire d'Immunochimie, 77, Rue Pasteur, 69365 Lyon Cedex 2 (France)

(First received September 6th, 1983; revised manuscript received October 21st, 1983)

SUMMARY

Serological methods can be applied to the diagnosis of systemic candidiasis, but cell wall mannans can detect antibodies occurring in several cases unrelated to candidiasis. The present study proposes a procedure for the preparation of specific antigens obtained from an experimental infectious model. The specific immunoglobulins were obtained from rabbits with chronic systemic candidiasis. After precipitation by ammonium sulfate and purification by ion-exchange chromatography on DEAE-Sephadex A-50, these immunoglobulins were fixed on cyanogen bromide-activated Sepharose 4B and used for the preparation of an affinity chromatography column. This column allowed isolation of specifically bound fractions of *Candida albicans* soluble antigens. When analyzed by quantitative immunoelectrophoresis with a polyvalent hyperimmune antiserum, these fractions showed eight precipitation lines, whereas the complete soluble antigen exhibited 48 lines. Possible applications of these antigens to specific serodiagnosis of systemic candidiasis are being evaluated.

INTRODUCTION

Systemic candidiasis remains a major infectious problem for high-risk patients [1]. Direct diagnosis is extremely difficult; clinical signs are poorly evocative and hemocultures may be negative. The serological diagnosis can

0378-4347/84/$03.00 © 1984 Elsevier Science Publishers B.V.

be helpful, particularly detection of precipitins to *Candida albicans* soluble cytoplasmic antigen. However, antigenic extracts used to this purpose contain contaminants of cell wall origin [2—6] which give rise to false-positive reactions for sera of apparently normal subjects [1, 6—9]. As a consequence, it seems necessary to utilize antigenic fractions specific for systemic candidiasis. Elimination of cell wall mannans and characterization of specific mycelial antigens have been tried [3, 4, 10—17].

The present paper reports the preparation of specific antigens of deep candidiasis using an original chromatographic procedure: antibodies obtained from rabbits with experimentally induced chronic systemic candidiasis were fixed on cyanogen bromide (CNBr) activated Sepharose 4B [18]. Antigens thus prepared were then analyzed by quantitative immunoelectrophoresis.

MATERIALS AND METHODS

Antibody preparation

Experimentally induced infection. Twelve hybrid albino rabbits in the weight range 3.5—4 kg were used. Three rabbits were inoculated with saline by injection into the external auricular vein, nine were inoculated with *Candida albicans*, six received 1.38×10^7 colony-forming units (CFU) per kg ($\equiv 0.1\ LD_{50}$), and the remaining three received 2.8×10^7 CFU/kg ($\equiv 0.2\ LD_{50}$).

From day 20 after inoculation, a 10-ml blood sampled was drawn from each rabbit every seven days for a three-month period. The course of infection was controlled by line immunoelectrophoresis of antibodies [19]. At the end of the three-month period, the rabbits were sacrificed and autopsies were done. Organs exhibiting macroscopic alterations were removed under sterile conditions and were used for post-mortem cultures after homogenization and for histopathologic tests performed in the Department of Anatomo-Pathology of the Institut Pasteur (Lyon, France).

Specific immunoglobulin G (IgG) purification. Only rabbit sera rich in antibodies were selected, mixed, precipitated by 25% ammonium sulfate and purified by ion-exchange chromatography on DEAE-Sephadex A-50 (Pharmacia Fine Chemicals), as described by Harboe and Ingild [20], in pH 5 acetate buffer, flow-rate 25 ml/h. Purification was checked by cellulose acetate electrophoresis, immunoelectrophoresis, line immunoelectrophoresis and fused rockets, using anti-rabbit whole protein antiserum and anti-rabbit IgC antiserum (Institut Pasteur de Lyon).

