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NATURAL KILLER CELL ACTIVITY IN EXPERIMENTAL PARACOCCIDIOIDOMYCOSIS OF THE SYRIAN HAMSTER

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SUMMARY

The study evaluated the activity of NK cells during the course of experimental infection of hamsters with *Paracoccidioides brasiliensis*. Eigthy hamsters were infected with *P. brasiliensis* by intratesticular route and sacrificed at 24h, 48h, 96h, 1, 2, 4, 8 and 11 weeks of infection and compared to 40 noninfected hamsters employed as controls. These animals were submitted to the study of NK cytotoxic activity by a single-cell assay and humoral immune response by immunodiffusion and ELISA tests. The production of macrophage migration inhibitory factor in the presence of Phyto-hemagglutinin and *P. brasiliensis* antigen and histopathology of the lesions were evaluated at 1, 4, 8 and 11 weeks of infection. The infected animals displayed significantly high levels of NK activity during the four weeks of infection that decreased from the 8th week on when compared to controls. This impairment of NK activity was associated with depression of cell-mediated immune response and with increase in the extension of the histopathologic lesions. There was an inverse correlation between NK cell activity and specific antibody levels. The results suggest that after initial activation, NK cells were unable to control the fungus dissemination. The impairment of NK activity in the late stages of the infection might be related to immunoregulatory disturbances associated with paracoccidioidomycosis.

KEYWORDS: Paracoccidioidomycosis; Hamster; Cell-mediated immunity; NK cell activity; Antibodies.

INTRODUCTION

Paracoccidioidomycosis is a deep mycosis that leads to a depressed cell-mediated immune response of the host [19, 20]. In experimental models the severity of the disease depends, among other factors, on the route of infection [28]. Hamsters infected by the intratesticular route develop disseminated paracoccidioidomycosis with depression of cell-mediated immunity, associated with loosening of granulomata, intense proliferation of fungi and amyloidosis [23]. This model allowed to demonstrate that depression of cellular immune response was secondary to and dependent on the mycosis itself [6, 23].

When the fungus penetrate the host and stablishes itself deep within the tissues, natural and immunological mechanisms of host defense are triggered.

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Natural killer (NK) cells are an important subset of lymphocytes involved in tumour and infection resistance [30]. They are reported to be important effector cells against fungi [3, 14, 15] and other microorganisms [13, 33]. Murine NK cells limit the "in vitro" growth of *P. brasiliensis* [16] suggesting that these cells may play an important defensive role during the early phases of *P. brasiliensis* infection.

Studies of NK cell cytotoxic activity in hamsters demonstrated that these effector cells possess similar functional and morphological properties as NK cells described for other species [29].

Recently we observed that NK cell activity was depressed in patients with active paracoccidioidomycosis [24]. The experimental testicular inoculation of *P. brasiliensis* in hamsters is a good model of the human progressive disease. In this model, the infection is also characterized by depression of cell-mediated immune response [6], allowing the study of the infection and immune response since the early phases of the disease.

The aim of the present work was to study NK cell activity in hamsters infected with *P. brasiliensis* by testicular route. Cytotoxic activity was correlated with cell-mediated and humoral immune responses and with histopathology of lesions in different periods of the infection.

MATERIAL AND METHODS

Animals

Male hamsters (*Mesocricetus auratus*) aged 8-12 weeks were obtained from the animal house of the Botucatu University Campus and were maintained on pelleted food and water *ad libitum*.

Fungi

We used a *P. brasiliensis* isolate, a gift from Dr. Roberto Naiff, INPA, Manaus, obtained from hamster inoculated with extracts of visceral organs of armadillos [21]. The fungi were cultured in Fava Netto's medium [9] at 37°C during 6 days. This *P. brasiliensis* isolate was designed PbT.

Experimental design

Eigthy hamsters were inoculated into the left testis with 2 x 10^5 viable yeast cells of 6 day-old culture of *P. brasiliensis* isolate PbT. Viability test was performed according to CALICH et al. [4]. After infection the animals were followed for 11 weeks to study the survival rate. Seven animals were sacrificed at 24h, 48h, 96h, and 1, 2, 4, 8 and 11 weeks after inoculation when NK cell activity and humoral immune responses were determined. As controls 5 normal noninfected hamsters were sacrificed at the same periods and submitted to the study of NK cell activity. Histopathology of the lesions and cell-mediated immune response were studied in 5 infected animals sacrificed at 1, 4, 8 and 11 weeks after inoculation.

