MYCOLOGY

Antibody isotypes to a *Paracoccidioides brasiliensis* somatic antigen in sub-acute and chronic form paracoccidioidomycosis

MICHELANGELO JUVENALE, GILDA M. B. DEL NEGRO, ALBERTO J. S. DUARTE and GIL BENARD

Laboratório de Alergia e Imunologia Clínica e Experimental, Faculdade de Medicina da Universidade de São Paulo and Laboratório de Micologia Médica, Instituto de Medicina Tropical de São Paulo, Brazil

This report describes the differences in isotype antibody reactivity against a crude *Paracoccidioides brasiliensis* antigenic preparation in the sub-acute (SAF) and chronic (CF) forms of paracoccidioidomycosis before treatment. IgG antibodies were detected in all patients, with a slightly but not significantly higher reactivity in the SAF. IgG1 antibodies were present, frequently at high levels, in both forms, whereas IgG3 was always low or absent. IgG2 antibodies were detectable in most patients, but at high levels in only a few CF patients. IgG4 was found mainly in SAF patients, whereas IgA was detected almost only in CF patients, probably due to a Th2 pattern of immune response in the more severe SAF, and the characteristic mucosal involvement of the CF, respectively. Immunoblot analysis showed that, in addition to the 43-kDa immunodominant fraction, other less well-characterised fractions were also recognised differentially by the isotypes and deserve further investigation.

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* and has an important social and economic impact, for it affects healthy men during their productive years, causing respiratory tract mucosal or cutaneous lesions, or both [1]. The infection is acquired by inhalation of conidiospores present in the soil, but its reservoir is still not fully characterised [2]. The mucosal lesions can vary from isolated oral ulceration to diffuse interstitial pulmonary involvement. However, most patients present multiple lesions at different sites of the respiratory tract. This form of the disease, called the chronic form (CF), mainly affects adult men and may develop many years after the patients have left the endemic area. In contrast, the less frequently observed sub-acute form (SAF) affects both sexes, mainly under 30 years of age, and probably develops soon after exposure to the fungus. Patients with the SAF present

with visceral involvement, predominantly of the mononuclear-phagocytic system [1].

PCM has been associated with variable degrees of depression of the cellular immune response and enhancement of the humoral immune response. The latter has been characterised by B-cell polyclonal activation, hypergammaglobulinaemia, and high serum levels of anti-*P. brasiliensis* antibodies [3–5]. Evaluation of the humoral immune response has been an important tool for diagnosis and follow-up of PCM patients. The level of specific antibody production has been used as a correlate of disease severity as well as for assessment of treatment response [1]. Various techniques and antigenic preparations have been described in the literature to assess anti-*P. brasiliensis* antibody response [6]. This laboratory has been using a counterimmuno-electrophoresis (CIE) test [7], and more recently, an enzyme-linked immunosorbent assay (ELISA) [8] successfully. The ELISA was introduced because it allows the processing of greater numbers of samples and uses a crude preparation in which at least 18 antigenic fractions are present [8], thus being more representative of the whole fungus, whereas the CIE employs a culture filtrate antigen whose major component is the 43-kDa fraction [6].

The present study aimed to characterise the reactivity

of total IgG, its subclasses, and IgA against the crude *P. brasiliensis* preparation by ELISA and immunoblotting.

Materials and methods

Patients and control sera

Sera from 47 untreated PCM patients were used. Sera were generally collected before treatment, as part of the diagnosis of PCM. Sera were divided and frozen in small volumes at -20°C until use. Patients were classified as SAF ($n = 14$) or CF ($n = 33$) according to the clinical presentation of the disease [1]. Sera from 40 healthy blood donors were also used to determine cut-off values of the ELISA.