Soluble antigen preparation

Culture was carried out in Sabouraud medium (Institut Pasteur Production), 30 g/l, enriched with 3 g/l of yeast extract (Difco), for 48 h at 30°C in a shaker incubator (GFL, Touzart et Matignon, Vitry-sur-Seine, France). After centrifugation for 15 min at 15,000 *g* the yeasts were collected and washed twice with sterile distilled water. Then a mixture of 40 g of 0.5 mm glass beads and 10—15 g of yeast was suspended in 15 ml of sterile distilled water in a special 70-ml flask (Braun Melsungen). Disruption of cells was done under liquid carbon dioxide for 2 min at 2800 cycles/min in a Braun Melsungen homogenizer. Breakage was checked under the light microscope and if the

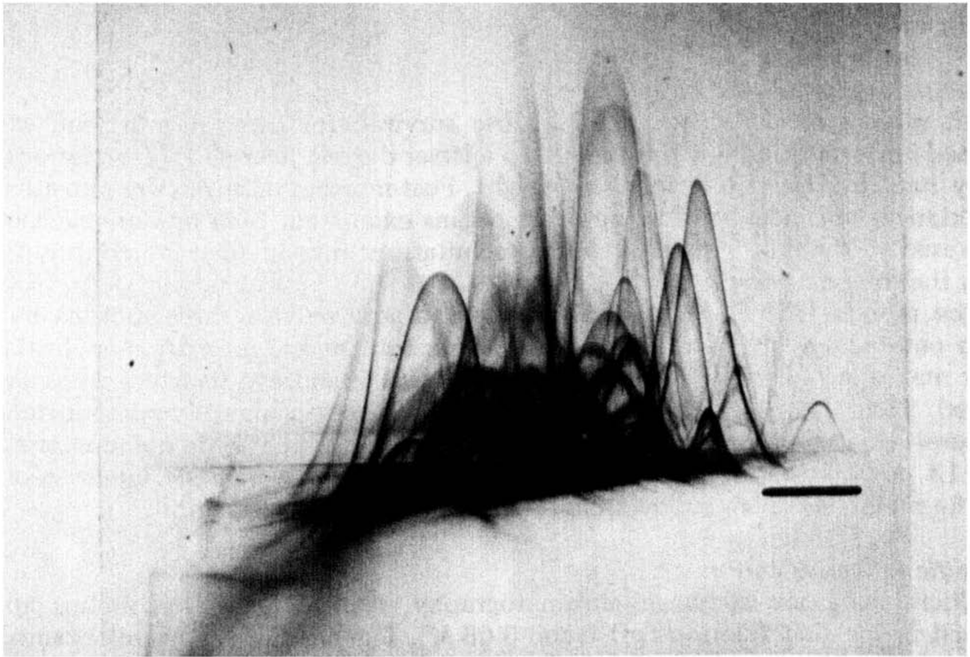

Fig. 1. Soluble *Candida albicans* antigen showing 65 fractions by crossed immunoelectrophoresis with the corresponding polyvalent antiserum.

amount of intact cells exceeded 30%, a second 1-min breakage was carried out.

The supernatant was clarified by a 30-min double centrifugation at 48,000 *g* (Beckman J2-21). The soluble antigen obtained had a protein concentration of 30 g/l (determined by refractometry) and exhibited 65 fractions on crossed immunoelectrophoresis (Fig. 1). The antigen was transferred to flasks and lyophilized.

Specific antigen purification

Immunoabsorbent preparation. A 15-g quantity of CNBr-activated Sepharose 4B (Pharmacia) was made to swell for 15 min in 10^{-3} *M* HCl solution. Then 500 mg of purified IgG were added to the 15 g of activated gel and coupling was achieved in pH 8.3 buffer (0.1 *M* $NaHCO_3$, 0.5 *M* NaCl) within 2 h at room temperature and by gentle rotary shaking. Remaining active groups were blocked by 1 *M* glycine at pH 8. The gel was then washed several times alternately in pH 4 buffer (0.1 *M* acetate, 0.5 *M* NaCl) and in pH 8.3 buffer (0.1 *M* $NaHCO_3$).

