Optimization of a Mouse Immunization Protocol with *Paracoccidioides brasiliensis* Antigens

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The objectives of the present study were to optimize the protocol of mouse immunization with Paracoccidioides brasiliensis *antigens (Rifkind's protocol) and to test the modulation effect of cyclophosphamide (Cy) on the delayed hypersensitivity response (DHR) of immunized animals. Experiments were carried out using one to four immunizing doses of either crude particulate* P.brasiliensis *antigen or yeast-cell antigen, followed by DHR test four or seven days after the last immunizing dose. The data demonstrated that an immunizing dose already elicited response; higher DHR indices were obtained with two or three immunizing doses; there were no differences between DHR indices of animals challenged four or seven days after the last dose. Overall the inoculation of two or three doses of the yeast-cell antigen, which is easier to prepare, and DHR test at day 4 simplify the original Rifkind's immunization protocol and shorten the duration of the experiments.*

The modulation effect of Cy on DHR was assayed with administration of 2.5, 20 and 100 mg/kg weight at seven day intervals starting from day 4 prior to the first immunizing dose. Only the treatment with 2.5 mg Cy increased the DHR indices. Treatment with 100 mg Cy inhibited the DHR, whereas 20 mg Cy did not affect the DHR indices. Results suggest an immunostimulating effect of low dose of Cy on the DHR of mice immunized with P. brasiliensis *antigens.*

Key words: *Paracoccidioides brasiliensis* - immunization - paracoccidioidomycosis

Paracoccidioidomycosis is a systemic mycosis caused by *Paracoccidioides brasiliensis* which is endemic in Latin America, especially Brazil, Argentina and Venezuela.

The disease presents two polar forms: (1) *Hyperergic positive pole,* characterized by a benign localized disease with intact cell immune response and formation of compact granulomata with few parasites, and (2) *Anergic negative pole,* characterized by a malignant disseminated disease with depressed cellular immunity and loose granulomata with large numbers of fungi. In general, the higher the level of immunosuppression, the greater the severity of the clinical presentation, as demonstrated by numerous clinical and experimental investigations (Franco et al. 1993).

In previous studies we have described mouse and hamster immunization protocols with *P. brasiliensis* antigens in order to exacerbate the specific cell immune response and eventually protect the animals against an infectious challenge (Moscardi & Franco 1981, Prestes et al. 1983, Bacchi & Franco 1985, Biagioni et al. 1986, Kamegasawa et al. 1988, 1992, Defaveri et al. 1989a, b). In murine models we have utilized the immunization protocol described by Rifkind et al. (1976) standardized for inducing a delayed hypersensitivity reaction (DHR) in animals immunized by the intradermal route with particulate crude fungal antigen. Although efficient, this protocol is time-consuming (four weeks) and requires large amounts of antigen.

Cyclophosphamide (Cy) is an anti-cancer agent with a cytotoxic action on rapidly multiplying cells (Ahmed & Hombal 1984). Its use in experimental animals has revealed that, depending on the dose and administration schedule, the cell immune response may be exacerbated or depressed. Thus, the drug has been utilized experimentally as an immunomodulator of host resistance against microorganisms, in particular pathogenic fungi (Askenase et al. 1975, Berd & Mastrangelo 1988, Oratz et al. 1991).

In view of the above considerations, the objectives of the present study were to optimize the protocol of mouse immunization with *P. brasiliensis* antigens and to test the modulating effect of Cy on the response of immunized animals.

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MATERIALS AND METHODS

Experimental design - As described by Rifkind et al. (1976), the protocol of mouse immunization for the development of DHR to fungal antigens is based on four cutaneous injections of *P. brasiliensis* particulate antigen administered at seven-day intervals; seven days after the last immunizing dose, the animals are challenged in the foodpad with soluble fungal antigen and evaluated by the footpad test (FPT).

To optimize this protocol by reducing the time of immunization and the time between the last immunizing dose and the challenge, and to simplify the method for the preparation of the immunizing antigen, we performed experiments using one to four immunizing doses of the *P. brasiliensis* particulate antigen or a yeast-cell antigen, and evaluated DHR four or seven days after the last immunizing dose.

Animal - Thirty-day-old Swiss male mice weighing on average 35 g, obtained from the Animal House of the Campus of Botucatu, UNESP, were used in all experiments.

