MYCOLOGY

Evaluation of tests for antibody response in the follow-up of patients with acute and chronic forms of paracoccidioidomycosis

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Several serological tests have been used successfully in the diagnosis of paracoccidioidomycosis (PCM). In contrast, data about the use of these tests in the follow-up of PCM patients have been heterogeneous. In this study, serum samples from 43 PCM patients with different clinical forms were analysed by counter-immuno-electrophoresis (CIE), complement fixation (CF) and ELISA before treatment. With CIE and ELISA, the chronic unifocal form showed significantly lower antibody levels compared with chronic multifocal and acute forms. Acute form patients had significantly higher titres than patients with multifocal disease by CIE but not by ELISA. No significant differences were observed with CF. Twenty-seven of these patients were followed-up for 2 years and showed a decline in antibody levels by all three tests, paralleling clinical improvement. However, only patients with unifocal disease cleared their antibodies after 1 year of treatment as analysed by CF and ELISA and after 2 years by CIE, suggesting that these patients may need shorter courses of therapy. Patients with the other clinical form of the disease needed ≥ 2 years of therapy to clear their antibodies. Sera from a further five patients who presented with a relapse were analysed. At the time of relapse all showed increases in antibody levels by CIE and ELISA, but only three showed increases by CF tests. Therefore, CIE and ELISA demonstrated a better clinical correlation than CF, probably reflecting the fungal burden of PCM patients more accurately.

Introduction

Paracoccidioidomycosis (PCM) is one of the most important endemic deep mycoses in Latin America [1]. It is a chronic granulomatous disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*. It has an important social and economic impact because it affects previously healthy men during their productive years, causing cutaneous or respiratory tract mucosal lesions, or both [1]. The infection is acquired by inhalation of conidiospores from the soil, but its reservoir has not yet been fully characterised [2]. The mucosal lesions can vary from isolated oral ulceration to diffuse interstitial pulmonary involvement. However, most patients present with multiple lesions located at

different sites of the respiratory tract. This form of the disease, called chronic form (ChF), may develop many years after the patient has left the endemic area. In contrast, the less frequently seen acute form (AF) affects both sexes <30 years of age and probably develops soon after exposure to the fungus. Patients with the AF have visceral involvement, predominantly of the mononuclear-phagocytic system [1].

Assessment of the serological response has long been used in PCM with two main purposes. Since its introduction by Moses in 1916 [3], it has been used mainly as a diagnostic tool, because in a significant proportion of the patients it is difficult to obtain clinical specimens to visualise or cultivate the fungus, or both, because only deep-sited lesions are present. Also, it has been used to follow up patients under treatment. Several techniques employing different antigenic preparations have been developed for diagnostic purposes and tested in different centres with

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good results [4]. In contrast, data on the use of serological tests in the follow-up of patients have been very heterogeneous. In the 1950s, Fava Neto first standardised the serological tests for both diagnosis and follow-up of PCM patients, the CF test and tube precipitation [5]. In the following decade, Restrepo introduced the double immunodiffusion (DID) test [6]. Counter-immuno-electrophoresis (CIE) and indirect immunofluorescence (IFI) tests appeared later [7, 8] and, more recently, ELISA and immunoblotting techniques have also been applied to the diagnosis and follow-up of PCM patients [9–13].

This study compared the CIE and complement fixation (CF) tests to an ELISA with a *P. brasiliensis* somatic antigen. While the CIE and CF tests have been used routinely for monitoring PCM patients receiving treatment [14], the ELISA has been introduced because it has the potential advantage of being more sensitive and allowing the processing of greater numbers of samples. Also, anti-*P. brasiliensis* antibody levels measured by the three assays were correlated with the different clinical presentations of the disease, and the literature on the use of serological tests to follow up PCM patients under treatment was reviewed.

Materials and methods

Patients and sera

This study used serum samples from 43 patients with proven PCM: 27 patients were assayed before treatment and 6, 12 and 24 months after the initiation of therapy. Sera from the remaining patients were assayed only before treatment. The 27 patients were on regular anti-fungal therapy and were showing signs of clinical and mycological improvement, according to their clinical records. Samples were collected between 1991 and 1996 and maintained at -20°C until the time of the study. The study also selected, from this same period, serum samples from five other patients who had relapses of the disease. Samples from these five patients were obtained at the start of therapy, when clinical improvement was observed, and when relapse was diagnosed clinically and mycologically. The patients were grouped according to the clinical forms of the disease, based on the classification proposed at the 3rd International Meeting on Paracoccidioidomycosis [15], as follows: 30 patients with the chronic (adult) form, comprising 23 with multifocal and 7 with unifocal lesions, and 13 patients with the acute (juvenile) form of PCM.