Double immunodiffusion and counterimmuno-electrophoresis tests

A culture filtrate from a 10-day culture of the yeast phase of *P. brasiliensis* strain 113 (Culture Collection, Instituto de Medicina Tropical de São Paulo) was used for these assays, as described previously [7, 8]. The double immunodiffusion test (DID) was performed in agar 1% gel in buffered saline (pH 6.9) containing sodium citrate 0.4% and glycine 7.5% and the serum samples were tested undiluted, as described previously [7, 8]. Counterimmuno-electrophoresis (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 in the anodic side, and the antigen in the cathodic side of the slide. The sera were diluted in a two-fold series and tested from the undiluted sample. Samples that reacted at least undiluted were considered positive [7].

P. brasiliensis somatic antigen

A cellular yeast extract (somatic antigen) prepared as described previously [8] was used for the ELISA. Briefly, *P. brasiliensis* yeast cells (strains 113 and 339, the latter kindly supplied by A. Restrepo, CIB, Medellin, Colombia) were cultivated in Fava Netto's agar medium at 36°C for 7 days. Then, the cells were resuspended in 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 10 mM phenylmethylsulphonylfluoride as protease inhibitor. 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 by sterilising membranes, divided into small volumes and stored at -20°C . The protein content was 1.97 mg/ml as determined by Lowry's method [9].

ELISA for IgG, IgG subclasses and IgA anti-P. brasiliensis somatic antigen

ELISA for IgG anti-*P. brasiliensis* somatic antigen was done as described previously [10], with a few

modifications [8]. Briefly, polystyrene high-binding microplates (Costar, Cambridge, MA, USA) were coated with *P. brasiliensis* somatic antigen (25 $\mu\text{g/ml}$) diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated at 37°C for 1 h and at 4°C overnight. Plates were washed with PBS and blocked with PBS-gelatin 0.05%-BSA 2% for 1 h at 37°C . After washing the plates with PBS-Tween 0.1%, 50 μl of the dilutions of the patients' sera were added and incubated for 2 h at 37°C . Plates were washed and an anti-human γ -chain biotinylated monoclonal antibody (MAb) (Sigma) diluted 1 in 800 in PBS-gelatin 0.01%-BSA 0.4% was added and incubated for 1 h at 37°C . Streptavidin- β -galactosidase (1 in 2500; Sigma) was added and incubated for 60 min at 37°C . The reaction was developed with 50 μl of ONPG (Sigma) for 60 min at 37°C . Optical density (OD) was determined at 415 nm in an ELISA reader (BioRad, Hercules, CA, USA). The same steps were followed for IgG subclasses and IgA determinations. The respective MAbs – anti-human γ -chains, $\gamma 1$, $\gamma 2$, $\gamma 3$, $\gamma 4$ (Sigma) – were used as recommended at dilutions of 1 in 600, 1 in 1000, 1 in 3000 and 1 in 2500, respectively. Preliminary titration assays with four positive control sera with different titres of anti-*P. brasiliensis* antibodies (2048, 512, 64 and 4 in the CIE test) were performed to determine the optimal dilutions of the patients' sera. The following working dilutions were then used in this study: 1 in 5000 for total IgG, 1 in 200 for IgG1, 1 in 600 for IgG2, 1 in 100 for IgG3, 1 in 25 for IgG4 and 1 in 100 for IgA. These results were further confirmed by dilution experiments with patients' sera (data not shown). Cut-off values were calculated from the mean $+3$ SD of sera from 40 healthy donors. The assays with the patients' sera incorporated positive and negative control sera.