Antigens - (1) *Yeast-cell antigen* - Yeast cells of isolates Bt1, Bt2 (from the Fungal Collection of the Department of Pathology, Faculty of Medicine of Botucatu, UNESP) and 192 (from the Fungal Collection of the Department of Microbiology and Immunology, Faculty of Medicine, USP) were cultured on Fava- Netto's medium (Fava Netto 1961). After seven days of culture, the fungi were suspended and inactivated in sterile 0.85% saline (SS) containing 2% formalin for 18 hr. The fungal cells were then washed three times in SS, rapidly sonicated (1 min; 80-90A) to break cell clusters, and adjusted to a concentration of 10^7 cells/ml. (2) *Particulate antigen* - This antigen was prepared as described previously (Bacchi & Franco 1985). Briefly, yeast cells from the same *P. brasiliensis* isolates were inactivated as described above, resuspended in a 10-fold larger volume than that of the initial sediment and ruptured by sonication (Thornton sonicator; 20x3-min cycles; 80-90 A; on an ice bath). Fungal rupture was monitored microscopically. The suspension was centrifuged at 700 g for 20 min at 4°C and half of the supernatant was discarded; the sediment was resuspended in the remaining volume and was used as the antigen for immunization. (3) *Soluble antigen* - This antigen was prepared as described above up to the sonication of yeast cells. Throughout this step, phenylmethylsulfonyl fluoride (a protease inhibitor) in a final concentration of 20 mM/ml was added and the suspension was centrifuged at 11.000 g for 30 min at 4°C. The supernatant was filtered by passage through a 0.22-µm Millipore filter, dialyzed against distilled water, and lyophilized.

Immunization - The animals were immunized into the dorsum with the yeast-cell antigen through a 0.05 ml subcutaneous injection containing 10 to 10^7 fungal cells. The animals immunized with the particulate antigen received subcutaneous injections of 0.025 ml antigen diluted 1:4 in SS.

Groups of animals were immunized during the administration of Cy (Laboratório Abbot do Brasil). The drug was administered intraperitoneally at the doses of 2.5, 20 and 100 mg/kg weight at 7-day intervals starting from day 4 prior to the first immunizing dose. The drug concentration was prepared fresh on the day of inoculation. As a control, animal groups were inoculated in a similar manner with SS.

Measurement of DHR - The response was evaluated by the FPT as described previously (Bacchi & Franco 1985). Each animal was inoculated with 0.05 ml of the soluble antigen into the right footpad and with 0.05 ml of SS into the left footpad.

After 24 hr, DHR was measured and expressed as the difference in weight (mg) between the two footpads.

Statistical analysis - Analysis of variance followed by multiple comparisons according to Tukey was used to detect differences between the means. Student's test for unpaired samples was employed to compare two means. Values of $p < 0.05$ were considered significant (Zar 1984).

RESULTS

Immunization with the yeast-cell antigen - Figure 1 shows the mean FPT indices for a group of eight animals immunized with a single injection of intact particulate antigen at the doses of 10 to 10^7 fungal cells and challenged seven days later. The control group was immunized with the crude particulate antigen. The animals immunized with concentrations higher than 10^5 fungal cells presented reactive indices in plateau, similar to those of the control group (Tukey test; $p < 0.05$).

Immunization with the particulate antigen - Groups of 15 animals immunized with one to four doses of the antigen and challenged seven days after the last immunizing dose presented FPT indices higher than those of the group of non-immunized animals (control group). The indices increased progressively up to the group immunized with three doses (Tukey test; $p < 0.05$) (data not shown).

Immunization with the particulate antigen and challenge after four or seven days after immunization - Groups of mice (n=10) immunized with two or three doses of the particulate antigen and challenged four or seven days after immunization presented similar FPT indices that were significantly higher than those of the control group (ani-

