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CYCLOPHOSPHAMIDE EFFECT ON PARACOCCIDIOIDOMYCOSIS IN THE RAT

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SUMMARY

Paracoccidioidomycosis is an endemic fungal disease widely distributed throughout Latin America. The potent immunosuppressor cyclophosphamide (CY) has been used to modulate host immune response to *Paracoccidioides brasiliensis* in an experimental model. Inbred male Buffalo/Sim rats weighing 250-300 g were inoculated with 5 x 10^6 *P. brasiliensis* cells of the yeast phase form by intracardiac route. One group of animals was treated with 20 mg/kg body weight at days +4, +5, +6, +7, +11 and +12 post-infection (pi.), while a control group was infected alone. No mortality was recorded in either group. Treated rats presented: a) a decrease in granuloma size, which contained less fungal cells; b) a lack of specific antibodies up to 35 days pi., and c) a significant increase in the footpad swelling test (DTH) against paracoccidioidin. Splenic cell transfer from CY-treated *P. brasiliensis*-infected donors to recipients infected alone led to a significant increase in DTH response in the latter versus untreated infected controls. Likewise, in treated infected recipients transferred with untreated infected donor spleen cells, footpad swelling proved greater than in controls. Thus, it would seem that each successive suppressor T lymphocyte subset belonging to the respective cascade may be sensitive to repeated CY doses administered up to 12 days pi.. Alternatively, such CY schedule may induce the appearance of a T cell population capable of amplifying DTH response.

KEYWORDS: Cyclophosphamide; Delayed type hypersensitivity; Paracoccidioidomycosis; Splenocyte transfer

INTRODUCTION

Paracoccidioidomycosis is a fungal disease caused by a thermally dimorphic fungus *Paracoccidioides brasiliensis*. Humans most likely acquire the infection by inhaling airborne propagules of saprophytic mycelial phase, which change into the yeast phase in host tissue [13, 24].

Diverse clinical forms have been described: asymptomatic infection; self-limited primary respiratory disease; chronic progressive pulmonary, acute disseminated and chronic disseminated diseases [4, 10].

Paracoccidioidomycosis is endemic from Mexico (20° N latitude) down to Argentina (35° S), with the greatest prevalence in Brazil and Venezuela, followed by Colombia and Argentina [22].

In most mycotic diseases, it has been shown both in man and in experimental animals that cell-mediated immunity plays a leading role in mounting the host's defence, particularly by means of T lymphocytes [3, 14, 27].

Patients with the disseminated form of the disease

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present depressed delayed type hypersensitivity (DTH) response, as measured by the routine skin reaction to paracoccidioidin, in the presence of raised anti-*Paracoccidioides* antibody levels [17, 19]. However, in the case of focal lesions, DTH response proves positive not only for the specific antigen, but also for several T cell mitogens [15].

In the rat model infected with *P. brasiliensis* by the intracardiac route, pulmonary granulomas are not dissimilar to those observed in human patients, though rarely leading to death.

In a wide diversity of experimental animals, cyclophosphamide (CY) has proven a valuable tool to modulate host immune response to numerous infections [14, 20, 21, 26]. According to the treatment schedule with this immunosuppressor, immunological mechanisms may be enhanced or inhibited due to the drug's alkylating properties acting on rapidly-dividing cells [6, 23].

The aim of our present study was to evaluate CY effect on the course of disease in *P. brasiliensis*-inoculated rats, including: a) histology of lung lesions, b) serum antibody titers, c) DTH determination by footpad swelling, d) rat survival and e) transfer of spleen cells from CY-treated infected donors to infected recipients and viceversa.

MATERIAL AND METHODS

Animals: Inbred Buffalo/Sim rats raised at the bioterium belonging to the Department of Microbiology were employed. For each experimental point, 3-4 month-old male animals weighing 250-300 g were used.

Microorganisms: *P. brasiliensis* Uterus strain from the Center of Mycology, isolated from a uterine cervix biopsy in 1989, was maintained in the yeast phase by subculturing in 2% dextrose, broth agar every 10 days at 37°C. *Coccidioides immitis,* Acosta strain from Mycology Center Culture Collection, was maintained in Sabouraud's honey-agar medium at 28°C.

