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CYCLOPHOSPHAMIDE EFFECT ON COCCIDIOIDOMYCOSIS IN THE RAT

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SUMMARY

Coccidioidomycosis is a systemic mycosis, endemic in arid areas of the American continent.

The rat was employed as an experimental host, since it had been shown to reproduce human lesions and present a chronic course of disease with granulomas mainly restricted to lungs.

Given the influence of immunosuppressive therapy on the clinical course of human coccidioidomycosis, we studied the effect of cyclophosphamide (CY) in the experimental rat model. Accordingly, animals were inoculated with 400 **Coccidioides immitis** arthroconidia of the Acosta strain, by intracardiacal route. As single CY doses failed to alter the course of disease, three schedules were used: A) 4 daily doses of 20 mg/kg each, prior to **C. immitis** inoculation; B) 4 similar daily doses after infection; and C); 6 doses of 20 mg/kg each, given from day +1 to +4, then on days +8 and +9, post infection (pi), taking day 0 as the time of fungal inoculation. The first two schedules inhibited antibody formation up to day 28 pi, without modifying cellular response to coccidioidin as measured by foodpad swelling. Initially, there was greater fungal spread than in controls receiving **C. immitis** alone, which proved self-limiting in the latter. In contrast, schedule C led to 55% mortality, with both humoral and cellular response abrogation, accompanied by extensive **C. immitis** dissemination. Histology disclosed significant alterations, such as the persistence of primary infection sporangia, corresponding to the acute stage of coccidioidomycosis in the absence of granuloma development.

Therefore, the observed depression in cellular immunity seems responsible for the lack of inflammatory reaction capable of restricting sporangia proliferation in tissues which, in turn, enhances pathogen spread and mortality rate.

KEY WORDS: Cyclophosphamide; Coccidioidomycosis; Immunosuppression.

INTRODUCTION

Coccidioidomycosis is a systemic mycosis which is endemic to arid areas in the American continent[6]. It is acquired by inhalation of contaminated soil, affecting both man and several animal species[2, 8, 15].

Asymptomatic or subclinical infection presents a high incidence among endemic area dwellers but is mostly self-limited, although many individuals exhibit latent coccidioidomycosis foci in lung parenchyma and hilar lymph nodes[8].

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[10, 17]. Patent cell-mediated immune response has been shown essential to circumscribe infection[21, 22].

Rodents are particularly susceptible both to natural and experimental infection[16]. In fact, we have already shown[16] that rats regularly develop widespread coccidioidomycotic lesions in lungs, liver, spleen and thymus, following intracardiac (ic) inoculation, with the appearance of granulomas limited to the lungs in the chronic stage.

The multifunctional alkylating agent cyclophosphamide (CY) has been extensively employed in experimental trials to modulate the host's immune response and often exacerbates latent infections.

Since immunosuppresive treatment is known to modify the clinical course of human coccidioidomycosis[7, 12, 23], here we attempted to determine the effect of suitable doses and adequate schedules of CY in the **C. immitis**-rat model.

Both humoral and cellular immune response, histopathological alterations and organ colony-forming unit (CFU) count served to evaluate the degree of immunosuppression.

MATERIAL AND METHODS

Animals

Buffalo/Sim inbred adult male rats raised in our bioterium were used. For each separate experiment, 3-4 month-old animals weighing 200-250 g were employed.

Microorganism

Coccidioides immitis (Acosta strain), was originally obtained from a patient. Cultures were maintained in Sabouraud's honey agar medium at 28°C and repeatedly passaged in rats at the Centre of Mycology Faculty of Medicine, Buenos Aires University.

Inoculation procedure

Arthroconidia were harvested in saline from well-sporulated cultures and counted in a hemocytometer. Each rat received 400 arthrospores by intracardiacal route (ic) suspended in 0.1 ml of isotonic saline solution.

Tests performed

Animals were killed at days 7, 14, 21 and 28 post-infection (pi). At least 3 rats were used for each experimental point to determine: 1) viable **C. immitis** CFU count in tissue, 2) serum antibody formation, 3) footpad swelling increase and 4) organ histopathological alterations.

1 — Viable *C. immitis* CFU count in tissue

Lungs were removed aseptically and their weight determined. Organs were separately placed in glass homogenizers containing a suitable volume of sterile saline. Each homogenized organ was serially diluted in saline and 0.1 ml seeded on a Sabouraud's honey Petri dish. After 3-4 days incubation at 37°C, CFUs were counted and their number expressed as $\log_{10}$ CFU per gram of organ. Two weeks later these colonies were microscopically controlled searching for the presence of typical arthroconidia.