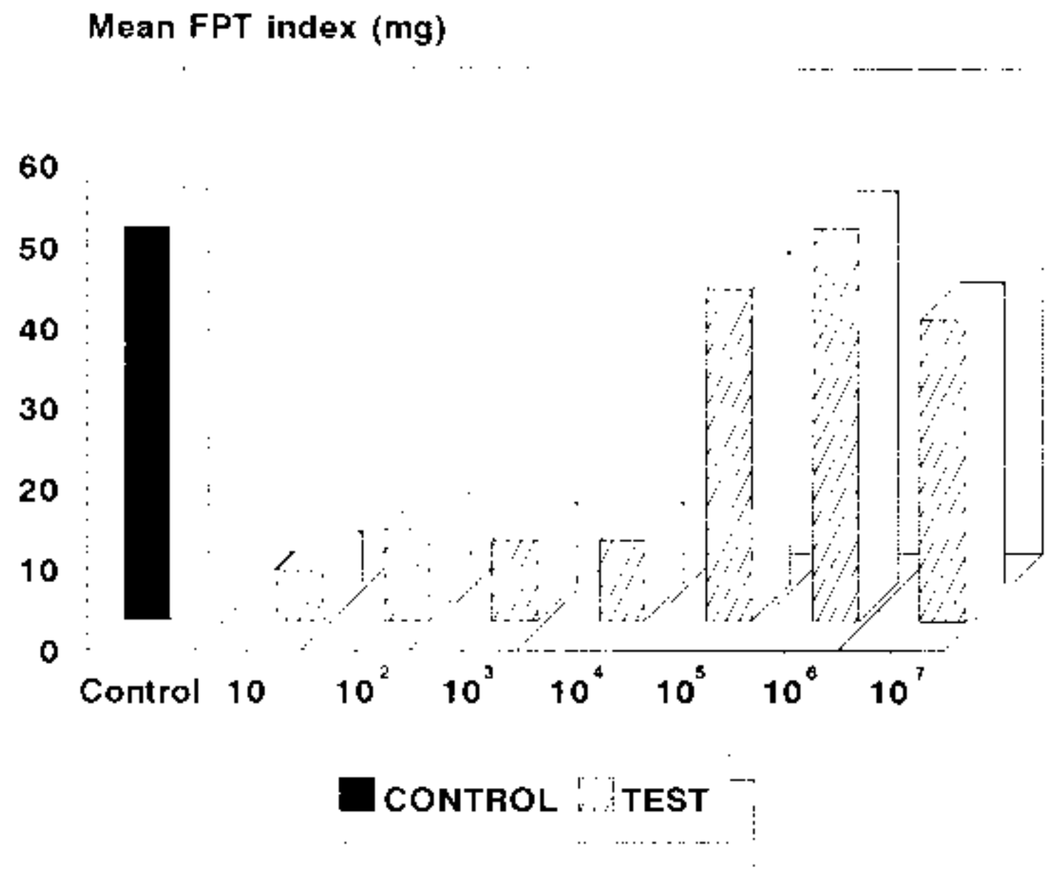

Fig. 1: mean FPT (footpad test) indices of mice (n = 8) immunized with a single dose at either the yeast-cell antigen (10-10^7 *Paracoccidioides brasiliensis* yeast cells) (test group) or the particulate antigen (control group). DHR was measured seven days post-immunization.

