Humoral Immune Response in Chromoblastomycosis during and after Therapy

P. ESTERRE,1,2* M. JAHEVITRA,1 AND A. ANDRIANTSIMAHAVANDY1
Parasitology Unit, Institut Pasteur de Madagascar, Antananarivo 101, Madagascar,1 and International Network of Pasteur Institutes, Institut Pasteur, F-75724 Paris cédex 15, France2

Received 1 November 1999/Returned for modification 24 January 2000/Accepted 13 March 2000

A longitudinal study was carried out in Madagascar, the most important focus of chromoblastomycosis (P. Esterre, A. Andriantsimahavandy, E. Ramarcel, and J. L. Pecarrere, Am. J. Trop. Med. Hyg. 55:45–47, 1996), to investigate natural immunity to this disease. Sequential blood samples were obtained before, during, and at the end of a successful therapeutic trial with terbinafine, a new antifungal drug. Using enzyme-linked immunosorbent assay and immunoblot methods, detailed analyses of antibody concentration and antigen mapping were conducted for 136 serum samples and tentatively correlated to epidemiological and pathobiological data. Two different cytoplasmic antigens, corresponding to the two fungal species involved (Fonsecaea pedrosoi and Cladophialophora carrioni), were used to analyze the distribution of different classes of immunoglobulins. This was done with respect to the origin of the isolates, clinical and pathobiological. Although strong individual variations were noticed, some major antigens (one of 18.5 kDa specific for F. pedrosoi and two of 23.5 and 33 kDa, respectively, specific for C. carrioni) corresponded to high antibody prevalence and concentration. As some antigenic components were also detected by immunoglobulin M (IgM) and IgA antibodies, the role that these specific antibodies could play in the immune response is discussed.

The pigmented fungi belonging to the Dematiaceae family are considered an emerging group of pathogenic fungi, at least in westernized countries (22). This increasing medical problem is also linked with the AIDS epidemic (8), and a case of disseminated chromoblastomycosis evolving in an AIDS patient has been recently published (9). Chromoblastomycosis is a cosmopolitan chronic mycosis infecting humans after inoculation by trauma. In the absence of prompt medical intervention, typical cauliflower-like verrucous lesions develop, sometimes over a period of more than 30 years, and show a highly organized granulomatous reaction associated with an extensive fibrosis in the dermis and subcutaneous tissues (11, 12). The disease has a high morbidity, with Madagascar described as the most important focus in the world (12). Available drugs are not very effective, except for the new terbinafine drug which was recently tested in a multicentric therapeutic trial (13). Thirty-five patients were infected with F. pedrosoi and followed during 1 year of specific therapy in the hospital of Andapa located in the rainy, northern part of Madagascar. Five patients were infected with C. carrioni and enrolled in a study of the same design organized in the hospital of Mananambary, located in the semidesertic southern region of Madagascar. For each patient, the two immunosassays were performed on the serum before (t0), during (at 4 [t1] and 8 [t2] months), and at the end (t3) of chemotherapy with terbinafine. The potential correlations of immunological results (levels of specific IgGs and IgG, IgM, and IgA immunoblot profiles) with the different clinopathological data collected during the therapeutic trial (15) were studied with this first group of 136 serum samples.

In addition, serum samples from 24 healthy controls (20 males and 4 females; mean age ± standard deviation [SD], 48.8 ± 15.3 years) presenting chronic lesions (mean evolution time of lesion, 10 years and 3 months) from two areas of chromoblastomycosis endemicity (12) were studied during a multicenter therapeutic trial (13). Thirty-five patients were infected with F. pedrosoi and followed during 1 year of specific therapy in the hospital of Andapa located in the rainy, northern part of Madagascar. Five patients were infected with C. carrioni and enrolled in a study of the same design organized in the hospital of Mananambary, located in the semidesertic southern region of Madagascar. For each patient, the two immunosassays were performed on the serum before (t0), during (at 4 [t1] and 8 [t2] months), and at the end (t3) of chemotherapy with terbinafine. The potential correlations of immunological results (levels of specific IgGs and IgG, IgM, and IgA immunoblot profiles) with the different clinopathological data collected during the therapeutic trial (15) were studied with this first group of 136 serum samples.