Inoculation procedure: A total of 10^7 yeast *P. brasiliensis* cells/ml were suspended in physiological solution after 72 h incubation. Viability control was carried out with acrydine orange. Following ether anesthesia, each rat received 0.5 ml of the suspension by the intracardiac route.

Serology: Agar gel immunodiffusion assay was performed with Difco Noble Agar and Polyethyleneglycol 6000, as described elsewhere [18].

The *P. brasiliensis* antigen was the cytoplasmatic supernatant obtained according to the technique of NEGRONI et al., [17] employing a concentration of 100 mg lyophilized antigen per ml of destilled water.

Footpad swelling test: Skin tests were carried out by inoculating 0.1 ml of paracoccidioidin, prepared as described elsewhere [17], containing 2 mg/ml of protein, in the hind footpad, and the same volume of sterile saline in the contralateral footpad.

Thickness was measured 24 h later by means of an Oditest precision caliber and percentage swelling calculated as below:

$$\% \text{ Swelling} = \frac{\text{Thick. inoc. footpad - Thick. control footpad}}{\text{Thick. control footpad}} \times 100$$

Histology: Histopathological studies were carried out on lung section, fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosine. At 50 x magnification a semiquantitative technique was employed to count blindly: 1) the number of follicullar epithelioid granulomas in each microscopic field; 2) the number of mini granulomas made up by small clusters of epithelioid cells in each microscopic field; and 3) the number of *P. brasiliensis* with buds (blastoconidiae) in each granuloma. Collapsed fungal cells those lacking buds or greatly altered in shape, were disregarded.

Immunosuppression: Cyclophosphamide (CY) (Endoxan Asta, Labinca, S.A.), was dissolved in sterile destilled water and injected by the intraperitoneal route in doses of 20 mg/kg body weight. Animals received 6 doses (total dosage 120 mg/kg) at days +4, +5, +6, +7, +11 and +12 pi.

A control group of *P. brasiliensis*-infected animals received sterile physiological solution by the above schedule.

Spleen cell collection: Spleens were aseptically harvested and disrupted by passing through a fine steel mesh. Red blood cells were removed with 0.85% ammonium trichloride solution for 40 seconds and macrophages by adherence in plastic Petri dishes for 1 h at 37°C in an atmosphere of 95% air and 5% CO_2;

spleen cells were then employed for transfer experiments.

Experimental design: Spleen cell suspension was transferred at day +19 pi. from CY-treated *P. brasiliensis*-infected animals to untreated infected rats, as well as from untreated infected donors to CY-treated infected recipients. In both cases, DTH response was elicited by challenge at day +20 pi. with paracoccidioidin and measured by footpad swelling 24 h later.

In order to confirm DTH specificity for *P. brasiliensis*, a further control group of *C. immitis*-infected rats received spleen cells from CY-treated *P. brasiliensis*-infected animals.

RESULTS

Mortality and *P. brasiliensis* from lung: Mortality was nil for *P. brasiliensis*-infected animals, whether treated or untreated with CY. Lung samples harvested from 15-35 days pi. showed *P. brasiliensis* growth both in mycelial and yeast forms.

Serum antibody (Ab) determination: Ab production became positive in *P. brasiliensis*-infected controls by day 25, but was only detectable in CY-treated infected rats at 35 days pi.

Histopathology: Lungs rather than liver, spleen or kidney were employed for semiquantitative *P. brasiliensis* evaluation because, although the mycosis in our experimental model is initially multiorganic, as from 3-4 weeks after infection spontaneously resolves in all organs except lungs. It was found that both infected untreated control and those infected but also treated with CY followed a similar histopathological course in all organs except lung, where the former group presented widespread alterations (Table 1). As shown in Fig. 1, lung from untreated *P. brasiliensis*-infected rats presented typical epithelioid granulomas laden with fungal yeast cells at 30 days pi. In contrast, at this time tissue from immunosuppressed animals exhibited small isolated granulomas harboring scanty *P. brasiliensis* cells (Fig. 2).