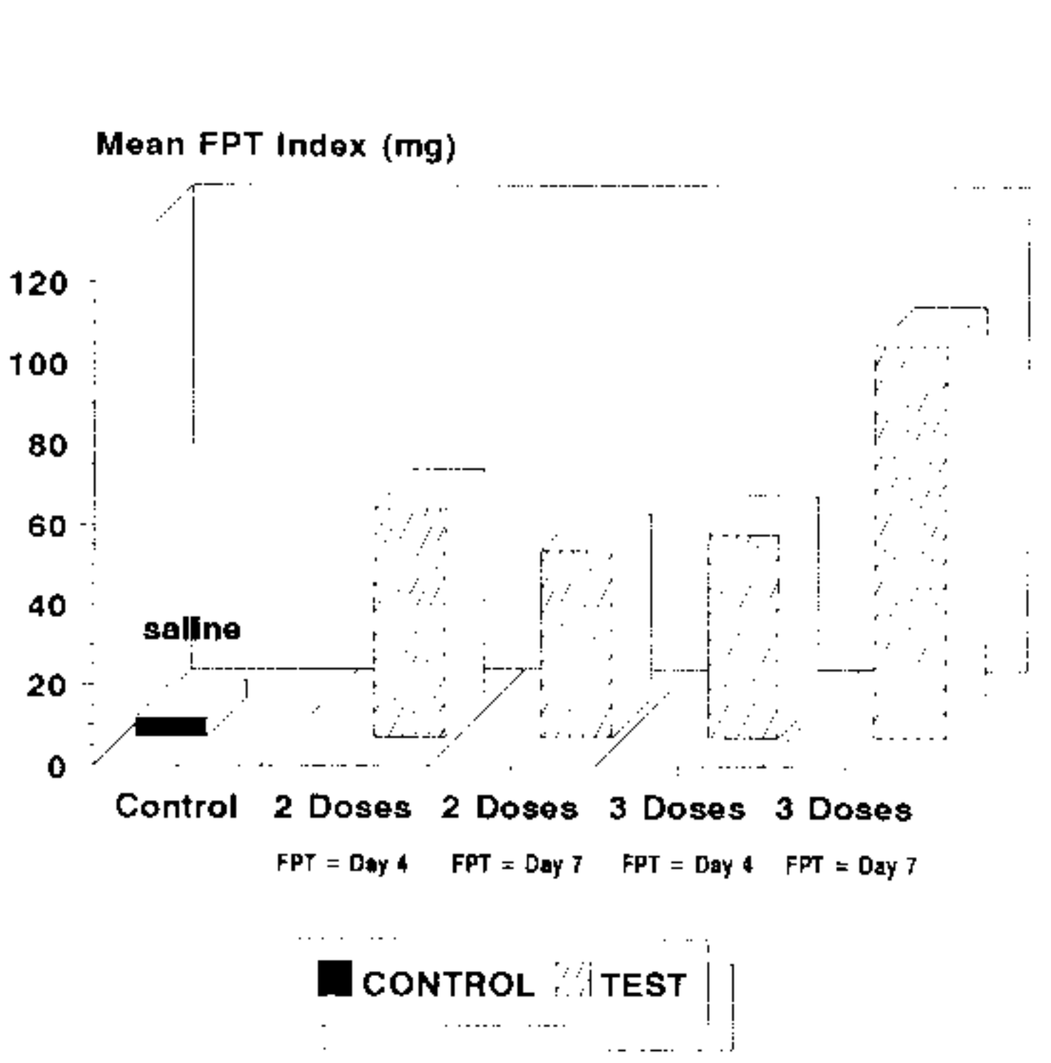

Fig. 2: mean FPT (footpad test) indices of mice (n = 10) immunized either with the particulate antigen (two or three doses) (test group) or with sterile saline (control group). DHR was measured four or seven days after immunization.

mals immunized with SS)(Fig. 2)(Tukey test; $p<0.05$).

Immunization with the yeast-cell antigen and challenge after four or seven days after immunization - Groups of mice (n=6), immunized with two or three doses of the yeast-cell antigen (10^7 *P. brasiliensis* yeast cells) and challenged