P. brasiliensis *antigen*

P. brasiliensis antigen (PbAg) was prepared by sonication of 7 day-old yeast culture of PbT isolate in Fava Netto's medium [9], centrifuged at 10,000 g at 4°C for 30 min, filtered through a 0.22μm Millipore membrane and submitted to sterility tests. The protein concentration was determined by Lowry's method [17].

Sacrifice and histopathology

For sacrifice each animal was anesthetized with ether and exsanguinated by cardiac puncture. Blood and peritoneal exsudate were collected and tissue samples were taken from the inoculated testis, lungs, liver, kidneys, spleen and lymph nodes. Blood samples were centrifuged and the sera stored at - 20°C. Tissue samples were fixed with formalin, paraffin embedded, cut into 4μm sections and stained with hematoxylin-eosin.

The histological lesions observed were scored semiquantitatively as follows: 1 when they covered less than 1/3 of the histological section, 3 when they covered more than 1/3 but less than the hole section, and 5 when they covered the whole section. An overall "lesion index" was calculated by adding the scores for all animals from each group and time, dividing by the number of organs examined and multiplying by 100.

Macrophage migration inhibition assay

The direct macrophage migration inhibition (MIF) assay was performed according to PERAÇOLI et al. [23] employing peritoneal exsudate cells and a polyethylene capillary tube in liquid medium. In the test, Phytohemagglutinin (PHA - P, Difco Laboratories, Detroit, MI) was used at a concentration of 10μg/ml and PbAg at a concentration of 200μg protein/ml. The results were expressed as "macrophage migration inhibition index" (MMI index) as follows:

$$\text{MMI index} = \left(1 - \frac{\text{migration area with PbAg or PHA}}{\text{migration area without PbAg or PHA}}\right) \times 100$$

Based on the 95th and 5th percentiles of the response of normal control hamsters (n = 25), PbAg MMI indices higher than 20 and PHA indices higher than 55 were considered to be positive.

Humoral immunity

Specific antibodies to *P. brasiliensis* were detected in sera collected from infected hamsters employing immunodiffusion and ELISA tests. The agar-gel immunodiffusion test was employed as described previously [23]. All sera were tested in two-fold dilutions and PbAg was employed at a concentration of 6 mg protein/ml. Results were expressed as the reciprocal of the highest serum dilution resulting in a precipitation band. In the ELISA assay, performed according to MENDES-GIANNINI et al. [18], PbAg concentration was 20 µg/ml and commercial goat-anti-hamster total immunoglobulin peroxidase conjugate (Cappel Organon Teknika Corp. West Chester, PA) was used in all experiments.

Target cells for natural killer cell assay

The human myeloid lymphoma line K562 was maintained as a suspension culture in medium RPMI 1640 (Gibco Laboratories, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 40 µg/ml gentamicin and 10% heat-inactivated fetal calf serum (56°C, 1 h) (complete medium). They were subcultured twice weekly and the day before testing. Prior to the tests, viability was assessed by trypan blue exclusion. Xenogeneic target cells, including K562, have previously been used to demonstrate cytolytic reactivity [26] and these cells were highly susceptible to hamster spleen-cell cytotoxicity [29].

Preparation of effector lymphoid cells

Effector cells were prepared from spleen of normal and infected hamsters. The spleens were aseptically removed and cell suspensions prepared by teasing the material through a stainless-steel sieve in cold RPMI 1640 medium. The cells were washed twice in cold RPMI. After counting the number of lymphocytes in a Neubauer chamber, the cells were resuspened to a concentration of 3×10^6 cells/ml. Plastic adherent cells were depleted by incubating mononuclear cells on a plastic Petri dishes (n. 3003, Falcon, Oxnard, California, USA) at 37°C for 60 min. Non-adherent cells were poured from plates after gentle shaking, washed and resuspended in complete medium. These non-adherent spleen cells were used as effector cells for the NK cytotoxic assay.

Single-cell cytotoxicity assay

Non-adherent spleen cells and K562 cells were used as effector and target cells, respectively. The single-cell cytotoxicity assay on Poly-l-lysine (PLL) coated coverslips was performed as described by PERAÇOLI et al. [24].