SDS-PAGE and immunoblotting procedure

Electrophoresis in polyacrylamide 10% gels with a stacking gel of 5% were run with a buffer system as described by Laemmli [11]. The somatic antigen was solubilised in buffer containing 62.5 mM Tris-HCl (pH 6.8), SDS 2%, glycerol 20% and 0.5 M DL-dithiothreitol and then heated at 100°C for 2 min. For each sample, c. 20 μg were applied to the gel. Gels were stained with silver and molecular mass was determined by the use of a 20–94-kDa standard kit (Pharmacia, Uppsala, Sweden). After separation of the somatic antigen in SDS-PAGE, blots were prepared as described by Towbin *et al.* [12]. Nitrocellulose paper (BioRad, Hercules, CA, USA) was hydrated in transfer buffer (methanol 20%, 25 mM Tris base, 192 mM glycine) at pH 8.3, then aligned with the gel and placed in an electroblot apparatus (25 V, overnight). After transfer, the paper was cut into strips and incubated with a solution of skim milk 5% in PBS (SM-PBS) at room temperature for 2 h. Strips were then incubated with sera diluted 1 in 20 in SM-PBS for 18 h at room temperature. After washing in PBS, the strips were

incubated with mouse anti-human heavy chain-specific IgG1, IgG2, IgG4 and IgA conjugate (Sigma) at room temperature. Strips were washed and bands were visualised with diaminobenzidine- H_2O_2 substrate.

Statistical analysis

Values obtained for juvenile and adult patients were compared by the Mann-Whitney test. Differences were considered significant at $p < 0.05$. The Spearman test was used in the correlation studies.

Results

Sera from 47 untreated patients with PCM were tested for anti-*P. brasiliensis* antibodies by CIE and DID assays. Of these patients, 14 presented with SAF and 33 had CF. Anti-*P. brasiliensis* antibodies were detected in all 47 patients by both CIE and DID, whereas none of the 40 serum samples from apparently healthy blood donors was reactive in these assays.

Fig. 1 shows the antibody levels in CIE and ELISA for both groups of patients. Patients with the SAF showed

Fig. 1. Results of anti-*P. brasiliensis* culture filtrate antibody titres measured by CIE (a) and IgG (b), IgG1 (c), IgG2 (d), IgG4 (e), and IgA (f) antibody reactivity to *P. brasiliensis* somatic antigen, measured by ELISA, in the sera of SAF (S; ○) and CF (C; ●) paracoccidioidomycosis patients. Each dot represents the value from a single patient and the horizontal lines represent the median value from the group. The shaded area corresponds to the mean + 3 SD of the results from 40 healthy donors' sera. The statistical significance level, when present, is indicated for each comparison between SAF and CF patients.

higher amounts of specific antibodies than patients with the CF, as detected by CIE. IgG antibodies were also detected in all patients by ELISA, with slightly higher levels in SAF patients than in CF patients (OD₄₁₅ medians 2.4 and 1.7, respectively); however, these results did not reach statistical significance. IgG1 subclass antibodies were detected above the cut-off level in all SAF patients and in 94% of the CF patients; a high reactivity (OD₄₁₅ > 1.0) was generally detected within this subclass in both groups (SAF, 86%; CF, 85%). For the IgG2 subclass antibodies, although the reactivity was above the cut-off level in the majority of the patients (93% in SAF patients and 94% in CF patients), a few patients, with the CF only (n = 7, 21%), had OD₄₁₅ values > 1.0. IgG3 antibodies were either not detected above the cut-off point or were detected at low levels (data not shown). Conversely, IgG4 antibodies were found in all SAF patients, usually with high reactivity, but were not detectable in a majority (58%) of the CF patients. The median OD₄₁₅ was significantly higher in the former than in the latter (1.4 versus 0.0, $p < 0.0001$). Reactivity for IgA was found above the cut-off level mainly in the CF group (73%), but only in a minority of patients (29%) in the SAF group. Their median OD₄₁₅ were significantly different: (0.64 versus 0.26, $p = 0.007$).