Materials and Methods

Study area and patients. A total of 40 patients (34 males and 8 females; mean age ± standard deviation [SD], 48.8 ± 15.3 years) presenting chronic lesions (mean evolution time of lesion, 10 years and 3 months) from two areas of chromoblastomycosis endemicity (12) were studied during a multicenter therapeutic trial (13). Thirty-five patients were infected with F. pedrosoi and followed during 1 year of specific therapy in the hospital of Andapa located in the rainy, northern part of Madagascar. Five patients were infected with C. carrioni and enrolled in a study of the same design organized in the hospital of Mananambary, located in the semidesertic southern region of Madagascar. For each patient, the two immunosassays were performed on the serum before (t0), during (at 4 [t1] and 8 [t2] months), and at the end (t3) of chemotherapy with terbinafine. The potential correlations of immunological results (levels of specific IgGs and IgG, IgM, and IgA immunoblot profiles) with the different clinopathological data collected during the therapeutic trial (15) were studied with this first group of 136 serum samples.

In addition, serum samples from 24 healthy controls (20 males and 4 females; mean age ± SD, 41.7 ± 13.8 years; P = 0.4 for controls versus patients) and 13 patients infected with diseases endemic to the area (one for each of the following diseases: candidiasis due to Candida albicans, Aspergillus fumigatus-associated aspergillosis, Trichophyton rubrum infection, fungal mycetoma, malaria, schistosomiasis mansoni and haematobium, Wuchereria bancrofti-associated lymphatic filariasis, distomatosis, ascariasis, cysticercoisis, Trichina infection, hydatidosis, and taeniasis) were included in the analysis. All serum samples had been kept frozen (−80°C) and were examined under uniform laboratory conditions to avoid internal variations.

Fungal cultures and antigens. Two reference strains, one of F. pedrosoi (IPM-AS) and one of C. carrioni (IPM-M8), were obtained from skin biopsies of two patients enrolled in the therapeutic trial. They were cultivated in 500 ml of Sabouraud’s liquid medium, mechanically agitated (300 rpm for 10 to 15 days) in a roller-type cell culture system (Bellco New Technology, Ltd., Vineland, N.J.). Typical growth curves of the two fungi were obtained, and the antigens were prepared from the log phases (1, 19). We obtained two somatic antigens after 0.5% formaldehyde extraction, disintegration with a Polytron homogenizer (Kinematica, Ltd., Kriens, Switzerland), and sonication (20 kHz) with a VibraCell apparatus (Sonics & Materials Inc., Danbury, Conn.). The antigenic extracts were finally lyophilized (in 3-ml vials) and the protein contents were determined...
by the Bradford technique (Bio-Rad, Richmond, Va.) before and after the final step (4).

ELISA technique. The ELISA technique was performed as previously described (1, 26), with only slight modifications in order to obtain optimal conditions with the fungal antigens: plates were coated with antigens (concentration, 1.0 μg/ml) and incubated for 1 h; serum dilutions were 1/200; the conjugate was peroxidase-labeled anti-human Ig (Sanofi-Diagnostic-Pasteur, Marnes-la-Coquette, France) at a 1/8,000 dilution; and measurements of optical density at 492 nm were done with a UV spectrophotometer (Multiskan Plus; Labsystems, Helsinki, Finland) driven by a computer (Prolinea 486; Compaq Ltd., Houston, Tex.). Each assay was referenced by including a positive reference sample obtained from five pooled positive serum samples, and the results were expressed in arbitrarily defined immunoenzymatic units (IEU), as previously described (14, 26). Sera were classified as positive when the assay result was greater than 25 IEU, as previously described (14).

RESULTS

We obtained two antigenic extracts, one of F. pedrosoi (IPM-A8 antigen; yield, 21 to 66 μg/ml) and the other one of C. carrionii (IPM-M8 antigen; yield, 19 to 35 μg/ml). The specificity was first tested on 19 historical serum samples studied by ELISA, with good reproducibility (86%) within this limited sample group (1). The results presented here are the results obtained with a larger collection of 136 serum samples, collected every 4 months from 42 patients monitored for 1 year after receiving specific antifungal chemotherapy (13, 15), and were compared with results obtained with serum samples from 24 healthy Malagasy people.