The number of target/binding cells (TBC) and cytotoxic effector cells was calculated according to the method of WAHLIN et al. [31]. Spontaneous target cell death was determined on lymphocyte-free control coverslips by scoring the fraction of dead (trypan blue stained) target cells in 300 cells. The percentage of lymphocytes bound to target cells was determined by counting 500 lymphocytes (%TBC). The fraction of conjugates containing dead target cells was determined by scoring 100 conjugates. The fraction of target-cell binding lymphocytes that were cytotoxic (A) was calculated as $A = B - (B \times C)$, where B is the fraction of conjugates containing dead target cells, and C is the fraction of spontaneously dead target cells as determined from control coverslips. The percentage of NK-effector cells present in the lymphocyte sample was calculated as A x % TBC.

Statistical analysis

Data were analysed statistically by Student's t test. Correlation studies among NK cell cytotoxicity and humoral and cell-mediated immunity were done using Spearman's multiple correlation method [35]. Probability of $P < 0.05$ was considered significant.

RESULTS

Histopathology of the lesions

The histological lesions observed at the inoculation site (testis) or at the dissemination sites such as liver, spleen, lungs and lymph nodes presented the same pattern. In the testis they were characterized by confluent granulomas made up of epithelioid cells, fungi and numerous giant cells surrounded by a mononuclear infiltrate (Fig. 1A). This pattern was maintained in all periods studied. Lesions observed at liver, spleens and lungs were made up of isolated epithelioid granulomas dispersed by the parenchima. These granulomas with defined limits, contained giant

cells, and in some of them it was possible to identify fungi (Fig. 1B).

Table 1 shows the survival rate and the extension of the organ lesions during the infection. The number of surviving animals decreased from the 4th week on. Contrariwise extension of the lesions increased gradually from week one to week 11, when the higher indices of extension of the lesions were seen. At this time, in the inoculation site there was an extensive area of necrosis involved by numerous confluent granulomas (Fig. 1C).

TABLE 1

Percentage of surviving animals and extension of the granulomatous lesions presented by hamsters inoculated with *Paracoccidioides brasiliensis* in relation to time of infection evolution.

characteristics	Weeks of infection			
	1	4	8	11
Survival	100[a]	91	75	25
Extension of lesions	79.2[b] ±6.9	133.4 ±20.4	161.2 ±28.9	207.8 ±44.6

[a] percentage of surviving animals per week.

[b] results are expressed as mean values of the lesion index for 5 animals sacrificed per week.