Determination of antibody titres by CIE has been used routinely by our group as a good correlate of disease severity. Hence, this raised the question as to whether the results obtained by ELISA would correlate with those obtained by CIE. Of all the subclass determinations, only IgG4 and IgA (the latter in CF patients only) gave a correlation between the two methodologies (n = 46, $\rho = 0.49$, $p < 0.001$ and n = 33, $\rho = 0.44$, $p = 0.01$, respectively). The presence of any correlation between the ELISA results for IgG and its subclasses was also investigated. Correlation was found for IgG1 (n = 44, $\rho = 0.76$, $p < 0.001$), IgG2 (n = 44, $\rho = 0.45$, $p = 0.002$) and IgG4 (particularly in the SAF group, n = 11, $\rho = 0.92$, $p < 0.001$). Finally, a correlation was observed in CF patients between IgG and IgA ($\rho = 0.62$, $p < 0.001$) and, to a lesser extent, between IgG and IgG2 ($\rho = 0.46$, $p = 0.007$).

The study then evaluated the pattern of recognition of the IgG1, IgG2, IgG4 and IgA antibodies directed to the *P. brasiliensis* somatic antigen by Western blotting. As shown in Fig. 2a, IgG1 from both patient groups recognised several antigen fractions, including one of 43 kDa that corresponds to the main *P. brasiliensis* antigen used for diagnosis (data not shown). It can also be seen that low mol. wt fractions were preferentially recognised by sera from the sub-acute group, except for a 27-kDa fraction that was also recognised by serum samples from two CF patients. The 43-kDa fraction was recognised by IgG2 antibodies together with higher mol. wt fractions (>94 kDa), especially in the CF patients with high IgG2 reactivity in the ELISA (Fig. 2b). The pattern of recognition by IgG4 antibodies was

heterogeneous even in sera from the SAF patients, which had a strong IgG4 reactivity in the ELISA (Fig. 2c). Finally, IgA-rich sera from CF patients recognised not only the immunodominant 43-kDa fraction (four of eight sera), but several other fractions of variable molecular size (Fig. 2d). The serum samples tested from SAF patients showed a weak reactivity in agreement with the low ELISA reactivity.

Discussion

Higher antibody reactivity against the crude filtrate antigen by CIE in SAF than CF form patients was observed in the present study, which is in agreement with the results of an earlier study [7] and with the notion that SAF disease is generally more severe [1]. In contrast, although SAF patients had slightly higher IgG reactivity against the somatic antigen by ELISA, there was no statistical difference between these and the CF patients. In an earlier study [8], CF patients showed lower antibody reactivity than SAF patients. In that study, the CF group comprised patients with multifocal and unifocal disease, whereas in the present study, unifocal patients were rare (only five patients). Patients with CF unifocal usually present with low anti-*P. brasiliensis* antibody titres, which explains the lower reactivity in the previous results. It is interesting to note that, although both the ELISA-IgG and CIE have already been proved useful to monitor severity of disease and treatment follow-up [8], no correlation was found between them in the present study. This could be due to differences in the sensitivity of the two methods or in the nature of the antigen, as the somatic antigen contains a higher number of fractions [6, 8].

The analysis of the isotype representation of the IgG reactivity indicated that the two forms of the disease have distinct profiles. The present study employed an antigenic preparation that was more representative of the whole fungus and extended earlier observations with the specific 43-kDa antigen [13]. In the present study, whereas a high level of IgG1 reactivity was detected in the majority of patients in both groups, IgG4 reactivity was mainly associated with the SAF group. A high level of IgG2 reactivity was only seen in some CF patients. Based on the knowledge that a conversion to IgG4 is dependent on interleukin (IL-4) [14, 15] and that a conversion to IgG2 is influenced by interferon (IFN)- γ [15, 16], it is suggested that the SAF of the disease is related to a Th2-type pattern of immune response. This pattern of humoral immune response would be associated with the more intensely depressed cellular immune function of this form of the disease. Conversely, in some patients with the CF, a Th1-type pattern of immune response would be expected, because sufficient IFN- γ would have been produced to promote the conversion to IgG2 during the immune response.