The ELISA technique was first developed for the purpose of diagnosis. Using mycological or histopathological examinations as reference tests for the diagnosis, we calculated a sensitivity of 86.9%, a specificity of 92.5%, and a global efficiency of 89.1% (14), results better than the ones obtained recently with double diffusion (25) and counterimmunoelectrophoresis (29). It is interesting to note that the best results were obtained with the F. pedrosoi antigen in all the combination experiments (Fig. 1).

Concentrations of specific Igs in serum were measured before treatment (concentration at \( t_0 \pm SD, 72.9 \pm 46.8 \) IEU), after 4 months (concentration at \( t_4 \pm SD, 66.1 \pm 44.4 \) IEU) and 8 months (concentration at \( t_8 \pm SD, 59.4 \pm 43.5 \) IEU), and at the end (concentration at \( t_{12} \pm SD, 59.3 \pm 40.7 \) IEU) of the trial (Fig. 2). The Ig levels gradually declined during the first 8 months of therapy and then remained stable until un-
The demonstration of circulating antibodies in chromoblastomycosis was first made using double immunodiffusion (5, 25, 28, 29) or indirect immunofluorescence (6, 17) techniques, with inconsistent results. More recently, the antigen profile of *F. pedrosoi* was characterized by immunoprecipitation and immunoblotting (26) together with the partial characterization of fungal antigens (1, 19). Our laboratory developed an ELISA, the efficiency of which for diagnostic purposes was carefully evaluated on reference patients (1, 14). The availability of ELISA and Western immunoblotting (26) together with the partial characterization of fungal antigens (1, 19) led us to carry out, for the first time with regard to this disease, a longitudinal study on 40 patients, monitored during 1 year of antifungal therapy (13, 15).

As for most of fungal pathogens, the role of natural immunity in protection is uncertain, and this is especially true for the antibody response (7). In addition, a part of the population in areas of endemity that has been exposed to the fungus will develop a specific humoral response but not the disease (2, 14). The time necessary for serology to become negative is variable in our experience, and some patients may have persistent positive results more than 1 year after the end of antifungal treatment. As there is no clear information on the evolution of the humoral immunity status of chromoblastomycosis patients receiving therapy, we monitored 40 reference patients during a 1-year therapeutic trial with terbinafine. In our sample and with the quantitative ELISA technique, it was always possible to make a diagnosis of infection after 12 months of successful therapy. Interestingly, the patients with the most severe form of the disease, that is, chronic multiple lesions, presented higher titers of specific Igs.

Western blotting with the two fungal lysates seems to be a powerful tool, since it allows a precise measurement of antibody pattern. Sera from people exposed to chromoblastomycosis-related fungal antigens responded to several bands in immunoblots, with wide intersubject variations in the pattern and in the number of bands observed. As has been done for other parasitic diseases (for a global overview, see references 20 and 21), we have documented heterogeneous humoral responses against species-specific antigens. Some antigenic determinants (so-called immunodominants) were strongly reactive in terms of high antibody frequencies: 18.5, 36, and 40 kDa for *F. pedrosoi* and 23.5, 26, 33, and 40 kDa for *C. carrionii*. Some minor antigens were recognized by both IgG and IgM or IgA. Surprisingly, three antigenic determinants, of 47-, 57-, and 84-kDa molecular masses, were only recognized by specific IgA. Both with ELISA and immunoblot techniques, *C. carrionii*

![Image](https://via.placeholder.com/150.png?text=Image)
nii-infected patients seemed to be worse responders than *F. pedrosoi*-infected patients.

The immunological parameters here presented (specific IgGs, most of them dealing with the IgG1 isotype; IgA and IgM levels; and their evolution during chemotherapy) seem to correlate with the clinical heterogeneity described for paracoccidioidomycosis, a two-pole disease with a Th2-like regulation of the humoral response (2, 3). The detailed Ig isotype mappings revealed a high degree of complexity for the stimulating antigen set, including high levels of IgM antibodies which may have been a consequence of constant antigenic stimulation due to continuously low background fungal degradation (11). Another explanation for the IgM binding is that many fungal proteins are actually glycoproteins, the glycosidic moiety evoking an IgM response. In the absence of any mucosal involvement in this pathology, IgA might represent only a potential marker of the immune status of patients with chromoblastomycosis. Even if the immune mechanisms involved in the control of the fungus seem to be of cellular origin, at least autologous antibodies could be linked with the chronicity and the extent of the lesions in this pathology (16). In areas of endemicity, a part of the population that has been exposed to the fungus will not develop the disease but will have a significant level of circulating specific antibodies (14). At the moment, many factors relevant to susceptibility or resistance mechanisms in humans are still unknown. A next step would be to correlate our information on humoral immunity and immunopathology in chromoblastomycosis with in vivo studies of the CD40-CD40 ligand interactions (18, 27).