Chromatography. Chromatography was performed in a $C_{16/20}$ column (Pharmacia) with a bed volume of 13 ml and at flow-rate of 25 ml/h. The washed gel was equilibrated with 200 ml of pH 8.3 buffer (0.2 *M* Tris—HCl). Then 150 mg of the soluble antigenic preparation were applied at the top of the column. Elution was performed successively with pH 8.3 buffer (0.2 *M* Tris—HCl, 0.5 *M* NaCl) and pH 2.8 buffer (0.2 *M* glycine—HCl). The various fractions were salted out by dialysis and lyophilized. Antigenic analysis of these fractions was made by line immunoelectrophoresis [19] and fused rockets [21, 22] using a polyvalent anti-*Candida albicans* antiserum.

RESULTS

Experimentally induced infection

All rabbits receiving a 0.1 LD_{50} dose survived for three months and exhibited important kidney lesions and, to a lesser degree, liver and spleen lesions. They had lost 15% of their initial weight. Post-mortem cultures were positive for kidneys and negative for the other organs examined. Line immunoelectrophoresis of their sera showed 4—8 precipitation lines in relevant continuity with the reference line system.

The rabbits receiving a 0.2 LD_{50} dose also survived for three months but their pathologic conditions were more severe: tubular and interstitial nephritis, liver and spleen lesions, and lung, brain (paralysis) and eye damages were observed. They had lost 40% of their initial weight. Here again, only post-mortem cultures of kidney were positive. Line immunoelectrophoresis demonstrated 12—13 precipitation lines, also in continuity with the reference line system.

The rabbits receiving saline did not present any signs of infection.

Specific IgG purification

When using ion-exchange chromatography, type G immunoglobulins appeared in the first fraction (pH 5 and 0.06 *M*). The purified IgG were obtained with nearly 99% purity as checked by cellulose acetate electrophoresis. Line immunoelectrophoresis (Fig. 2) and fused rockets (Fig. 3) showed that only IgG were present in this fraction.

