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EXPERIMENTAL COCCIDIOIDOMYCOSIS IN THE IMMUNOSUPPRESSED RAT.

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SUMMARY

C. immitis inoculated rats are known to develop infection restricted to lung whereas cyclophosphamide (CY) treatment leads to widespread dissemination with considerable mortality. In this study, an attempt was made to elucidate the mechanisms involved in such behaviour. With this aim, spleen cells were transferred from infected CY-treated to infected untreated rats, achieving significant specific inhibition in footpad swelling to coccidioidin in recipients, attributable to a suppressor T cell subpopulation induced by greater fungal antigen concentration arising from widespread *C. immitis* dissemination in immunosuppressed animals. NK activity proved similar regardless of CY treatment. Lastly, chronically infected rats presented increased colony forming units count after several weekly doses of CY, as happens in immunosuppressed patients harbouring a previous infection.

KEY WORDS: Rat; Coccidioidomycosis; Cyclophosphamide; Immunosuppression; T suppressor cells; Reactivation.

INTRODUCTION

Coccidioidomycosis is a systemic mycotic disease, endemic in desertic areas of the American continent[15].

Over the last decade, fungal infections have increased due to immunosuppressive therapy for organ transplant and because of virus-induced immunodeficiency[13].

Among non-human mammals, rodents are particularly susceptible to both natural and experimental infection.

Previous work on rats inoculated by intracardiac (ic) route has demonstrated infection mainly located by lungs, as happens in man[12]. Immunosuppressive treatment in this model leads to fungal dissemination in several organs, high mortality, absence of granulomas and depressed cellular response to coccidioidine[17].

In the present study an attempt was made to elucidate the mechanisms involved in the behaviour of infected immunosuppressed animals, by measuring suppressor lymphocyte and NK cell activities. Besides, an effort was made to reactivate chronic *C. immitis* infection.

MATERIALS AND METHODS

Animals. Buffalo/Sim inbred adult male rats raised in our bioterium and weighing 250-300g were used.

Microorganisms. *Coccidioides immitis* (Acosta strain) was originally obtained from a patient. Cultures were maintained as previously described[17]. Each rat received 400 arthrospores in 0.1ml of isotonic saline solution by ic route. As specificity control, 0.5ml of *Paracoccidioides brasiliensis* having an optical density of 0.3, was also inoculated ic.

Variable *C. immitis* Colony Forming Units (CFU) count in tissue. It was performed as described elsewere[17].

Delayed-type hypersensitivity elicitation. Skin tests were carried out by inoculating 0.1ml of coccidioidin prepared as already described[8] in

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the hind footpad, and the same volume of sterile saline in the contralateral footpad. As specificity control, 0.1ml of paracoccidioidin from disrupted yeast-shaped cell supernatant, was injected into the hind footpad. Thickness was measured by the standard technique[17].

Immunosuppression. Cyclophosphamide (CY) (Endoxan Asta, Labinca, S.A.) was disolved in sterile distilled water and injected by intraperitoneal route in doses of 20 mg/kg body weight. Animals received 6 doses (total dosage 120 mg/kg) at days 1, 2, 3, 4, 8 and 9 post infection (pi) as described elsewere[17].

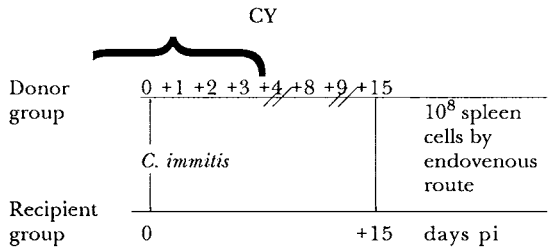
Spleen cell collection. Spleens were aseptically harvested and disrupted by passing through a fine steel mesh. Red blood cells were removed with ammonium trichloride solution and macrophages by adherence in plastic petri dishes; spleen cells were then employed for transfer experiments, as well as for NK determination.