In summary, the ELISA and immunoblot techniques allowed the analysis of 136 serum samples taken from well-studied patients with chromoblastomycosis lesions. Large numbers of antigenic determinants with various molecular masses were observed in this humoral approach. The observed polymorphism corresponds to the well-known wide repertoire of *F. pedrosoi* and *C. carrionii* phenotypes that is due to dimorphic transformation from the saprophytic to the parasitic phase and associated fungal growth (19). Although natural immunity to fungal infections in general, and to chromoblastomycosis in particular, is not well understood, it is known to have no protective effect, as the disease has a very chronic and debilitating pattern of evolution. Our data confirm that high levels of specific antibodies are identified in the serum of patients and that IgG levels correlate positively, as do antineutrophilic antibody levels (16), with the chronicity and the extent of the lesions.

ACKNOWLEDGMENTS

We thank the Andapa (Mahatahata Ratsioharana) and Manambaro (Emmanuelson Randrianiaina) hospital teams for technical assistance during serum collection and clinical examinations.

REFERENCES

1. Andriantsimahavandy, A., P. Michel, H. Rasolofonirina, and J. Roux. 1993. Apport de l'immunologie au diagnostic de la chromoblastomycose à Mada- gascar. J. Mycol. Med. 3:30–36.

2. Baida, H., P. J. Biselli, M. Juvenale, G. M. B. Del Negro, M. J. S. Mendes-Giannini, J. S. Durate, and G. Bernard. 1999. Differential antibody isotype expression to the major Paracoccidioides brasilensis antigen in juvenile and adult form paracoccidioidomycosis. Microbes Infect. 1:273–278.

3. Benard, G. M. H. Horta, G. M. B. Del Negro, L. Batista, M. A. Shinakai-Yasuda, and A. J. S. Duarte. 1996. Antigen-specific immunosuppression in paracoccidioidomycosis. Am. J. Trop. Med. Hyg. 54:7–12.

4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye bind- ing. Anal. Biochem. 72:248–254.

5. Buckley, H., and I. Murray. 1966. Precipitating antibodies in chromoblastomycosis. Sabouraudia 5:78–80.

6. Coundou, P., A. Mayoux, E. R. Brygoo, and M. Rakoto. 1970. Utilisation de l’immunofluorescence indirecte dans la chromoblastomycose humaine et expérimentale à *Phialophora pedrosii* et *Cladosporium carrionii*. Etude préliminaire. Arch. Inst. Pasteur Madagascar 39:65–76.

7. Dixon, D. M., R. Cox, J. Cutler, and G. Deepe. 1996. Researchers use molecular immunology and technology to combat fungal pathogens. ASM News 62:81–84.

8. Dromer, F., and B. Dupont. 1996. The increasing problem of fungal infec- tions in the immunocompromised host. J. Mycol. Med. 6:1–6.

9. Duggan, J. M., M. D. Wolf, and C. A. Kaufmann. 1993. *Phialophora verru- cosus* infection in an AIDS patient. Mycoses 36:215–218.

10. Espinel-Ingroff, A., S. Shadomy, D. Dixon, and P. Goldson. 1986. Exoantigen test for *Cladosporium bartianum*, *Fonsecaea pedrosii*, and *Phialophora verru- cosus*. J. Clin. Microbiol. 23:305–310.

11. Esterre, P., S. Pyrol, D. Sainte-Marie, R. Prudain, and R. Grimaud. 1993. Granulomatous reaction and tissue remodelling in the cutaneous lesion of chromoblastomycosis. Virochows Arch. 422:285–291.

12. Esterre, P., A. Andriantsimahavandy, E. Ramarcel, and J. L. Pecareurre. 1996. Forty years of chromoblastomycosis in Madagascar: a review. Am. J. Trop. Med. Hyg. 55:45–47.