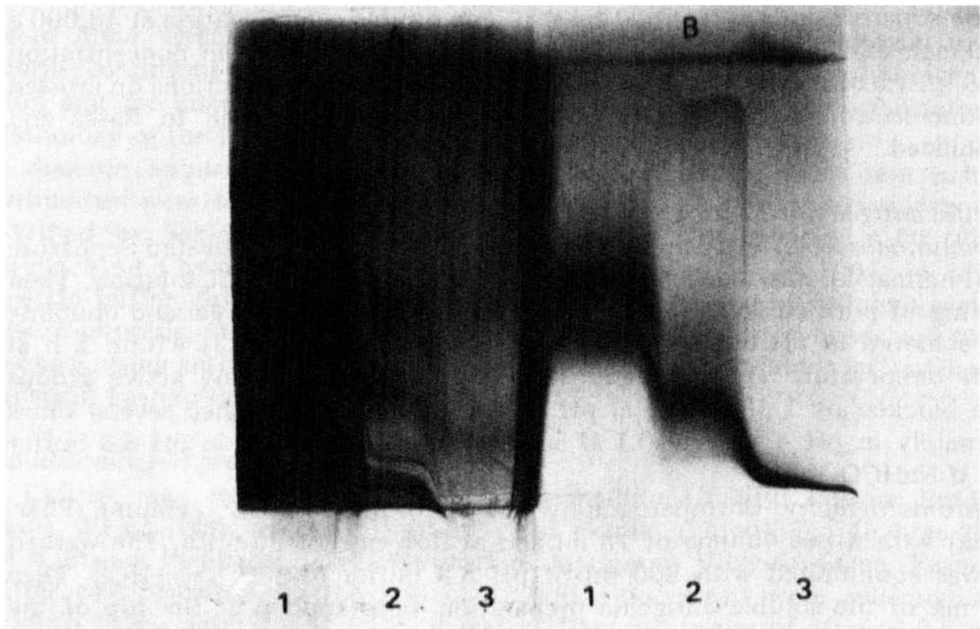

Fig. 2. IgG purification from sera of experimentally infected rabbits: control of separation steps by line immunoelectrophoresis. (A) 450 μl of anti-rabbit whole protein antiserum in 4.7 ml of agarose; (B) 450 μl of anti-rabbit IgG antiserum in 4.7 ml of agarose. 1 = 25 μl of serum from rabbit No. 7 in 250 μl of agarose; 2 = 25 μl of the same serum treated with ammonium sulfate; 3 = 25 μl of IgG purified by ion-exchange chromatography.

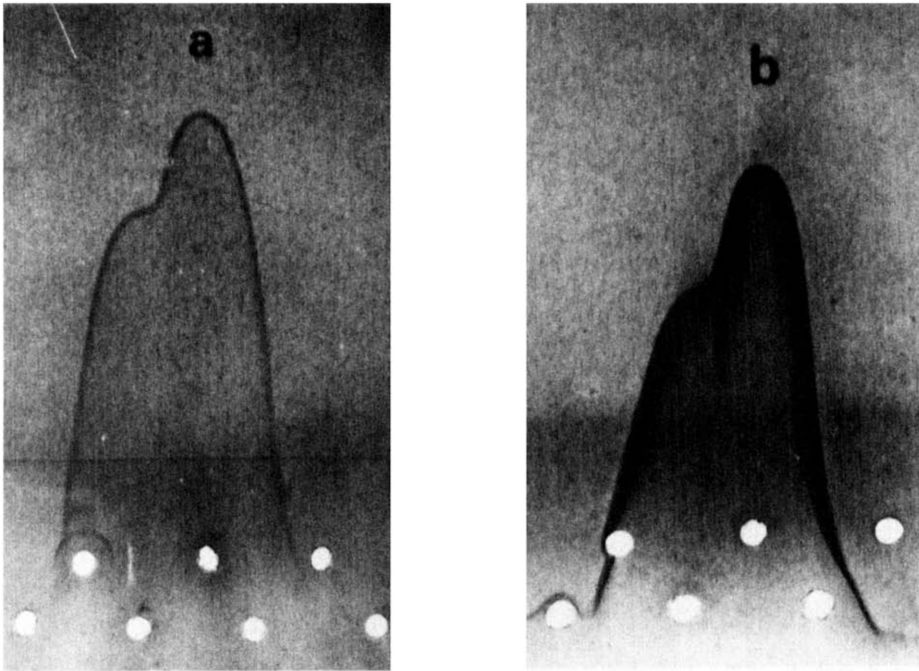

Fig. 3. IgG purification from sera of experimentally infected rabbits: control of separation steps by fused rockets (each well contained 2 µl of antigen). (a) 1 ml of anti-rabbit whole protein antiserum in 10 ml of agarose; (b) 1 ml of rabbit IgG antiserum in 10 ml of agarose.

Specific antigens purification

Using antibody affinity chromatography, unbound antigens were eluted with a total volume of 200 ml of pH 8.3 buffer and the eluate was collected in 28 fractions. The first eight fractions contained the greatest part of these