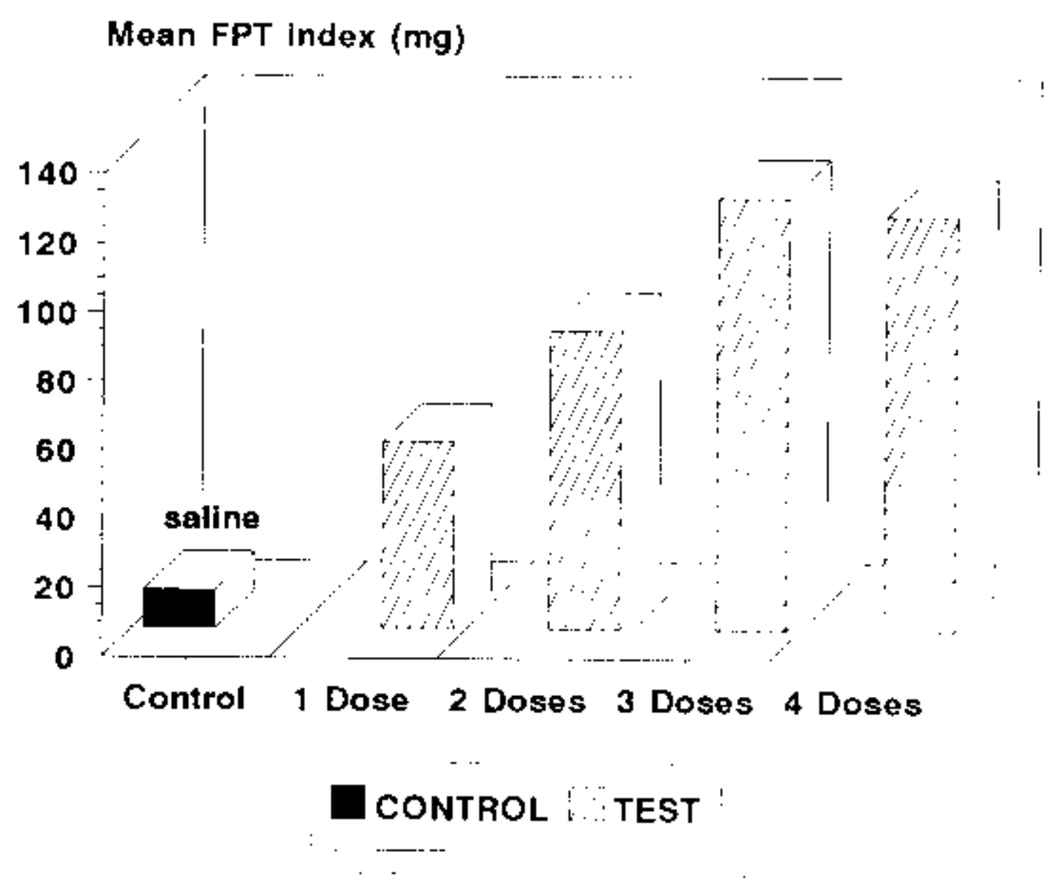

Fig. 3: mean FPT (footpad test) indices of mice (n = 6) immunized with two or three doses of either the yeast-cell antigen (10^7 *Paracoccidioides brasiliensis* yeast cells) (test group) or with sterile saline (control group). DHR was measured four or seven days after immunization.

four or seven days after immunization, presented similar FPT indices that were significantly higher than those of the control group (animals immunized with SS) (Fig. 3) (Tukey test; $p < 0.05$).

Immunization with Cy - Groups of six mice received three intraperitoneal inoculations of 2.5, 20 or 100 mg Cy at 7-day intervals; the first inoculation was administered four days prior to the first immunizing dose with the particulate antigen. Seven days later, the animals received the second immunizing dose and were then challenged on day 14 (Fig. 4).

The FPT indices of the immunized animals treated with 2.5 mg Cy were higher than those of the control groups (Student t test; $p <0.05$).

The FPT indices of the animals immunized and treated with 20mg Cy were slightly lower than those of the control group, but the difference was not significant (Student test; $p<0.05$).

The FPT indices of animals immunized and treated with 100 mg Cy were significantly lower than those of the immunized animals treated with SS (control group) (Student t test; $p < 0.01$) (Fig.4).

DISCUSSION

Immunization of mice with fungal antigens has been widely used in studies of protective immunity, antigen standardization and modulation of the immune response (Moscardi & Franco 1981, Prestes et al. 1983, Biagioni et al 1986, Bacchi & Franco 1989, Defaveri et al. 1989a, b). Most studies have used the protocol of Rifkind et al. (1976), which is time consuming and requires