Fig. 2. Antibody isotype response, (a) IgG1, (b) IgG2, (c) IgG4 and (d) IgA, from SAF and CF PCM patients to the *P. brasiliensis* somatic antigen, as analysed by immunoblot; (a–d): lane p, mol. wt standards; a, negative control serum; b, positive control serum. (a) Lanes c–h, SAF patients; i–n, CF patients. (b) Lanes c–e, SAF patients; f–n, CF patients. (c) Lanes c–j, SAF patients; k–n, CF patients. (d) Lanes c–f, SAF patients; g–n, CF patients.

Thus, in addition to the suggestion that it might be a marker of acute disease, IgG4 may also be a marker of disease severity and a good parameter for treatment follow-up, as it correlated well with CIE. IgA reactivity, on the other hand, was consistently seen only in the CF patients. This finding is not surprising because mucosal involvement of the respiratory tract, from lungs to the oral mucosa, is a main feature of this form and rare in the SAF [1]. Chronic antigen inflammation of mucosa is well known as a potent

stimulus for the secretion of IgA antibodies [17]. In fact, IgA reactivity correlated well with CIE in the CF patients and may also eventually serve as an additional parameter for monitoring treatment response. Studies are currently in progress to test this hypothesis.

An alternative factor that could also have contributed to the different results in SAF and CF is age. SAF patients were 5–28 years old, whereas CF patients were 35–72 years old. Indeed, significant changes in serum

Fig. 2. (continued)

immunoglobulin levels are found with ageing [18]. It has been shown that the serum levels of the IgA, IgG1, IgG2 and IgG3 isotypes, but not IgG4, increased in older individuals [19]. In contrast, the ability to respond to a specific antigen challenge with specific antibody production decreases with age [18]. Thus, it seems unlikely that age has, by itself, determined the differences in the two clinical groups. In human strongyloidiasis, for example, chronicity of infection did not correlate with specific IgA and IgG4 antibody levels, and IgG1 antibody levels were higher in younger patients [20]. The high levels of IgG4 were

probably the result of repeated antigen exposure through auto-infection, most commonly seen in the young. A similar hypothesis was also sustained for the high IgG4 levels in younger schistosomiasis patients [21, 22]. Therefore, more than a chronic antigen stimulus, these situations would represent a challenge with heavy antigen loads. This argument may also apply to SAF patients, in whom a heavier fungal load, compared with CF patients, is expected [1]. Moreover, the IgG4 subclass, by neither fixing complement nor having opsonising activity [23], could favour the successful establishment of a higher number of

parasites in the host and the progressive fungal dissemination.

Interestingly, the levels of IgG3 reactivity were very low or absent, as has already been seen with the *P. brasiliensis*-specific fraction of 43 kDa [13]. In other infectious conditions, in which the antibody profile is also predominantly influenced by a Th2 pattern of immune response and characterised by high levels of specific IgG4 or IgE antibodies, or both, there were low levels of the IgG3 subclass [24–26].

Up to now, the 43-kDa molecule has been considered the main diagnostic antigen in PCM and a possible inducer of protective immune response [27, 28]. However, several other glycoproteins, of 22–25 kDa [29], 27 kDa [30], 58 kDa [31], 70 kDa [32] and 87 kDa [33], have been described recently as being widely recognised by PCM patients' sera. Therefore, these antigens and probably others may all be relevant to the host immune response, as well as to the immunopathogenesis and diagnosis of the mycosis. This study then addressed the question as to whether different fractions of the somatic antigen were recognised in a specific manner by the subclasses of IgG or by IgA. Although a limited number of sera from each clinical presentation could be analysed by immunoblotting, the results indicate that the pattern of antigen fractions recognised by each presentation was dissimilar. This was more evident for IgG1, in which a number of low mol. wt fractions, including the 27-kDa fraction, were predominantly recognised by SAF sera, and for IgA, by which low mol. wt fractions were recognised by the CF sera. Even though the 43-kDa fraction remained the most frequently recognised fraction by all subclasses and by IgA, these results emphasise that other less well characterised fractions may be important and merit further investigation.