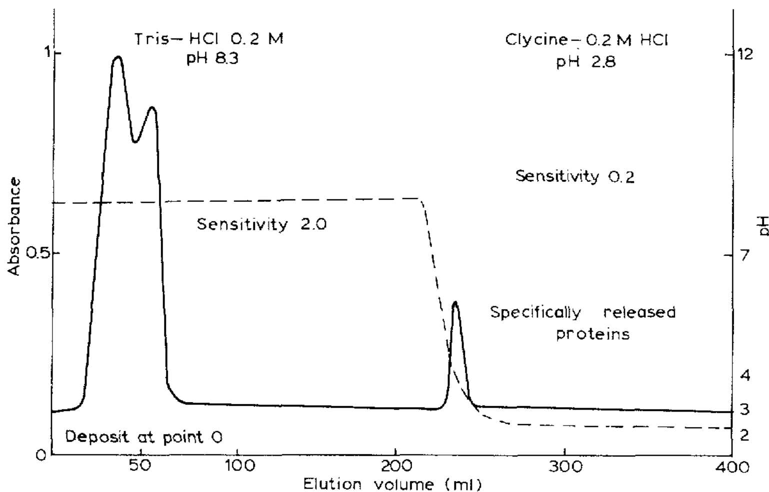

Fig. 4. UV absorbance chromatogram at 278 nm showing *Candida albicans* antigen separation by antibody affinity chromatography. The greater part of the antigens was not retained. The first peak recorded with low sensitivity was more important than the second peak recorded with maximum sensitivity, which implies that only a very small amount of antigen was retained by the affinity column.

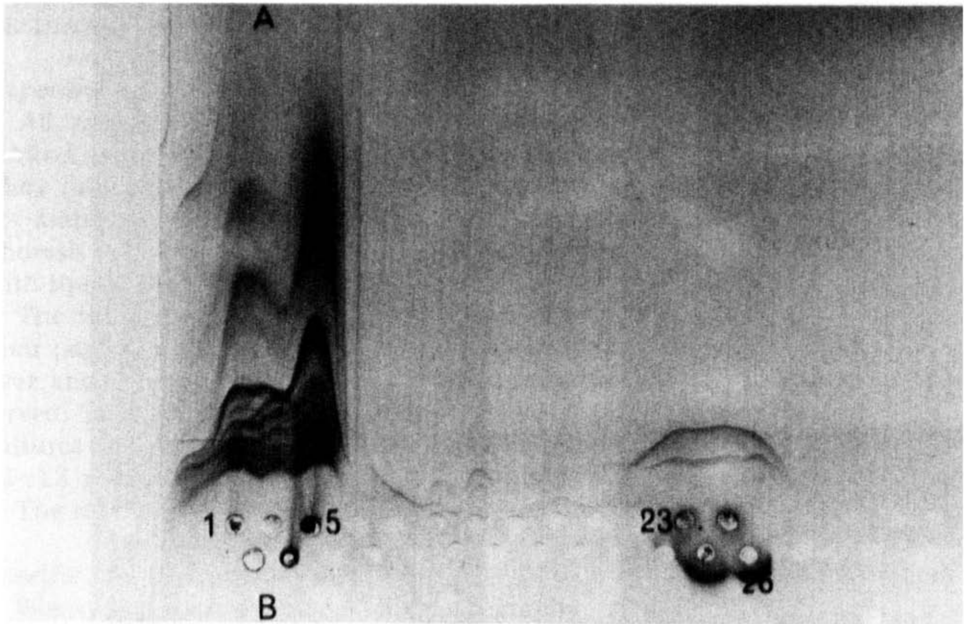

Fig. 5. Fused rockets of fractionated *Candida albicans* antigens by affinity chromatography. (A) 1 ml of anti-*Candida albicans* hyperimmune serum in 10 ml of agarose; (B) 4.2 ml of blind agarose. The distance between the wells was 4 mm. The wells received 2 μl of each fraction. Wells 1—5 corresponded to the first UV absorbance peak, wells 23—26 to the second peak (Fig. 4).

antigens. Then specifically bound antigens were eluted with a total volume of 30 ml of pH 2.8 buffer and the eluate collected in five fractions (Fig. 4).

Immunoelectrophoretic analysis and fused rockets of these fractions (Fig. 5) allowed antigen separation to be controlled during chromatography. In the line immunoelectrophoresis plate (Fig. 6) it was possible to count and identify the antigens that were present only in the second elution fractions. Eight precipitation lines could be identified; they all existed in the initial complete antigen showing 48 lines.