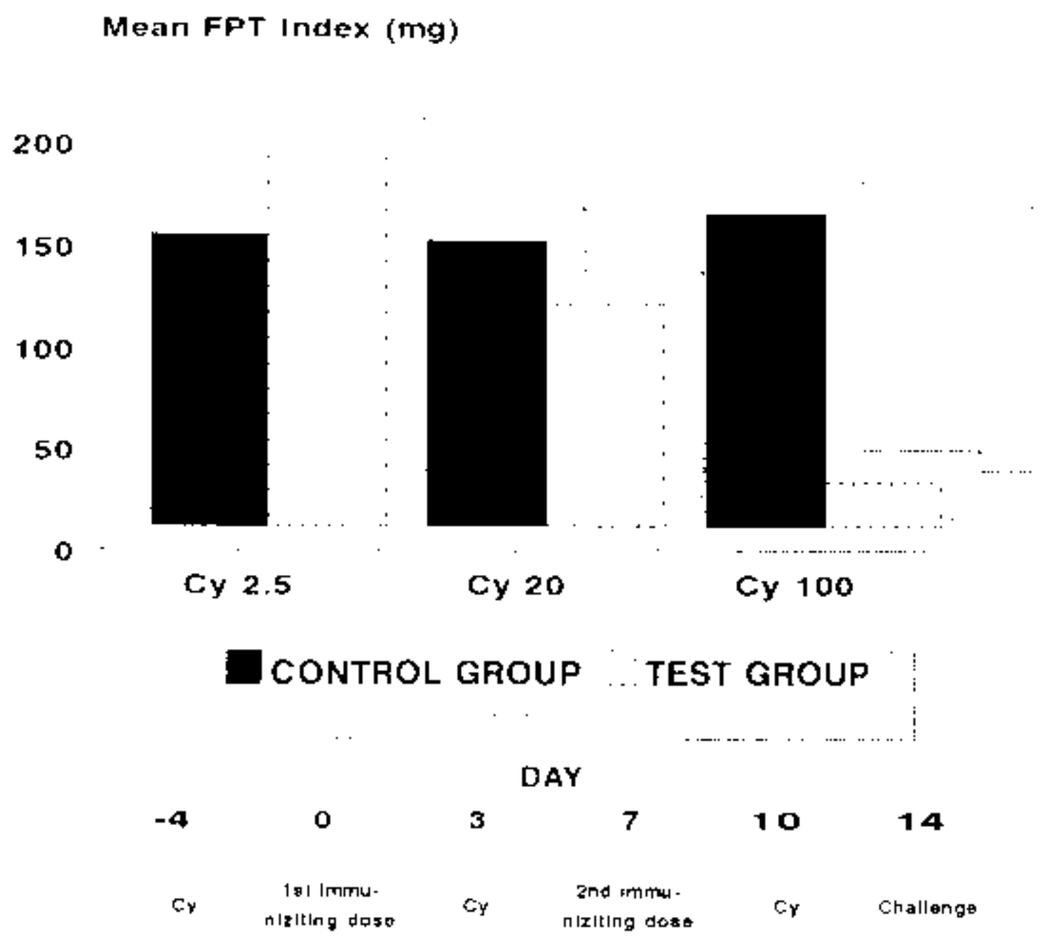

Fig. 4: immunization protocol and treatment with Cy (cyclophosphamide). FPT (footpad test) mean indices of controls animals (n = 7) and animals immunized with two doses of the yeast-cell antigen and treated with Cy 2.5, 20 or 100 mg ip, at Day -4, 4 and 7 (test groups; n = 6). Control animals were immunized in a same manner using sterile 0.85% saline instead of Cy.

the use of large quantities of an immunizing agent of cumbersome preparation.

The objective of our first experiment was to compare the efficacy of the standard immunizing antigen (particulate antigen) with that of an antigen of easier preparation represented by intact dead-*P. brasiliensis* yeast cells (yeast-cell antigen). Immunization with 10^5 to 10^7 intact yeast cells induced a DHR similar to that obtained with the standard antigen.

We then performed experiments directed at the optimization of the protocol, i.e., we immunized groups of mice with a smaller number of doses of either antigen and challenged the animals by the FPT over a shorter period of time. We observed that the animals presented DHR as early as after the first immunizing dose although in low levels. With two or three immunizing doses, the animals can be challenged four days after the last dose.

Taken as a whole, the experiments demonstrate that, using the present protocols of mouse immunization with *P. brasiliensis* antigens, less than four immunizing doses may be used; good results are obtained even with one or two immunizing doses and challenge four days after the last dose. The particulate antigen utilized for immunization can be replaced with an antigen of easier preparation. These modifications simplify the original Rifkind's protocol, and shorten the duration of the experiments (Rifkind et al. 1976).

Immunization protocols aiming at vaccination and protective immunity trigger complex cell mechanisms including suppressor responses. The efficacy of these protocols can be improved by the inactivation of the suppressor mechanisms that block the responses of the host to immunization. Immunomodulators that inhibit the induction of suppressor cells can increase the immune response to the immunizing antigen, favoring host protection against an infectious challenge. Cy is an immunomodulator that has been tested for this purpose on the basis of its ability to selectively inactivate subpopulations of suppressor cells (Askanase et al. 1975).

The use of Cy for this purpose has been described in several reports, with wide variation in the dose used (5 to 250 mg/kg weight), in the number of days of administration prior to the first immunizing dose as well as in the subsequent doses, and in the results obtained (Askanase et al. 1975, Ahmed & Hombal 1984, Berd & Mastrangelo 1988, Morikawa et al. 1989, Nakamura et al. 1989, Okuyama et al. 1989, Oratz et al. 1991). In our model, high Cy doses (100 mg/kg weight) induced a marked suppression of the delayed hypersensitivity response as measured by FPT. A lower dose (20 mg/kg weight) practically did not modify the animals' cell immune response. When lower doses were tested (2.5 mg/kg weight), there occurred an increase in DHR in immunized mice. This result suggests the immunostimulating action of low doses of Cy on the DHR of mice immunized with *P. brasiliensis* antigens. Future experiments should confirm this immunomodulating action of Cy in paracoccidioidomycosis and demonstrate its role as a potentiator of host defense against an infectious challenge in vaccination schedules.