2 — Serology

Assays were performed by agar gel immunodiffusion and by Counter-Immuno-Electrophoresis (CIE) in gel medium supplemented with Polyethyleneglycol 6000, as described elsewhere[24]. Briefly, gel for immunodiffusion was prepared with 1% Difco Noble Agar in phosphate buffer pH 7.2 plus 1% Polyethyleneglycol 6000. For CIE, 0.9% agarose in veronal buffer pH 8.2, supplemented as above was employed.

3 — Footpad Swelling test

Skin tests were carried out by inoculating 0.1 ml of coccidioidin, prepared as described elsewhere[20], 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.

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

4 — Histology

Lungs, liver and spleen were fixed in 10% formalaldehyde and paraffin embedded. Parafin sections were stained with hematoxylin and eosin (H & E).

CY (Endoxan Asta, Labinca SA) schedules

The drug was dissolved in sterile distilled water and injected by intraperitoneal route (ip) in daily serial doses of 20 mg/kg body weight for each inoculum. Three administration schedules were employed:

Schedule A: 4 doses (total dosage 80 mg/kg) at days −8, −6, −4 and −1, taking 0 as **C. immitis** infection day;

Schedule B: 4 doses (total dosage 80 mg/kg) at days 1, 2, 3 and 4 days post-infection (pi);

Schedule C: 6 doses (total dosage 120 mg/kg) at days 1, 2,3 and 4, then at 8 and 9 days pi. A 3-day (5-7 days pi) resting period was adopted to avoid drug toxicity to the highly CY-sensitive Buffalo rat.

For each animal group receiving one of the above CY schedules, a **C. immitis**-infected group receiving sterile saline alone according to schedules A, B and C served as controls.

The efficacy of CY schedules was tested by contact hypersensitivity. A 5% 2,4-dinitrofluoro benzene (DNFB) dose was given at day 0 (sensitization time) on the rats' shaven flank, triggering the reaction at day 7 on the pinna with 0.3% DNFB. Ear thickness vs contralateral pinna thickness was measured 24 h later. Mean values for schedules A and B were 0.129 ($n = 6$) and 0.177 ($n = 7$) respectively, vs 0.695 ($n = 6$) for a non-suppressed control group ($p < 0.001$ in every case).

All CY schedules patently abrogated DNFB contact hypersensitivity and were assayed to determine which one, if any, was capable of significantly modifying the course of disease, as shown by mortality, lung CFU count, footpad swelling test, antibody formation and histological alterations.

RESULTS

Mortality percentage

No mortality was recorded for animals infected with 400 **C. immitis** arthrospores, nor for those receiving CY schedules A or B. Rats treated by schedule C (120 mg CY/kg) were found to exhibit 55% mortality from 14 to 17 days pi.

C. immitis CFU count

Lung samples harvested at various times pi showed a significant increase in CFU count among CY-treated animals.

For schedules A and B, values peaked at day 14 pi, later approaching levels for non-suppressed rats (Fig. 1).

In contrast, CY schedule C showed the greatest colony count up to 28 days pi, together with gross and microscopical evidence of **C. immitis** proliferation in spleen, liver and kidney.

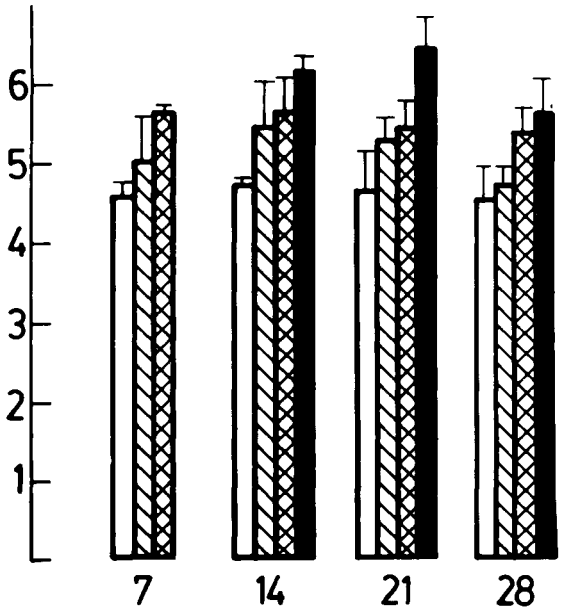


Fig. 1 — CFU lung count in **Coccidioides immitis** infected adult male rats treated with 3 different CY schedules (see Material and Methods) or left untreated: a) at 7 days pi; b) at 14 days pi; c) at 21 days pi; and d) at 28 days pi. White bars, results from infected untreated control rats; striped bars, schedule A; doubly striped bars, schedule B; and black bars, schedule C.
Values in abscissae represent days pi; in ordinates, $\log_{10}$ (**C. immitis** CFU/g lung).