Serum samples from patients with other deep mycoses were used as controls in the development of the ELISA. This group comprised 20 cases of histoplasmosis, 15 cases of lobomycosis, 10 cases of cryptococcosis and six cases of aspergillosis. Serum samples ($n = 65$) from normal blood donors were used to establish the cut-off value for the ELISA.

Antigens

Two types of antigen were used in the study. The first was a 10-day crude filtrate obtained from the yeast phase of *P. brasiliensis* strain 113 (Culture Collection, Instituto de Medicina Tropical de São Paulo). The fungus was cultured in neopeptone 1.6%, glucose 1.0%, thiamine 0.01% and asparagine 0.02% liquid medium for 10 days at 36°C with shaking. The culture supernates were filtered through Whatman paper, concentrated 10-fold and dialysed against distilled water [14]. This culture filtrate antigen was employed in the DID, CIE and CF tests. For the ELISA, a cellular yeast extract (somatic antigen) was employed. It was prepared following a technique described previously [16]. Briefly, *P. brasiliensis* yeast cells (strains 113 and 339) were cultured in Fava Neto's agar medium at 36°C for 7 days. The cells were resuspended in 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 10 mM phenylmethylsulphonylfluoride as a protease inhibitor. The cells were ruptured by maceration in the presence of glass beads and liquid nitrogen. After centrifugation at 10 000 rpm for 15 min, the supernate was filtered through sterilising membranes, divided into small volumes and stored at -20°C until use. Protein content was 1.97 mg/ml as determined by Lowry's method [17].

Routine serological tests

The DID test was performed in agar 1% gel in buffered saline (pH 6.9), containing sodium citrate 0.4% and glycine 7.5%. The antigen (12 μl) was placed in the central well and reference serum and patients' sera (12 μl) in the surrounding wells. The slides were incubated for 48 h, washed in saline, dried, and stained with Coomassie Brilliant Blue R (Sigma). The serum samples were tested undiluted [14]. CIE was performed in agarose 1% gel with electrophoresis in veronal-buffered saline, pH 8.2, at 120 V for 90 min. Serum samples were applied to the anodic side and the antigen to the cathodic side of the slide. The sera were diluted two-fold and tested from the undiluted sample. Samples that reacted at least undiluted were considered positive [14]. The CF test was performed according to the Centers for Disease Control (CDC, Atlanta, USA) [18]. The results were considered positive when the titre was ≥ 1 in 8. These three tests have been used routinely in our laboratories, DID being the test of choice for diagnosis and CIE and CF mostly used for follow-up of PCM patients during treatment [14, 19].

ELISA

This assay was performed as described previously, with few modifications [9, 10]. Briefly, polystyrene microplates (Corning, USA) were coated with 50 μl of the cellular yeast extract, diluted in 0.06 M carbonate buffer (pH 9.6) and incubated at 37°C in a humid chamber for 2 h at 4°C overnight. After five washes with PBS-Tween

20 0.05% (PBS-T), 50 μ l of the patients' sera diluted 1 in 200 in a solution of skim milk 5%-gelatin 0.1% in PBS-T were added. After incubation for 2 h at 37°C, plates were washed five times and 50 μ l of the goat anti-human IgG diluted 1 in 4000 (whole molecule; Sigma) conjugated with peroxidase were added to the wells. The plates were incubated at 37°C for a further 2 h, washed again and the reaction was developed by the addition of o-phenylenediamine-H₂O₂ as substrate. The absorbance was determined at 490 nm in a microplate reader (BioRad, USA) after stopping the reaction with 2 N H₂SO₄. All tests were performed in duplicate. The cut-off value was calculated from the mean +2 SD obtained with samples from normal blood donors (n = 65) tested at 1 in 200 dilution and was 0.1 OD units.

Statistical analysis

Serological titres were analysed as groups by the Kruskal-Wallis non-parametric test, after variance check. When appropriate, data were expressed as median of the groups. The comparison between groups was made by Dunn's test. All data were considered significant when $p < 0.05$.