DISCUSSION

The chronic systemic *Candida albicans* infection experimentally induced in rabbit was clinically close to that observed in man, which is in agreement with results previously reported [4, 5, 16]. The infection allowed antibodies detectable by immunoprecipitation to be obtained. These antibodies were obtained with a nearly 99% degree of purity by combining ammonium sulfate precipitation with ion-exchange chromatography, as described elsewhere [20]. The antigens that were specifically adsorbed onto the antibody affinity column were wholly released at pH 2.8 and were recorded in one single, low concentration peak (Fig. 4). The yield was rather low: it was necessary to run the same sample three times in the column in order to retain the greater part of the specific fractions.

The purification of cytoplasmic extracts of *Candida albicans* by eliminat-

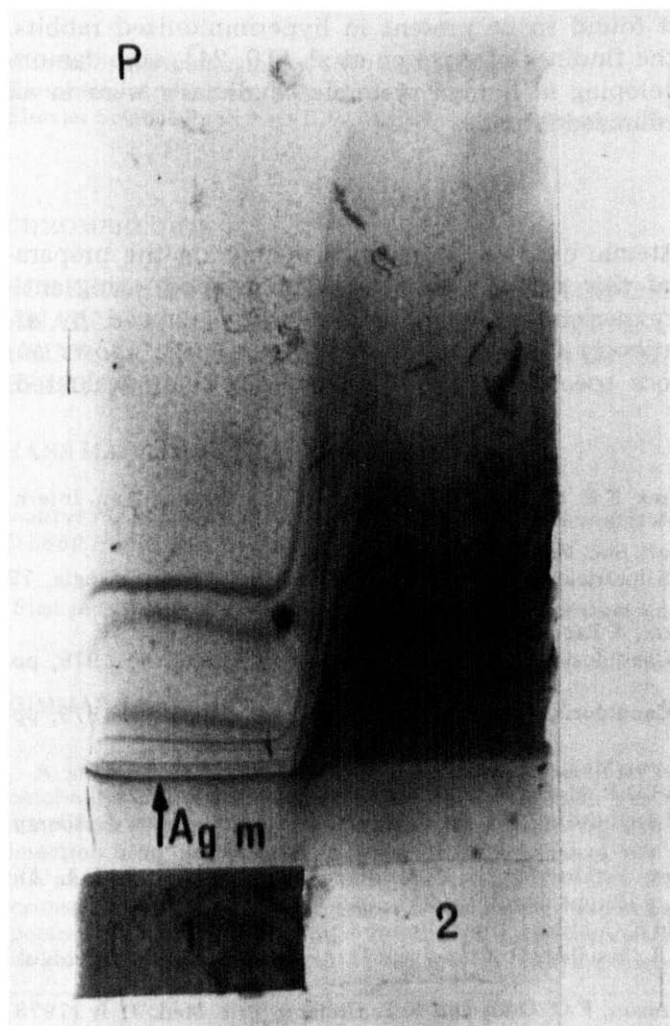

Fig. 6. Line immunoelectrophoretic identification of specific antigens of systemic candidiasis. P = polyvalent hyperimmune serum; 1 = purified specific antigen corresponding to the second peak in Fig. 4. Agm = major antigen forming a markedly intense precipitation line in the purified antigen as well as in the soluble complete antigen; 2 = soluble complete antigen.

ing mannans by affinity chromatography using concanavalin A [3, 14] improves the diagnostic specificity of systemic candidiasis. However, glycoproteins which play a role in candidal pathology are eliminated along with the mannans [4, 5]. Gabriel and Guinet [2, 23], using the immunoaffinoelectrophoresis method with concanavalin A Sepharose as ligand, discovered that 38% of the *Candida albicans* antigens studied showed marked affinity for this ligand. Our method for obtaining specific antigens of systemic candidiasis uses only their immunological properties, retaining exclusively those antigens that proved to develop immune response in animals with deep candidiasis.

The antibodies elaborated in the course of experimentally induced infec-

tion in the rabbit were all found to be present in hyperimmunized rabbits. This is in agreement with the findings of Axelsen et al. [10, 24], who demonstrated that antibodies developing in human systemic candidiasis were in all cases found in the hyperimmunized rabbit.