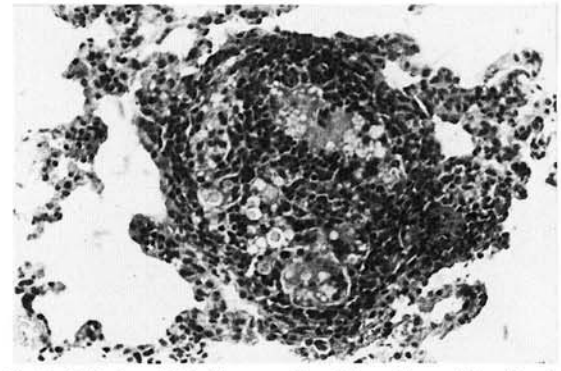

Fig. 1 - Follicular epitheliod lung granuloma laden with yeast phase fungal cells at 21 days post-infection, in a rat inoculated by the intracardiac route with *P. brasiliensis*. H & E 200 x.

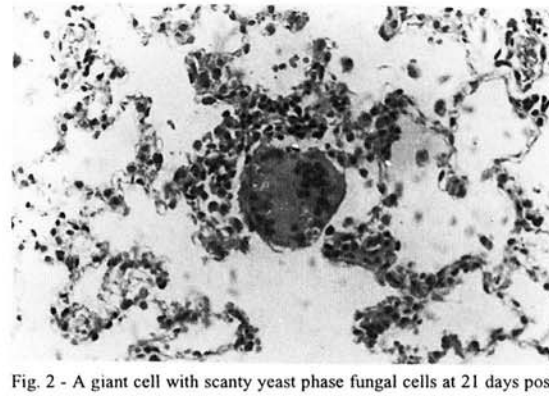

Fig. 2 - A giant cell with scanty yeast phase fungal cells at 21 days post-infection, in a cyclophosphamide-treated rat inoculated by intracardiac route with *P. brasiliensis*. H & E 200 x.

TABLE 1

Semiquantitative parameters for lung evaluation in Buffalo/Sim rats infected with *P. brasiliensis* (Pb) alone (control), versus those also treated with cyclophosphamide (CY)

Animal group	Nº typical granulomas/ Nº microscopic fields Means (± SD)	Nº mini granulomas/ Nº microscopic fields Means (± SD)	Nº Pb cells per granuloma Means (± SD)
Control Pb alone (n= 9)	1/4 (± 2)	0	15 (± 4)
Experimental Pb + CY (n=7)	1/15 (± 3)	1/5 (± 2)	5 (± 2)

Magnification was 50 X throughout. The fraction represents the mean number of granulomas per field. Student's t test disclosed significant differences with $p<0.05$ for all control versus experimental groups.