Results

The DID was positive in 95.3% of the 43 patients included in this study. The two patients with negative results had localised disease and their diagnosis was made by biopsy. The ELISA with the somatic antigen was based on a previous report showing good results with an assay with a similar antigenic preparation [11]. In the present study, the ELISA showed high (100%) sensitivity but, as described for other immunoenzymic assays, cross-reactions were observed with sera from patients with histoplasmosis and lobomycosis (Fig. 1) [9–11].

Results of the ELISA, CIE and CF tests on sera collected before therapy and grouped according to the clinical forms are summarised in Fig. 2.

Low OD values for patients with the unifocal form were obtained in the ELISA, with three patients exhibiting values near the cut-off value. These results differed significantly from those of patients with the acute and chronic multifocal forms ($p < 0.001$). The OD values were distributed similarly in these latter two groups and no statistical difference was found between them.

Sera from all 43 patients showed positive results in the CIE test. The unifocal group showed significantly lower antibody titres, ranging from 2 to 16. Of note, with this test there was no overlap in the antibody titres between the unifocal and the AF groups. In the latter group, the titres were always high, being ≥ 64 and occasionally reaching 4096. Patients with multifocal disease exhibited a broader range of antibody titres, with low,

Fig. 1. OD₄₉₀ values determined by ELISA in serum samples (diluted 1 in 200) from different clinical groups: (■, L) lobomycosis, n = 15; (▲, A) aspergillosis, n = 6; (▼, H) histoplasmosis, n = 20; (◆, C) cryptococcosis, n = 12; (●, P) paracoccidioidomycosis, n = 43. Dotted line represents the cut-off value, established as the mean + 2 SD obtained with sera from 65 healthy blood donors.

intermediate and high values (8–256). Significant differences were also observed in the median antibody titres between multifocal and AF groups ($p < 0.001$).

In the CF test, sera from c. 50% of the patients with unifocal disease and AF exhibited negative results or antibody titres at the cut-off level. Indeed, low titres of CF antibodies in sera from AF patients with high titres in other reactions have been described previously [14, 19]. As a result, AF patients presented either low or high antibody titres, with no intermediate values. The multifocal group, on the other hand, had a less heterogeneous distribution. As a consequence, significant differences were found only between unifocal and multifocal forms ($p < 0.05$).

For the follow-up studies, sera were collected after therapy for 6, 12 and 24 months from 16 patients with the multifocal form, four with the unifocal form and seven with the acute form of the disease. These patients were on regular treatment during this follow-up period and had shown evidence of clinical improvement according to the patients' records. There were no relapses during this period. All three diagnostic techniques showed a similar trend, with decrease in antibody titres associated with therapy (Fig. 3).

With the ELISA, the reactivity of the AF group decreased sharply between the time of diagnosis and 6 months of therapy. After that, no evident decrease was observed until 24 months of therapy. The multifocal group presented a gradual decrease in reactivity until 24 months, when the median values were around 0.3, as for the AF group. The unifocal group exhibited low values at the beginning of the investigation, values near

Fig. 2. Pre-treatment antibody levels determined by (a) CF, (b) CIE and (c) ELISA tests in sera of patients with different clinical forms of paracoccidioidomycosis. (∇ , M), multifocal form; ($\circ$, U), unifocal form; and ($\square$, A), acute form. Dotted areas represent the cut-off values for CF and ELISA.

the cut-off after 1 year, and negative values after 2 years.

Sera from patients with the unifocal form of the disease had a median antibody titre of 8 when tested by CIE before treatment and 12 months after treatment, decreasing by only one dilution to 4 at 24 months. In contrast, patients with the AF showed an evident decrease, ranging from a median of 1024 before treatment began to 16 after 24 months. A decrease was also observed for the antibody titres in the multifocal group, dropping from 32 to 4 after the same length of therapy. Although patients in the AF

Fig. 3. Median values of the antibody levels before and during treatment determined by (a) CF, (b) CIE and (c) ELISA tests in sera of patients with different clinical forms of paracoccidioidomycosis. (∇), multifocal form; ($\circ$), unifocal form; ($\square$), acute form. Dotted areas represent the cut-off values for CF and ELISA.

group showed a more marked decrease, after 2 years only patients with the chronic unifocal and multifocal forms had median antibody titres that could be considered as healing titres [20].

Sera from patients with all three clinical forms showed similar decrease with time in the antibody titres when the CF test was used, with unifocal patients showing the lowest titres. This last group exhibited negative results or titres near the cut-off value after treatment for 1 year. On the other hand, in the multifocal and AF groups titres were still positive after therapy for 12

months, but after 24 months, only the former presented titres that could be considered as healing titres [20].