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo #96/1129-0 and #96/5789-4, Conselho Nacional de Desenvolvimento Científico e Tecnológico #301487/96-0 and Laboratórios de Investigação Médica do Hospital das Clínicas da FMUSP.

References

1. Franco MF, Mendes RP, Moscardi-Bacchi M, Rezkallah-Iwasso M, Montenegro MR. *Paracoccidioidomycosis*. *Baillières Clin Trop Med Comum Dis* 1989; **4**: 185–220.
2. Restrepo-Moreno A. Ecology of *Paracoccidioides brasiliensis*. In: Franco M, da Silva Lacaz C, Restrepo-Moreno A, Del Negro G (eds) *Paracoccidioidomycosis*. Boca Raton, FL, CRC Press. 1994: 121–130.
3. Chequer-Bou-Habib D, Daniel-Ribeiro C, Banic DM, do Valle ACF, Galvão-Castro B. Polyclonal B cell activation in paracoccidioidomycosis. *Mycopathologia* 1989; **108**: 89–93.
4. Benard G, Durandy A, Assis CM *et al.* Responses of T and B lymphocytes to a *Paracoccidioides brasiliensis* cell wall extract in healthy sensitized and non-sensitized subjects. *Am J Trop Med Hyg* 1995; **53**: 189–194.
5. Benard G, Hong MA, Del Negro GMB, Batista L, Shikanai-Yasuda MA, Duarte AJS. Antigen-specific immunosuppression in paracoccidioidomycosis. *Am J Trop Med Hyg* 1996; **54**: 7–12.