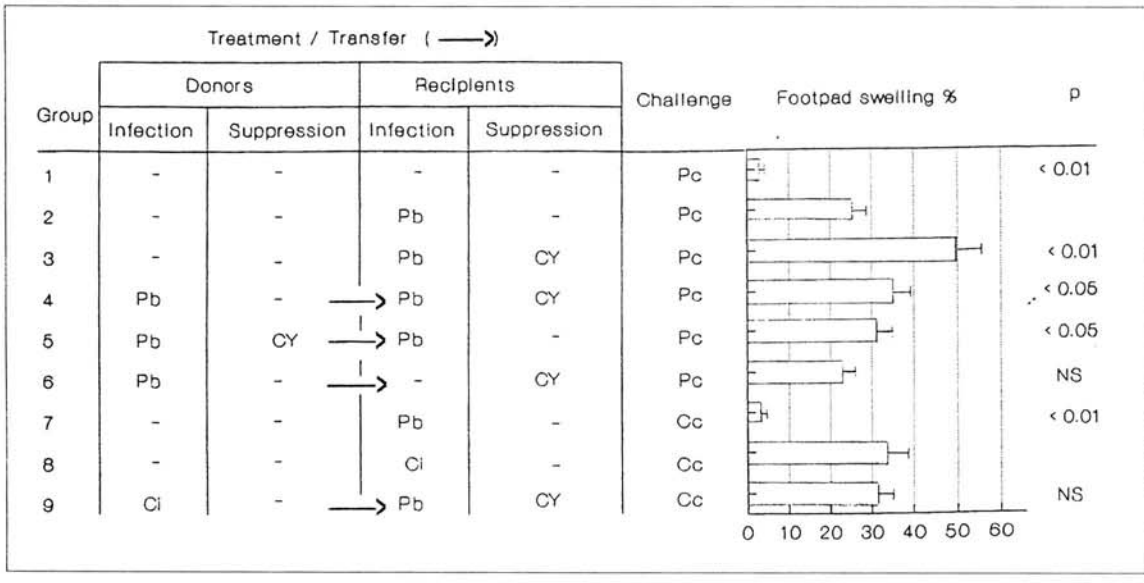

Fig. 3 - DTH response enhancement by adoptive spleen cell transfer performed at day + 19 pi. from untreated or cyclophosphamide-treated donor rats inoculated with sterile physiologic saline, *P. brasiliensis* (5.10^6 cells) or *C. immitis* (5.10^6 cells) by the intracardiac route to untreated or cyclophosphamide-treated recipients inoculated with either *P. brasiliensis* or *C. immitis*.
All recipients received 10^7 spleen cells by endovenous route and were challenged at day + 20 pi., while footpad thickness was measured 24 h later. Footpad swelling: bars indicate means ± SD of two experiments with 7 rats each. PC, Paracoccidioidin; Pb, *P. brasiliensis*; Cc, coccidioidin; Ci, *C. immitis*; Ns, not significant; Group 2 and 8 served as control.

Delayed type hypersensitivity (DTH) response: Footpad swelling test measurements were made 24 h after paracoccidioidin inoculation.

DTH response in CY-treated *P. brasiliensis*-infected rats (Group 3 in Fig. 3) proved significantly greater ($p<0.01$) than in untreated infected animals (Group 2). Besides, untreated infected recipients transferred with treated infected donor spleen cells (Group 5) presented significantly increased footpad swelling versus untransferred recipients ($p<0.05$) (Group 2).

Likewise, on performing inverse transference, that is, treated infected recipients transferred with untreated infected donor spleen cells (Group 4), footpad swelling proved significantly greater than in Group 2 ($p<0.05$).

As regards specificity, DTH in untreated *P. brasiliensis*-infected rats challenged with coccidioidin (Group 7) failed to differ statistically from that recorded in Group 2 animals, which was also the case with remaining controls (Groups 8 and 9).

It should be pointed out that CY treatment exerted an amplifying effect on DTH response fostering *P. brasiliensis* clearance, in spite of being considered an immunosuppressor.

DISCUSSION

Both in man and animals, cell-mediated immunity (CMI) is responsible for host defence in most systemic mycoses [8].

An immunosuppressor such as cyclophosphamide (CY) is capable of modifying the course of disease, as well as regulating T lymphocyte-mediated response mechanisms, thus shedding light on the nature of host defence. Therefore, the use of CY in the *P. brasiliensis*-infected rat model would seem to provide a valuable tool to elucidate the role of CMI in this fungal infection. According to the route of inoculation and the CY treatment schedule, the immune response may either be enhanced or inhibited [2, 7, 9].

It has been suggested that the regulatory mechanism involves a cell population undergoing rapid division and thus susceptible to the drug's DNA-alkylating properties [1]. Since a single CY dose had failed to modify the course of Junin virus [14] or *C. immitis* infection in rats, [20, 21] here we again resorted to

a fractional CY schedule totalizing 120 mg/kg body weight, known to lack toxicity for these animals [25].

In a previous work, serial CY doses in the Buffalo/Sim strain inoculated with *C. immitis* were found to inhibit CMI, leading to widespread lethal dissemination instead of granuloma formation restricted to the lung [20, 21]. In contrast, CY treatment in rats infected with *P. brasiliensis* by intracardiac route induced the formation of smaller pulmonary granulomas harboring fewer fungi, as shown by light microscopy sections, whereas untreated controls exhibited the typical large granulomas with plentiful fungi.