The serological evaluation of the five patients who relapsed during their follow-up is shown in Fig. 4. In both ELISA and CIE there was an evident increase in the antibody titres for all five patients, whereas in the CF test an increase in antibody titres was seen in only three patients.

Discussion

The first aim of this study was to compare the anti-*P. brasiliensis* antibody levels revealed by the three different tests in relation to the three clinical forms of the disease, i.e., AF, chronic multifocal and chronic unifocal. Of the three tests, only CIE distinguished the three groups, whereas the ELISA was able to distinguish only the chronic unifocal from the multi-

focal and AF groups. On the other hand, the CF test demonstrated significant differences in antibody titres only between the chronic multifocal and the chronic unifocal groups. Confirming previous results obtained by CF, half of the AF patients had low titres or no reactivity, similar to patients with the unifocal form of the disease [14, 19].

Correlation between the severity of the disease and high serological titres is commonly stated in the literature, but evidence is only anecdotal. Biagioni et al. used an indirect immunofluorescence assay and showed a tendency for an increase in IgG antibody titres from chronic localised to chronic disseminated and to AF patients [21]. Other authors found significantly higher IgG antibody levels in AF compared to ChF patients with ELISA [22]. The same authors also showed that antigen-specific IgM antibodies were associated with the AF, as suggested earlier by Barbosa et al. [23]. Although the present

Fig. 4. Serological follow-up of five (a-e) paracoccidioidomycosis patients who relapsed during treatment. The arrows indicate when the clinical relapse was diagnosed. (.....), CF; (—), CIE; (— —), ELISA.

study did not find a difference between the chronic multifocal and AF patients in the ELISA, a statistical difference was seen when the two groups of ChF patients were grouped together and then compared with the AF patients (data not shown). Robles et al. analysed 142 patients, all except two with ChF, and showed that severity of disease again correlated with higher antibody titres when three different methods were used – DID, CIE and CF [24]. Other authors did not find a relationship between antibody titres obtained by DID and involvement of specific organs in ChF patients [25]. However, in a recent study with DID, patients with severe disease presented a median titre of specific antibodies slightly lower than that of the control group, with less severe disease [26].

The association between severe disease and higher antibody response may be important in the assessment of patients' prognosis and treatment follow-up. As treatment of PCM lacks standardisation, it must be tailored to each patient. Even though patients are classified as having acute or chronic disease, a wide range of manifestations may appear in each form [15]. For example, patients with ChF disease may present with very localised to widely disseminated disease. In our experience, patients with localised disease are seen more rarely than those with multifocal disease, and usually present with oropharyngeal lesions and without significant involvement of the regional lymph nodes. The results of the present study reinforce the idea of a subgroup of patients whose disease results from reactivation of the fungus, in which the immune response checks, at least partially, the fungal dissemination and restricts it to only one site. These patients showed antibody titres consistently lower than patients with disseminated disease. The exclusion of the unifocal patients from the ChF group consequently left those patients with a pattern of disease that was closer to the AF cases. This was demonstrated in part by the lack of difference in the ELISA results between the chronic multifocal and the AF patients, although the same was not observed with CIE. Assuming that antibody titres correlate with antigenic load, the ELISA would indicate that both patient groups may have similar fungal burdens, whereas the CIE would indicate a higher fungal burden in the AF patients. In fact, many patients with the chronic multifocal form in this study had severely disseminated disease, and analysis by ELISA would provide a more reliable correlate of the fungal burden than analysis by CIE.

These differences between CIE and ELISA were also observed when the serological response during treatment was analysed. All three tests showed that specific antibody titres decreased progressively during the 24 months of follow-up. However, the reactivity of patients' sera fell differently depending on the clinical form of the disease and the test employed. The ELISA again showed that patients with chronic multifocal and AF disease had a similar response, distinct from those

with unifocal disease. The latter group already had negative results after therapy for 1 year, while the other patients presented significant antibody titres even after 2 years, although many of them could be considered clinically and mycologically cured. With the CIE assay, antibody titre curves were quite similar for patients with chronic unifocal and multifocal disease, with low titres after 1 year, and titres that may be considered as healing titres at 2 years. On the other hand, AF patients showed a more discrete reduction with low titres only after 2 years. Thus, the ELISA results would indicate that those patients with disseminated disease (chronic or acute) would need prolonged therapy (≥ 2 years), whereas patients with localised disease would need shorter therapy, for c. 1 year. The CIE results, in turn, would indicate treatment for ≥ 2 years for ChF patients and even longer for AF patients. In fact, immunologically, the ChF may represent a more subtle immune defect than the AF, since it results from reactivation of quiescent foci of the fungus [27, 28]. These patients had been able, at least once in the past, to mount an efficient immune response, when first exposed to the fungus.