Footpad swelling test

Footpad measurements were made 24 h after coccidioidin footpad inoculation. Values at day 7 pi for all 3 schedules were disregarded due to minimal swelling even in controls. There were no significant differences between animals receiving schedules A or B and infected untreated controls. However, in rats receiving schedule C, swelling barely reached 5% at 14 days pi with a slight increase at days 21 and 28 pi, when infected untreated control levels exceeded 40% (Fig. 2).

Serum antibody (Ab) determination

Ab production became positive in controls by day 21 pi, but in schedules A and B was only detectable at 28 pi. In schedule C, it remained undetectable at all evaluation times.

Histopathology

C. immitis-infected control animals exhibited two types of inflammatory response. Up to 7 days pi, the reaction featured leucocytes with numerous polymorphonuclear cells together with larger sporangia spherules having thin walls and numerous small endospores (primary infectious sporangia)[15]. As from day 14 pi, follicular giant cell granuloma formation became evident, accompanied by a decrease in sporangia number, whose size was smaller and walls thicker and which harboured larger but less numerous endospores (cystic sporangia)[15] (Fig. 3). Lung was more commonly affected though on occasion granulomas without **C. immitis** were observed in liver.

CY treatment by both schedule A and B led to contrasting alterations vs controls, mainly: a) no follicular giant cell granuloma formation was observed up to day 21 pi; b) primary infectious sporangia only became cystic at day 21 pi (Fig. 4); and c) there was greater spread to liver and spleen.

Findings proved much more severe following schedule C (Fig. 5a & 5b): a) instead of granulomas, hemorrhagic suppurative necrotic areas were widespread in lung; b) primary infectious sporangia persisted throughout; and c) specific lesions with typical sporangia were detected in liver and spleen and often in kidney.

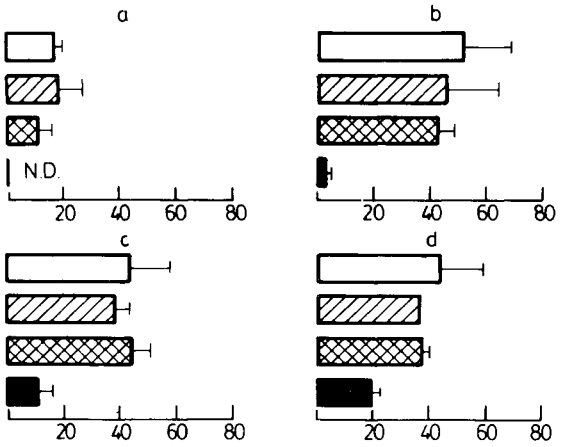


Fig. 2 — Footpad swelling reaction in **Coccidioides immitis** infected adult male rats treated as in Fig. 1.
Values in abscissae represent percentage swelling vs. contralateral footpad.

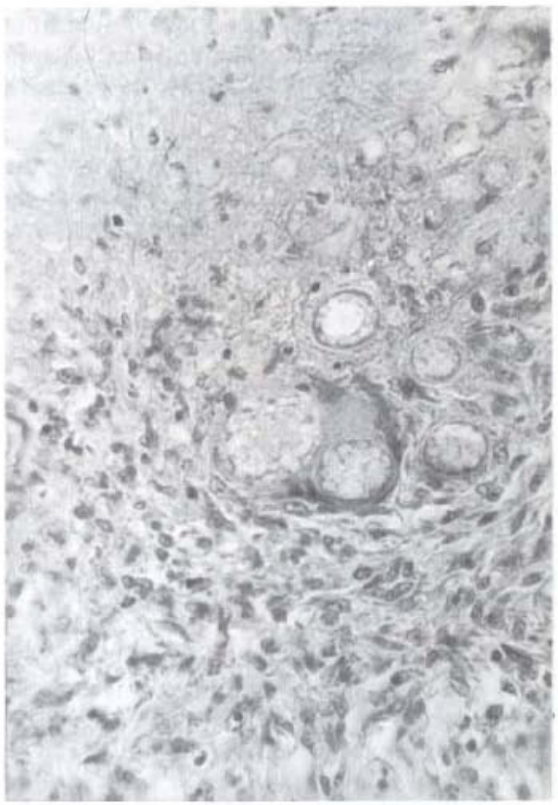


Fig. 3 — Giant granuloma with cystic **C. immitis** sporangia in CY untreated rat lung. H & E 250 X.