Furthermore, immunosuppression significantly increased DTH response in *P. brasiliensis*-infected rats, as measured by footpad swelling after paracoccidioidin challenge ($p<0.01$), as well as causing a delay in the appearance of anti-*P. brasiliensis* antibodies, as determined by immunodiffusion. The former finding would explain the remission of overt disease in immunosuppressed animals.

Comparing previous observations in the *C. immitis*-infected rat model, in which CY induces widespread infection, with our present findings in *P. brasiliensis*-inoculated animals, it would seem that the chosen drug does not necessarily affect each antigen-specific immune system in the same way. In support, the immunosuppressor cyclosporin A behaves likewise both clinically and in experimental models for certain fungal diseases [9].

In the case of *Histoplasma capsulatum*, DEEPE et al. [7] demonstrated immunoenhancement when CY was administered before or promptly after mouse infection with the fungal yeast phase. These authors contended that suppressor T cell precursors were more susceptible to CY effect than antigen-activated suppressor T cells. JIMENEZ-FINKEL & MURPHY [11, 12] showed that mouse inoculation with inactivated *P. brasiliensis* or with soluble *P. brasiliensis* culture antigen leads to a suppressor T cell population which, transferred to *P. brasiliensis*-antigen immunized recipients, inhibits DTH response to the antigen, as measured by footpad swelling both in the afferent and efferent limbs. In contrast, here spleen cells from *P. brasiliensis*-infected CY-treated donor rats transferred to infected untreated recipients 24 h before intradermal footpad paracoccidioidin challenge induced a significant increase in local swelling ($p<0.01$), thus disclosing an enhancing effect on the efferent expression limb of DTH response.

To sum up, two hypotheses are available for the *P. brasiliensis*-CY rat model. On one hand, each successive suppressor T lymphocyte subset belonging to the respective cascade, may be sensitive to repeated CY doses administered up to 12 days post-infection, causing abrogation of such suppressor cells and stimulating the DTH population. On the other hand, such CY schedule may induce the appearance of a T cell population capable of amplifying DTH response [5, 16].

RESUMEN

Efecto de la ciclofosfamida en ratas con paracoccidioidomicosis

La paracoccidioidomicosis es una enfermedad endémica fúngica ampliamente distribuida en Latino-América. La ciclofosfamida ha sido usada como potente inmunosupresor para modular la respuesta inmune, en un modelo experimental infectado con *Paracoccidioides brasiliensis*. Ratas machos Buffalo/Sim endocriadas de 250-300 gr. de peso, fueron inoculadas por via intracardiaca con 5.10^6 células de *P. brasiliensis* en fase levaduriforme. Un grupo de animales fue tratado con 20 mg/kg de peso de ciclofosfamida en los dias +4, +5, +6, +7, +11 y +12 p. i. mientras que un grupo control fue infectado solaménte. No se registró mortalidad en ninguno de los grupos. Las ratas tratadas presentaron: a) una disminución en el tamaño del granuloma, el cual contenia menor cantidad de células fúngicas; b) ausencia de anticuerpos especificos hasta los 35 dias p.i. y c) un aumento significativo en el test de hinchazón de la pata (DTH) contra la paracoccidioidina. Células esplénicas provenientes de dadores infectados con *P. brasiliensis* fueron transferidas a receptores infectados solamente, lo que produjo un aumento significativo de la DTH en estos últimos con respecto a los controles. Asi mismo, células esplénicas de dadores infectados solamente fueron transferidas a receptores infectados y tratados mostrando tambien un aumento en la DTH con respecto a los controles.

Cada uno de los linfocitos T supresores pertenecientes a la cascada de células T parecerian ser sensibles a dosis sucesivas de ciclofosfamida hasta los 12 dias p.i.. Por otra parte, el esquema de tratamiento con ciclofosfamida podria inducir la aparición de una población de células T capaces de amplificar la respuesta DTH.

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