Follow-up of the serological response with the CF test showed that, as with the ELISA, patients with unifocal disease had results near the cut-off level after treatment for 1 year. The other patients still had positive tests after treatment for 2 years, as seen with CIE and ELISA. In addition to peculiarities of the serological response of each clinical form, the differences in the three tests may be due in part to differences in the antigens used for the ELISA (somatic) and for the CIE and CF (culture filtrate). The somatic antigen displays several fractions, ranging from 15.6 to >94 kDa, which includes the 43-kDa glycoprotein, but with no major fraction (data not shown). The crude filtrate antigen displayed fewer bands, gp43 being the major component [13].

The role of the serological response in the follow up of PCM patients is still a matter of debate, although the first studies with this aim published >30 years ago had already shown that clinical improvement was associated with a fall in specific antibody titres [5, 6]. A large number of reports has been published since then; however, they have not added much to this initial statement. Restrepo et al. showed only partial improvement in the serological response after treatment for 6 months, with negative results for both the ID and the CF tests in only three of 16 patients studied. Although clinically and mycologically cured, most patients persisted with low antibody titres, a condition defined previously as healing antibody titres [29]. In fact, it was previously shown that PCM patients needed prolonged treatment to achieve complete cure and avoid relapses [30]. Lopes et al. showed that 72% of the patients cleared their anti-*P. brasiliensis* antibodies (by CF) after a mean length of treatment of c. 2 years, but some patients needed several years more of

Table 1. Serological response during and after therapy of PCM patients in studies conducted by Brazilian investigators

Ref. number	Serological tests	Number of patients acute/chronic	Therapy follow-up (months)	Serological response	Follow-up after therapy	Serological response
32	CF, TP	36 Chronic? 1 Acute?	3	Decrease at 3 months with TP and CF	37 patients for 7 months; nine relapses	Further decrease in TP and CF; serology vs relapse: NI
33	CF, TP, DID, CIE	10 Chronic 6 Acute	2–19	Significant drop in CF, TP and CIE; DID negativity in 57% at end of therapy	12 months, no relapses	...
34	TP, CF	7 Chronic 3 Acute	10–19	Significant drop in CF and negativity in TP at end of therapy	12 months, no relapses	...
35	TP, DID, IIF	11 Chronic 1 Acute	18	Significant decrease in titres, but still positive at low titres	1–7 months, three relapses	No correlation with relapses; but cured patients with negative or stable low titres
36	CF, TP, IIF	43 Chronic? 4 Acute?	...	CF was the best test to determine clinical cure, clinical improvement or worsening	...	...
37	CF, TP, DID, IIF	8 Chronic 1 Acute	3–24	Three cases still persisted positive at 24 months	...	...
38	CF, DID, IIF	37 Chronic 17 Acute	18	At 6, 12 and 18 months, persisted unchanged in, respectively, 61%, 52%, and 45% of the cases	...	...
39	DID	8 Chronic 2 Acute	3.5–7	...	48–81 months, two relapses	In 6 of 7 cured cases DID became negative; DID not done in relapses
40	DID	21 Chronic	6–18	Stable low titres at end of therapy	...	...
41	mELISA, CF, DID, CIE	14 Chronic? 2 Acute?	...	...	24 months	mELISA and DID became negative with clinical cure; FC and CIE tended to persist positive
42	CF, TP, DID, IIF	37 Chronic 8 Acute	...	Drop in DID was sharper than in PT and CF; IIF: NI	A) 39 patients for 5 years, four relapses; B) 23 patients for 6–13 years, three relapses	A) Negativity mainly in patients with maintenance therapy; low titres in 22; B) Negativity in 11, stable low titres in 9

CF, complement fixation; TP, tube precipitins; DID, double immunodiffusion; CIE, counter-immuno-electrophoresis; IIF, indirect immunofluorescence; mELISA, magnetic ELISA; NI, no information.