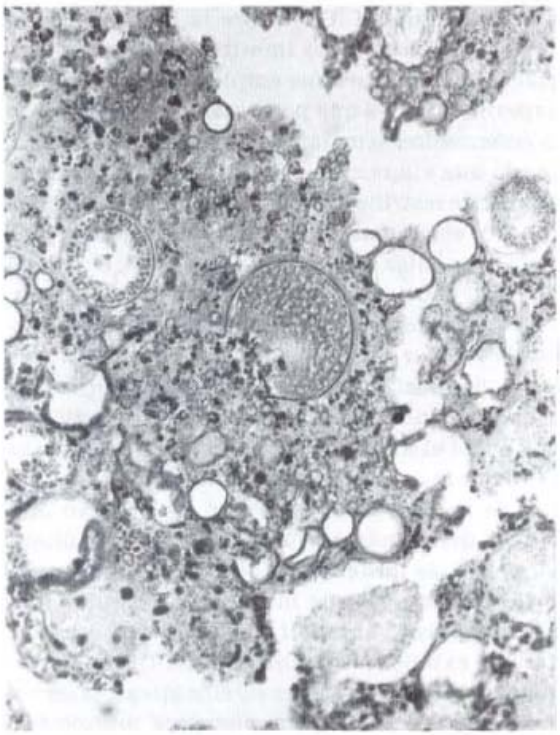


Fig. 4 — Lung inflammation showing primary infectious C. **immitis** sporangi and a few cystic sporangia, but without granulomas, in a CY-treated rat (Schedule A or B, see Materials & Methods). H & E 250 X.

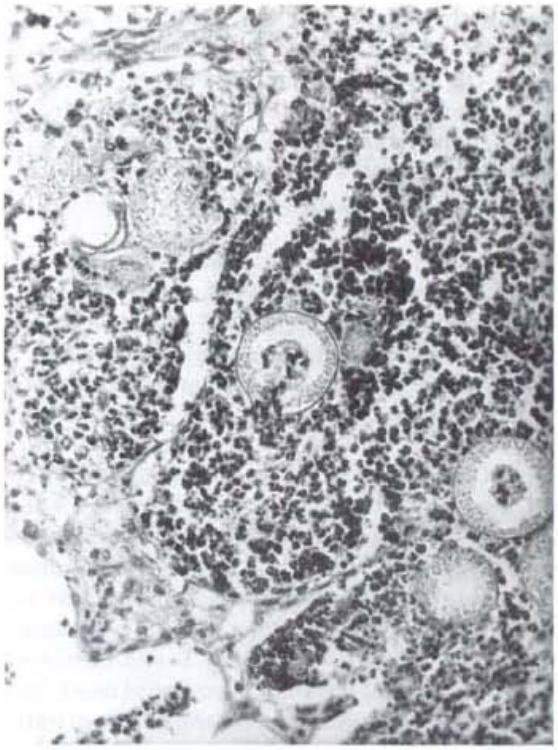
DISCUSSION

Human coccidioidomycosis infection is mainly asymptomatic or subclinical and reaches a high incidence among inhabitants of endemic areas.

Therefore, a satisfactory experimental model is essential to explain its infective mechanism. Previous work[16] has shown that the ic inoculated rat faithfully reproduces histological lesions found in man, with the development of widespread infection coursing chronically with granulomas mostly restricted to lung.

The exacerbation of infectious diseases due to immunosuppression, whether by drugs or radiation, has been amply studied[1, 3, 11]. Thus, irradiated mice[4] become more susceptible to **C. immitis** infection due to an impairment of cellular or T-dependent immunity.