13. Esterre, P., C. K. Inzan, E. Ramarcel, A. Andriantsimahavandy, M. Ratsioharana, J. L. Pecareurre, and P. Roig. 1996. Treatment of chromoblastomycosis with terbinafine: preliminary results of an open pilot study. Br. J. Der- matol. 134:33–36.

14. Esterre, P., M. Jahevitra, E. Ramarcel, and A. Andriantsimahavandy. 1997. Evaluation of the ELISA technique for the diagnosis and the seroepidemiology of chromoblastomycosis. J. Mycol. Med. 7:137–141.

15. Esterre, P., C. K. Inzan, M. Ratsioharana, A. Andriantsimahavandy, C. Raharisiolo, E. Randrianiaina, and P. Roig. A multicenter trial of terbinafine in patients with chromoblastomycosis: effects on clinical and biological cri-
teria. J. Dermatol. Treat. 9:529–534.

16. Galperin, C., Y. Shoenfield, B. Bilburd, P. Esterre, L. Meroni, N. Del Papa, G. M. Halpern, A. Andriantsimahavandy, and M. E. Gershwin. 1996. Anti- neutrophilic cytoplasmic antibodies in patients with chromoblastomycosis. Clin. Exp. Rheumatol. 14:479–483.

17. Gordon, M. A., and A. Doory. 1965. Application of fluorescent antibody procedures to the study of pathogenic dematocates fungi. II. Serological relationships of the genus *Fonsecaea*. J. Bacteriol. 90:551–556.

18. Growald, I. S., and R. A. Flavel. 1996. A central role of CD40 ligand in the regulation of CD4+ T cell responses. Immunol. Today 17:410–414.

19. Ibrahim-Granet, O., C. De Bièvre, and M. Jendoubi. 1988. Immunochemical characterisation of antigens and growth inhibition of *Fonsecaea pedrosii* by species-specific IgG. J. Med. Microbiol. 26:217–222.

20. Langley, J. G., H. C. Kariuki, A. P. Hammersley, J. H. Ouma, A. E. Butter- worth, and D. W. Dunne. 1994. Human IgG subclass responses and subclass reactions to *Schistosoma mansoni* egg antigens. Immunology 83:651–658.

21. Maizels, R. M., D. A. B. Bundy, M. E. Selkirk, D. F. Smith, and R. M. Anderson. 1993. Immunological modulation and evasion by helminth parasites in human populations. Nature 365:797–805.

22. Matsumoto, T., and T. Matsuda. 1985. Chromoblastomycosis and phaeohy- phomycosis. Semin. Dermatol. 4:240–251.

23. Ricard-Blum, S., D. Hartmann, and P. Esterre. 1998. Monitoring of extra- cellular matrix metabolism and cross-linking in tissue, serum and urine of patients with chromoblastomycosis, a chronic skin fibrosis. Eur. J. Clin. In- vestig. 28:748–754.

24. Rolland-Burger, L., X. Rolland, C. W. Grieve, and L. Monjour. 1991. Im-munoblot analysis of the humoral immune response to *Leishmania donovani* infantum polysaccharides in human visceral leishmaniasis. J. Clin. Microbiol. 29:1429–1435.

25. Romero, H., E. Guezed, and S. Magaldi. 1996. Evaluation of immunopre- cipitation technique in chromoblastomycosis. J. Mycol. Med. 6:83–87.

26. Simac, C., P. Michel, A. Andriantsimahavandy, P. Esterre, and A. Michault. 1995. Use of ELISA and EITB for the diagnosis and monitoring of neuro- cysticercosis. Parasitol. Res. 81:132–136.

27. Stout, R. D., and J. Sutcliffe. 1996. The many roles of CD40 in cell-mediated inflammatory responses. Immunol. Today 17:487–492.

28. Villalba, E., and J. F. Yepes. 1988. Detection of circulating antibodies in patients affected by chromoblastomycosis by *Cladosporium carrionii* using double immunodiffusion. Mycopathologia 102:17–19.

29. Villalba, E. 1989. Detection of antibodies in the sera of patients with chro- moblastomycosis by counterimmunoelectrophoresis. Preliminary results. J. Med. Vet. Mycol. 26:73–74.