CONCLUSION

The serodiagnosis of systemic candidiasis depends mainly on the preparation of specific antigens of this pathologic entity. Our method, using antibodies elaborated during experimental systemic infection followed by affinity chromatography (antibody CNBr-activated Sepharose 4B), allows antigens to be obtained whose specificity and efficiency are being evaluated.

REFERENCES

1 J.E. Edwards, Jr., R.I. Lehrer, E.R. Stiehm, T.J. Fischer and L.S. Young, Ann. Intern. Med., 89 (1978) 91—106.
2 S. Gabriel and R. Guinet, Bull. Soc. Fr. Mycol. Med., X, (1981) 307—312.
3 M. Munoz, G. Estes, M. Kilpatrick, A. Disalvo and G. Virella, Mycopathologia, 72 (1980) 47—53.
4 F.C. Odds and J.C. Hierholzer, J. Bacteriol., 114 (1973) 257—266.
5 F.C. Odds, Candida and Candidosis, Leicester University Press, Leicester, 1979, pp. 189—208.
6 F.C. Odds, Candida and Candidosis, Leicester University Press, Leicester, 1979, pp. 209—227.
7 C. Douchet and J. Muller, Lyon Méd., 227 (1972) 1129—1133.
8 P.H. Everall, C.A. Morris and D.F. Morris, J. Clin. Pathol., 27 (1974) 722—728.
9 J. Pepys, J.A. Faux, J.L. Longbottom, D.C. McCarthy and F.E. Hargreave, J. Allergy, 41 (1968) 305—318.
10 N.H. Axelsen, H.R. Buckley, E. Drouhet, E. Budtz-Jorgensen, T. Hattel and P.L. Andersen, Scand. J. Immunol., 4 (Suppl. 2) (1975) 217—230.
11 J.E. Cutler, L. Friedman and K.C. Milner, Infect. Immun., 6 (1972) 616—627.
12 J.H. Ellsworth, E. Reiss, R.L. Bradley, H. Chmel and D. Armstrong, J. Clin. Microbiol., 5 (1977) 91—99.
13 E.G.V. Evans, M.D. Richardson, F.C. Odds and K.T. Holland, Brit. Med. J., iv (1973) 86—87.
14 R.A. Greenfield and J.M. Jones, Infect. Immun., 34 (1981) 469—477.
15 J.L. Longbottom, W.O. Brighton, G. Edge and J. Pepys, Clin. Allergy, 6 (1976) 41—49.
16 F. MacDonald and F.C. Odds, J. Med. Microbiol., 13 (1980) 423—435.
17 R.E. Syverson, H.R. Buckley and C.C. Campbell, Infect. Immun., 12 (1975) 1184—1188.
18 H.G. van Eijk and W.L. van Noort, Clin. Chem. Clin. Biochem., 14 (1976) 475—478.
19 J. Kroll, in N.H. Axelsen, J. Kroll and B. Weeke (Editors), A Manual of Quantitative Immunoelectrophoresis, Methods and Applications, Universitetsforlaget, Oslo, 1973, pp. 61—67.
20 N. Harboe, A. Ingild, in N.H. Axelsen, J. Kroll and B. Weeke (Editors), A Manual of Quantitative Immunoelectrophoresis, Methods and Applications, Universitetsforlaget, Oslo, 1973, pp. 161—164.
21 C.B. Laurell, Scand. J. Clin. Lab. Invest., 29 (Suppl. 124) (1972) 21—37.
22 B. Weeke, in N.H. Axelsen, J. Kroll and B. Weeke (Editors), A Manual of Quantitative Immunoelectrophoresis, Methods and Applications, Universitetsforlaget, Oslo, 1973, pp. 217—230.
23 S. Gabriel and R. Guinet, Bull. Soc. Fr. Mycol. Med., IX (1980) 229—234.
24 N.H. Axelsen, Scand. J. Immunol., 5 (1976) 177—190.