6. Mendes-Giannini MJS, Del Negro GMB, Siqueria AM. Serodiagnosis. In: Franco M, da Silva Lacaz C, Restrepo-Moreno A, Del Negro G (eds) *Paracoccidioidomycosis*. Boca Raton, FL, CRC Press. 1994: 345–363.
7. Del Negro GMB, Garcia NM, Rodrigues EG *et al.* The sensitivity, specificity and efficiency values of some serological tests used in the diagnosis of paracoccidioidomycosis. *Rev Inst Med Trop Sao Paulo* 1991; **33**: 227–280.
8. Del Negro GMB, Pereira CN, Andrade HF *et al.* Evaluation of tests for antibody response in the follow-up of patients with acute and chronic forms of paracoccidioidomycosis. *J Med Microbiol* 2000; **49**: 37–46.
9. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
10. Mendes Giannini MJS, Bueno JP, Shikanai-Yasuda MA, Ferreira AW, Masuda A. Antibody response to the 43 kDa glycoprotein of *Paracoccidioides brasiliensis* as a marker for the evaluation of patients under treatment. *Am J Trop Med Hyg* 1990; **43**: 200–206.
11. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
12. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350–4354.
13. Baida H, Biselli PJC, Juvenale M *et al.* Differential antibody isotype expression to the major *Paracoccidioides brasiliensis* antigen in juvenile and adult form paracoccidioidomycosis. *Microbes and Infection* 1999; **1**: 273–278.
14. Fujieda S, Zhang K, Saxon A. IL-4 plus CD40 monoclonal antibody induces human B cells γ subclass-specific isotype switch: switching to $\gamma 1$, $\gamma 3$, and $\gamma 4$, but not $\gamma 2$. *J Immunol* 1995; **155**: 2318–2328.
15. Kitani A, Strober W. Regulation of $C\gamma$ subclass germ-line transcripts in human peripheral blood B cells. *J Immunol* 1993; **151**: 3478–3488.
16. Kawano Y, Noma T. Role of interleukin-2 and interferon- γ in inducing production of IgG subclasses in lymphocytes of human newborns. *Immunology* 1996; **88**: 40–48.
17. Fayette J, Dubois B, Vandenabeele S *et al.* Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA₁ and IgA₂. *J Exp Med* 1997; **185**: 1909–1918.
18. Burns EA, Goodwin JS. Immunodeficiency of aging. *Drugs Aging* 1997; **11**: 374–397.
19. Paganelli R, Quinti I, Fagiolo U *et al.* Changes in circulating B cells and immunoglobulin classes and subclasses in a healthy aged population. *Clin Exp Immunol* 1992; **90**: 351–354.
20. Atkins NS, Lindo JF, Lee MG *et al.* Humoral responses in human strongyloidiasis: correlations with infection chronicity. *Trans R Soc Trop Med Hyg* 1997; **91**: 609–613.
21. Hagan P, Blumenthal UJ, Dunne D, Simpson AJG, Wilkins HA. Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. *Nature* 1991; **349**: 243–245.
22. Demeure CE, Rihet P, Abel L, Ouattara M, Burgois A, Dessein AJ. Resistance to *Schistosoma mansoni* in humans: influence of the IgE/IgG4 balance and IgG2 in immunity to reinfection after chemotherapy. *J Infect Dis* 1993; **168**: 1000–1008.
23. Hamilton RG. Human IgG subclass measurements in the clinical laboratory. *Clin Chem* 1987; **33**: 1707–1725.
24. Kwan-Lim G-E, Forsyth KP, Maizels RM. Filarial-specific IgG4 response correlates with active *Wuchereria bancrofti* infection. *J Immunol* 1990; **145**: 4298–4305.
25. Kurniawan A, Yazdanbakhsh M, van Ree R *et al.* Differential expression of IgE and IgG4 specific antibody responses in asymptomatic and chronic human filariasis. *J Immunol* 1993; **150**: 3941–3950.
26. Tomee JFC, Dubois AEJ, Köeter GH, Beaumont F, van der Werf TS, Kauffman HF. Specific IgG4 responses during chronic and transient antigen exposure in aspergillosis. *Am J Resp Crit Care Med* 1996; **153**: 1952–1957.
27. Travassos LR, Puccia R, Cisalpino P *et al.* Biochemistry and molecular biology of the main diagnostic antigen of *Paracoccidioides brasiliensis*. *Arch Med Res* 1995; **26**: 297–304.
28. Taborda CP, Juliano MA, Puccia R, Franco M, Travassos LR. Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein of *Paracoccidioides brasiliensis* which induces a

- Th-1 response protective against fungal infection in BALB/c mice. *Infect Immun* 1998; **66**: 786–793.
29. Figueroa JI, Hamilton A, Allen M, Hay R. Immunohistochemical detection of a novel 22- to 25-Kilodalton glycoprotein of *Paracoccidioides brasiliensis* in biopsy material and partial characterization by using species-specific monoclonal antibodies. *J Clin Microbiol* 1994; **32**: 1566–1574.
 30. McEwen JG, Ortiz BL, Garcia AM, Florez AM, Botero S, Restrepo A. Molecular cloning, nucleotide sequencing, and characterization of a 27-kDa antigenic protein from *Paracoccidioides brasiliensis*. *Fungal Genet Biol* 1996; **20**: 125–131.
 31. Figueroa JI, Hamilton AJ, Allen MH, Hay RJ. Isolation and partial characterization of a *Paracoccidioides brasiliensis* 58 kDa extracellular glycoprotein which is recognized by human immune sera. *Trans R Soc Trop Med Hyg* 1995; **89**: 566–572.
 32. Camargo ZP, Unterkircher C, Travassos LR. Identification of antigenic polypeptides of *Paracoccidioides brasiliensis* by immunoblotting. *J Med Vet Mycol* 1989; **27**: 407–412.
 33. Gómez BL, Figueroa JI, Hamilton AJ *et al.* Use of monoclonal antibodies in diagnosis of paracoccidioidomycosis: new strategies for detection of circulating antigens. *J Clin Microbiol* 1997; **35**: 3278–3283.