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REFERENCES

Ahmed R, Hombal SM 1984. Cyclophosphamide (cytoxan). *J Am Acad Dermatol 11:* 1115.

Askenase PW, Hayden BJ, Gershon RK 1975. Augmentation of delayed type hypersensitivity by doses of cyclophosphamide which do not affect antibody responses. *J Exp Med 141:* 697-702.

Bacchi MM, Franco M 1985. Experimental paracoccidioidomycosis in the mouse. III. Histopathological and immunological findings after intravenous infection in the presence or absence of previous immunization. *Rev Soc Bras Med Trop 18:* 101-108.

Berd D, Mastrangelo MJ 1988. Effect of low dose cyclophosphamide on the immune system of cancer patients: depletion of CD44+. 2H4+ suppressor inducer T-cells. *Cancer Res 48:* 1671-1675.

Biagioni LMV, Sadatsune T, Franco MF, Mattos MCFI 1986. A comparative study of the immunoantigenicity of eight *Paracoccidioides brasiliensis* isolates. *Rev Inst Med Trop São Paulo 28:* 281-286.

Defaveri J, Coelho KIR, Rezkallah-Iwasso MT, Franco M 1989a. Hypersensitivity pneumonitis to *Paracoccidioides brasiliensis* antigens in mice. *J Med Vet Mycol 27:* 93-104.

Defaveri J, Martin LC, Franco M 1989b. Histological and ultrastructural study of the inflammation evoked by *Paracoccidioides brasiliensis* antigen in previously immunized mice. *Mycopathologia 105:* 53-58.

Fava Netto C 1961. Contribuição para o estudo imunológico da blatomicose de Lutz. *Rev Inst Adolfo Lutz 21:* 99-194.

Franco, M, Peraçoli MT, Soares A, Montenegro MR, Mendes RP, Meira DA 1993. Host-parasite relationship in paracoccidioidomycosis. *Curr Top Med Mycol 5:* 115-149.

Kamegasawa A, Rezkallah-Iwasso MT, Viero R, Franco M 1992. Evaluation of different immunization protocols with *Paracoccidioides brasiliensis* antigens in guinea pigs. *Rev Inst Med Trop São Paulo 34:* 243-249.

Kamegasawa A, Viero RM, Rezkallah-Iwasso MT, Franco M 1988. Protective effect of prior immunization on ocular paracoccidioidomycosis in guinea pigs. *Mycopathologia 103:* 35-42.

Morikawa Y, Kuribayashi K, Saito K 1989. Immunoregulatory effect of antibody on delayed-type hypersensitivity in mice. *Int Arch Allergy Appl Immunol 90:* 130-136.

Moscardi M, Franco MF 1981. Paracoccidioidomicose experimental do camundongo. II. Infecção intraperitoneal após sensibilização prévia. *Rev Inst Med Trop São Paulo 23:* 185-211.

Nakamura RM, Goto Y, Kitamura K, Tokunaga T 1989. Two types of suppressor T cells that inhibit delayed-type hypersensitivity to *Mycobacterium intracellulare* in mice. *Infect Immun 57:* 779-784.

Okyyama H, Matsunaga T, Kobayashi S, Hashimoto Y, Kawaguchi Y, Yamamoto K 1989. Effect of cyclophosphamide pretreatment on defective delayed-type hypersensitivity in autoimmune - prone MRL mice. *Int Arch Allergy Appl Immunol 88:* 394-401.

Oratz R, Dugan M, Roses OF, Harnis MN, Speyer JL, Hochster H, Weissman J, Henn M, Bystryn J 1991. Lack of effect of cyclophosphamide on the immunogenicity of a melanoma antigen vaccine. *Cancer Res 51:* 3646-3647.

Prestes FRC, Rezkallah-Iwasso MT, Franco M 1983. Contribuição ao estudo da imunidade celular ao *Paracoccidioides brasiliensis* no camundongo. *Rev Ciênc bioméd São Paulo 4: 77-84.*

Rifkind D, Frey JA, Peterson EA, Dinowitz M 1976. Delayed hypersensitivity to fungal antigens in mice. I. Use of the skin and footpad swelling tests as assays of active and passive sensitization. *J Inf Dis 133:* 50-56.

Zar JH 1984. *Biostatistical analysis,* Prentice-Hall. Inc., New Jersey, p. 50-58.