continuous treatment [31]. More recent studies have shown that 7 years may elapse after the cessation of therapy before 75% of the patients have negative serology [25], and that IgG antibodies against the immunodominant *P. brasiliensis* antigen may still be detected in healed patients by Western blot [12]. With the advent of the imidazoles in the therapy of PCM, many studies were conducted that used serology to evaluate the efficacy of the different therapy regimens available. However, direct comparisons are difficult because protocols varied widely. Table 1 summarises the studies conducted by Brazilian investigators, whereas Table 2 displays those done by investigators from other South American countries. It can be seen from the tables [32–51], as well as from some other studies that specifically focused on the performance of serological techniques [11, 12, 22, 25, 52–56], that antibody titres dropped with therapy but less frequently became negative when therapy was stopped or patients were considered clinically and mycologically cured, or both. In many reports, the fall in the antibody titres persisted even after withdrawal of the anti-fungal therapy. One report suggested that the titres after therapy correlated with the severity of the clinical manifestations before therapy [24]. This was also suggested in the present study: patients with more severe manifestations persisted with higher titres.

Of note is the observation that, by comparing the two tables, the patients enrolled in Brazilian studies tended to be more seriously ill, since in most series there were patients with the acute, more severe form of the disease. The AF seems to be very rare in the most southern part of and outside Brazil [57]. Also, treatment was generally more prolonged and, in some series, included maintenance therapy with sulphamides. Even so, relapses were more frequently recorded with the same drug treatments. The reasons for this higher severity of disease, whether related to regional variability in the virulence of the fungus or to the characteristics of the population at risk, are not known.

With few exceptions, serological documentation of the relapses was sparse and heterogeneous. Relapses or treatment failures could be either associated [22–25, 35] or not [22] with increase in serological titres. In fact, relapses have already been documented for a long time in a few cases, despite CF negative results with therapy [20]. Therefore, the role of serology in the management of these cases remains unclear, and a particular titre or range of titres that would indicate when to stop therapy with no risk of relapse has not yet been defined in clinical practice [56]. The results of the present study, although based on a limited number of cases, suggest that when ELISA and another test such as CIE are used in combination, relapses or treatment failures can indeed be detected.

In conclusion, the assessment of the antibody response in PCM patients by CIE and ELISA showed a better

Table 2. Serological response during and after therapy of PCM patients in studies conducted by South American investigators (excluding Brazil)

Ref. number	Serological tests	Number of patients acute/chronic	Therapy follow-up (months)	Serological response	Follow-up after therapy	Serological response
43	DID, CF	13 Chronic	3–12	Significant drop mainly after 4–6 months; some patients persisted with stable low titres	Four patients for 4–10 months, no relapses	...
44	DID, CF	5 Chronic	12	Decrease, but patients persisted with stable low titres	...	...
45	CF	8 Chronic	6	Still low titres after 6 months	...	...
46	DID, CF	38 Chronic?	6–12	Significant drop only after 3 months, but 75% of the cases remained with positive titres after 6 months	...	...
47	DID, CF	24 Chronic?	...	...	12–24 months, two relapses	CF low titres in 19 and DID weakly positive in 14 from 24 patients at 12 months; CF with stable low titres in and DID negative in 6 from 10 patients at 24 months
48	DID, CF	45 Chronic 2 Acute	3–24	Significant drop but still stable low titres at end of therapy	15 patients for 12 months, no relapses	DID negativity in 53% of CF; low titres in 60% of the patients
49	DID, CF	33 Chronic	3–18	Negativity in 45.5% cases only at end of therapy	...	...
50	DID, CF, CIE	17 Chronic	≥6	Negative results or decrease in ≥2 dilutions in 68%	≤2 years, two relapses	...
51	DID, CF	19 Chronic? 1 Acute	3–7	Negativity in 18 of 20 cases after 6 months	3–12 months	Persisted negative?

DID, double immunodiffusion; CF, complement fixation; CIE, counter-immuno-electrophoresis.

clinical correlation than CF. The former two tests correlated with the fungal burden at the time of diagnosis, distinguishing the different clinical forms. With therapy, the specific antibody titres fall, but less commonly disappear at the end of treatment or with clinical and mycological cure, irrespective of the serological test used. This was evident for the disseminated forms of the mycosis, either chronic or acute, which probably need more prolonged follow-up. Patients with unifocal disease could again be distinguished from those with disseminated disease by their serological response, as they needed a shorter period of therapy to clear their antibodies (≤ 1 year). The results also suggest that relapses are associated with rising titres in the CIE or ELISA tests, or both. Finally, the definition of cure in PCM remains a matter of debate, and more prospective studies are needed. Other laboratory parameters, such as antigenaemia in urine or serum [16, 58, 59], may help to define the time points to change or stop therapy more accurately.

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