NK activity determination. It was performed as previously described[9]. Briefly, the YAC-1 cell line was used as target: 5 x 10^6 cells/ml were incubated with 100 µCi of ^{51}Cr at 37°C for 1 h. After 4 washings, 10^4 cells in 50µl medium aliquots were seeded in 96 well plates and 10^6 spleen cells in 50µl added. Tests were performed by quadruplicate and 10^4 ^{51}Cr-labelled YAC-1 cell aliquiots seeded as controls. After 4 h incubation at 37°C in 5% CO_2 plates were spun and the supernatant collected and counts per minute (cpm) quantified by a gamma counter. Total cpm was determined by counting 10^4 ^{51}Cr-labelled YAC-1 cells. Cytotoxicity percentage was calculated according to the formula below:

$$\% \text{ Cytotoxicity} = \frac{\text{released cpm-background cpm}}{\text{total cpm-background cpm}}$$

Experimental design. Spleen cell transfer: Spleen cells were transferred from *C. immitis* – infected CY-immunosuppressed to infected untreated rats. To rule out potential drug effect, a second donor group consisted of uninfected CY-treated rats. Cell transfer was carried out at day 15 pi for both donor and recipient groups, according to the schedule below.

***C. immitis* reactivation**: Chronically infected animals received CY treatment starting at day 60 pi in order to achieve fungal reactivation. Immunosuppressive treatment consisted of weekly 100mg/kg doses during 4 weeks and killed 7 days later by ether overdose.



Statistical studies. The analysis of variance test was employed, regarding $p<0.05$ values as significant.

RESULTS

Spleen cell transfer. When spleen cells were transferred from infected immunosuppressed donors to infected untreated recipients, there was significant inhibition ($p<0.01$) in footpad swelling to coccidioidine in the latter (Table 1). To confirm specific inhibition, the above transference was carried out to recipients inoculated with *P. brasiliensis* instead of *C. immitis:* footpad swelling with paracoccidioidin failed to differ significantly from that observed in non-transferred *P. brasiliensis* infected rats.

Determination of natural killer (NK) activity. NK assay was performed to determine protective activity in the course of the infection, as well as to check whether CY treatment induced modifications in NK activity. There were no significant differences in NK activity in infected CY-treated versus infected untreated rats or in uninfected CY-treated versus naive (control) animals. NK activity was considerably lower in the two uninfected groups (Table 2).

***C. immitis* reactivation.** Immunosuppressive treatment led to an increase in CFU in lung, without spread to other organs (Fig. 1). Other CY schedules (20 mg/kg daily for 2 weeks or two 100 mg/kf dosis one week apart) failed to reactivate *C. immitis* (data not shown).

DISCUSSION

The role of cellular immunity in resistance against systemic mycoses has been pointed out by several authors[1,7].

In our experimental model, widespread *C. immitis* infection was readily achieved by means of immunosuppressive treatment, whose mechanism was the goal of this work.

Prior studies[17] have shown severe alterations in CY-treated rats, including 50% mortal-

TABLE 1
Transference of splenocytes from immunosuppressed infected donors to untreated infected recipients.

Animal group	Infection	CY Treatment	Transference	Challenge	% Footpad swelling (means±SEM)	p value compared to infected group
1	–	–	–	Coccidioidine	2.8±0.9	p<0.01
2	Ci	–	–	Coccidioidine	34.2±7.2	
3	Ci	+	–	Coccidioidine	4.3±2.0	p<0.01
4	Ci	–	–	Paracoccidioidine	3.5±1.7	p<0.01
5	Ci	–	Ci + CY[a]	Coccidioidine	9.1±8.6	p<0.01
6	Ci	–	CY[b]	Coccidioidine	32.1±4.0	NS
7	Pb	–	–	Paracoccidioidine	28.4±6.1	
8	Pb	–	Ci + CY[a]	Paracoccidioidine	31.5±5.4	NS

a. 10^8 splenocytes from treated infected rats according to schedule in Materials and Methods.
b. 10^8 splenocytes from treated uninfected rats.
Footpad swelling percentage was measured 24 h after challenge. Each experimental group consisted of at least 7 animals.
NS: not significant.

TABLE 2
Natural killer (NK) activity in untreated versus CY-treated infected rats.