Here we employed CY as immunosuppressive drug and found that a single dose failed to



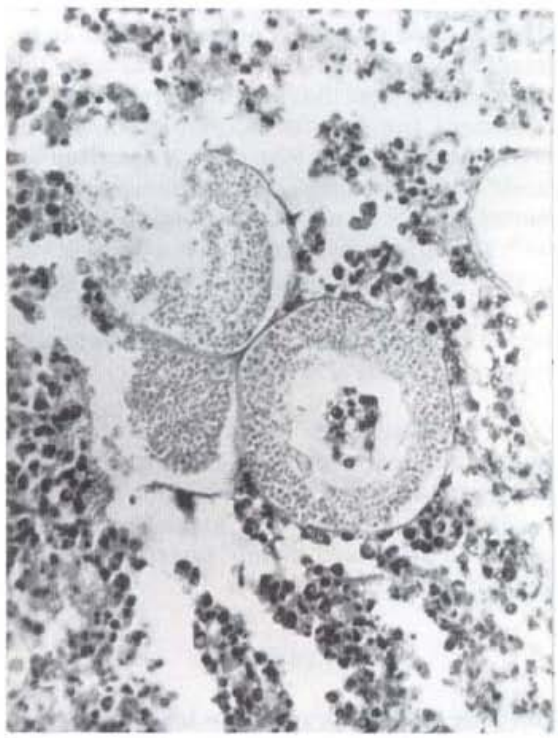


Fig. 5 — a) Necrotic suppurative hemorrhagic lung areas with primary infectious C. **immitis** sporangia in a CY-treated rat (Schedule C, see Materials & Methods) H & E 250 X. b) Same as above, at higher magnification.

modify the course of disease (data not shown). Since previous work with this host had required serial CY doses to alter the development of a viral infection[13], we applied similar schedules to the **C. immitis**-rat model, after confirming that such treatment was capable of abrogating DNFB contact hypersensitivity.

There was no mortality among **C. immitis**-inoculated rats receiving CY doses up to 80 mg/kg. In contrast, 120 mg/kg CY in 6 doses with a 3-day resting period to avoid drug toxicity[19], induced overt illness and 55% mortality at day 14 pi.

As regards humoral immunity, an 80 mg/kg CY dose proved sufficient to abrogate response up to 21 days pi, since antibodies only became detectable at 28 days pi. On raising the dose to 120 mg/kg, inhibition was maintained at all times tested. These findings were not unexpected as CY is known to impair B cell function, without affecting the clinical course of most fungal diseases. Besides, the protective role of antibodies against systemic mycosis is little known. Although all three schedules led to wider fungal dissemination in several organs, as well as increased colony count in lung, it was only the 120 mg/kg CY dose that inhibited granuloma development and specific footpad swelling. Our overall findings agree with reports on histoplasmosis[9] and candidiasis[5, 14] in immunosuppressed murine models as well as on **C. immitis** in the rat[18]. The appearance of granulomas closely correlated with T cell mediated immunity: thus, their scarcity in rats receiving CY during the second week pi, seems to correlate well with the impairment of DTH reactions.

In conclusion, the recorded lack of inflammatory response capable of circumscribing tissue sporange dissemination leading to subsequent fungal spread and higher mortality should be attributed mainly to cellular immunity inhibition.

RESUMEN

Efecto de la ciclofosfamida en la infección por Coccidioides immitis **en la rata**

El propósito de este trabajo fue estudiar el efecto de la inmunosupresión causada por la droga ciclofosfamida (CY) sobre la infección de la rata con **Coccidioides immitis** por vía intracardíaca. Este huésped fue empleado como modelo experimental, ya que presenta una evolución de la enfermedad semejante a la del hombre, alcanzando una etapa crónica con granulomas principalmente restringidos a los pulmones. Se utilizaron tres esquemas de CY: A) 4 dosis de 20 mg/kg cada una, antes de la inoculación de Ci; B) 4 dosis de igual cantidad de CY, luego de la infección; y C) 6 dosis de 20 mg/kg cada una, administradas desde el día +1 hasta +4 y continuando los días +8 y +9 post-infección (pi). Los dos primeros esquemas inhibieron la formación de anticuerpos hasta el día 28 pi, sin modificar la respuesta celular a la coccidioidina, medida como hinchazón de la almohadilla plantar. Se observó una mayor diseminación fúngica inicial, autolimitándose más tarde. Por el contrário, el esquema C provocó un 55% de mortalidad, disminución de la respuesta humoral y celular, acompañada de una extensa diseminación del Ci. La histología mostró alteraciones significativas, tales como persistencia de esporangios de primoinfección, correspondientes al estadío agudo de la coccidioidomicosis, con ausencia de desarrollo de granulomas. Por lo tanto, la depresión observada en la respuesta celular debido al tratamiento con CY sería la responsable de la ausencia de la reacción inflamatoria capaz de restringir la proliferación de esporangios en los tejidos, lo cual a su vez favorece la diseminación del microorganismo patógeno y el aumento de mortalidad.

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