Group \ Days pi	Percentage of citotoxicity 11	15	17
Naive (Control)	2,03	5,57	1,92
Uninfected CY-treated	4,14	3,1	2,8
Infected untreated	10,03	26,5	15,5
Infected CY-treated	9,0	27,1	11,1

Values are means of 3 samples, each one determined by quadruplicate. (See Materials and Methods).

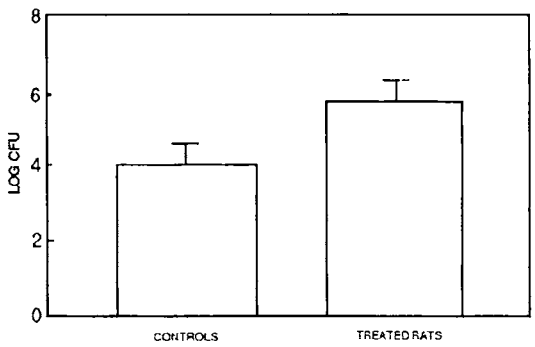


Fig. 1 – *C. immitis* reactivation determined in CFU after CY administration. Controls, *C. immitis*- infected rats killed at day 95 pi by ether overdose; Treated rats, infected animals receiving 4 weekly doses of 100 mg/kg CY, starting at day 60 pi.

ity, antibody synthesis inhibition, abrogated footpad swelling to coccidioidin and greater fungal dissemination, as well as lack of granuloma development, focal necrosis in lung tissue and persistence of active sporanges.

In the present research, the generation of a T cell subpopulation having suppressive activity in CY-treated rats with widespread infection was taken as our working hypothesis. To test this assumption, splenocytes from donor animals receiving the drug during the first two weeks pi were transferred to recipients previously infected with *C. immitis,* which showed patent footpad swelling inhibition to coccidioidin. This findings supports the development of a specific suppressor T lymphocyte subpopulation[2,4,14].

It may be speculated that in immunosuppressed rats with disseminated organ infection, the great concentration of fungal antigen is responsible for the induction of suppressor T cells, as already suggested in the literature[5,6,13].

The protective role of NK cells in mycotic infections has been stressed[9]. Our results show that *C. immitis*-infected animals, whether or not treated with CY, exhibited greater splenic NK activity versus uninfected controls. This finding seems to rule out drug effect on NK activity, in agreement with the work of CLEMONS et al[3].

NK cells play a role in murine resistance against *C. neoformans*[10] during the early stage. However, in mice treated with monoclonal anti NK 1.1. antibody, survival rate remains unaltered.

In vitro research has shown that NK cells are capable of inhibiting the growth of both *C. immitis*[16] and *C. neoformans*[11] endospores, whereas the role of NK cells in vivo is still controversial.

On attempting to reactivate fungal chronic infection in infected rats given CY as from 60 days pi, a considerable increase in CFU was recorded 35 days later. In support, clinical immunosuppression whether in transplant cases or in patients with severe immune system impairment, almost invariably leads to such findings.

RESUMO

Coccidioidomicose experimental em ratos imunossuprimidos.

Ratos adultos inoculados com *C. immitis* desenvolveram infecção circunscrita ao pulmão sem apresentar mortalidade; no entanto, ao serem imunossuprimidos com CY apresentaram disseminação fúngica em vários órgãos, ausência de granulomas e depressão na resposta celular à coccidioidina junto com uma mortalidade de 50%. Tentou-se determinar os mecanismos envolvidos neste comportamento. Para isso foram medidas as atividades dos linfócitos supressores e células NK. Ao serem transferidos esplenócitos de animais infectados e tratados com CY a ratos somente infectados conseguiu-se significativa inibição específica da resposta à coccidioidina. Este efeito seria devido a uma subpopulação de linfócitos T supressores induzida por maior concentração de antígeno nos animais imunossuprimidos. A atividade NK foi semelhante nos ratos infectados independentemente do tratamento com CY. Por outro lado, tentou-se a reativação da infecção crônica com *C. immitis*. Os animais infectados apresentaram maior quantidade de unidades formadoras de colônias nos pulmões depois de várias doses semanais de CY, assim como ocorre em pacientes que apresentam deficiências a nível imunológico.

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