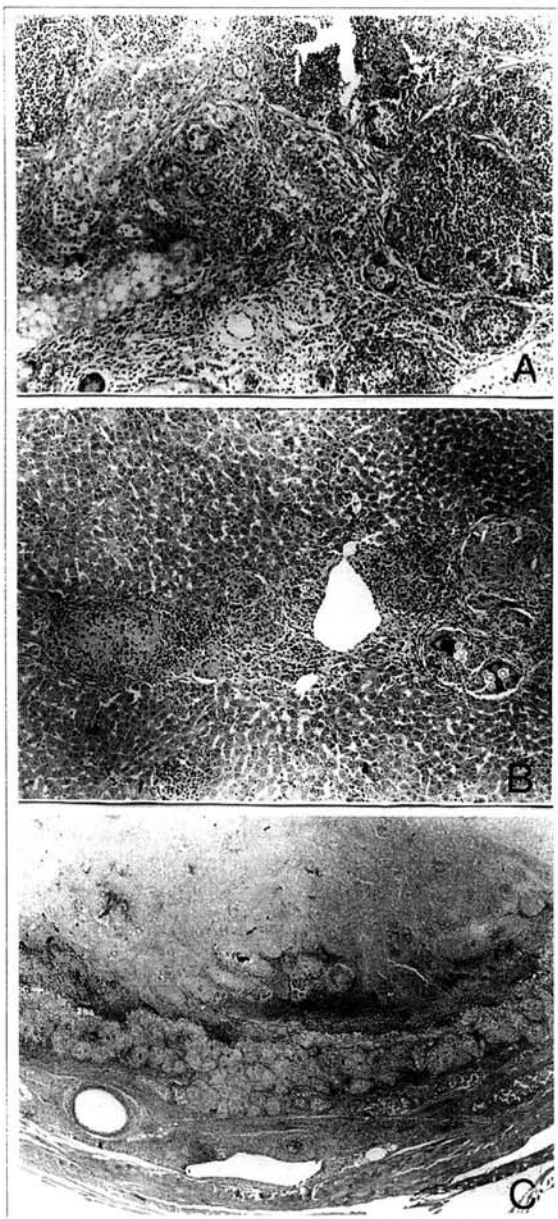

Fig. 1. Histopathology of the lesions. A. Testis - Confluent epithelioid granulomas containing numerous fungi, giant cells surrounded by mononuclear cell infiltrate. Granulomas were observed diffusely among degenerated seminipherous tubules. (HE - 250x). B. Liver - Epithelioid granulomas were involved by mononuclear infiltrate. Fungi were present inside the giant cells. (HE - 250x). C. Testis - Extensive necrosis area substitute the most part of the parenchima and was involved by numerous confluent granulomas. The capsule of the organ was thickened by fibrosis. (HE - 100x).

Cell-mediated immune response

The mean values of MMI indices in response to PHA and PbAg are shown in Fig. 2. The infected animals presented positive response to PHA and to PbAg up to 4th week of infection, with MMI indices decreasing thereafter to become negative (PHA-MMI indices below 55 and PbAg indices bellow 20) at 8 and 11 weeks of infection.

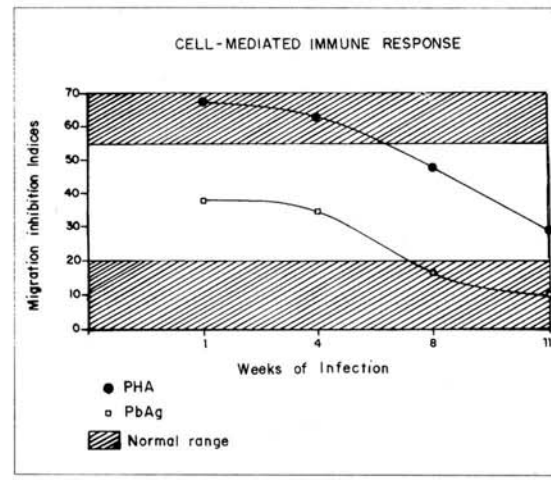

Fig. 2. Macrophage migration inhibition (MMI) indices in response to PHA and to PbAg in hamsters infected with *Paracoccidioides brasiliensis*. Each point represents the mean value for 5 animals sacrificed per week. The normal range represents the MMI indices observed in noninfected hamsters.

Humoral immunity

Serum specific antibodies to *P. brasiliensis* were detected by immunodiffusion and Elisa tests. (Fig. 3). Antibodies detected by Elisa test are represented by optical density at 492 nm. Values above 0.061 which is the mean + 2 SD obtained from 10 noninfected hamsters were considered positive. Antibody levels of infected animals increased from the 2nd week of infection and were elevated up to the end of infection. Both methods employed for the study of humoral immune response showed similar profile of antibody reactivity and a positive correlation (r = 0.82).

Comparison of MIF production and humoral immune response by Spearman's method revealed a negative correlation between MMI indices to PHA and immunodiffusion (r = - 0.64) or Elisa (r = - 0.60) tests. The same negative correlation was observed between MMI indices to PbAg and immunodiffusion (r = - 0.62) or Elisa (r = - 0.53) tests.

NK cell activity

Figure 4 shows the levels of cytotoxic activity of NK cells in infected and in control animals. Noninfected animals maintained the percentage of NK effector cells between 2.19 and 2.79% during all the experiment. Values of NK cell activity in infected hamsters were significantly elevated after 24h up to the second week of infection. From the 8th week on the cytotoxic activity was significantly lower. Comparison of levels of NK cell activity and humoral and cell-mediated immune responses during the infection by Spearman's method, revealed a positive correlation between cytotoxic activity and MIF production to PHA (r = 0.74) and to PbAg (r = 0.56). With respect to humoral immune response, there was a negative correlation between NK cell activity and antibody levels: immunodiffusion (r = - 0.67) and Elisa (r = - 0.80).

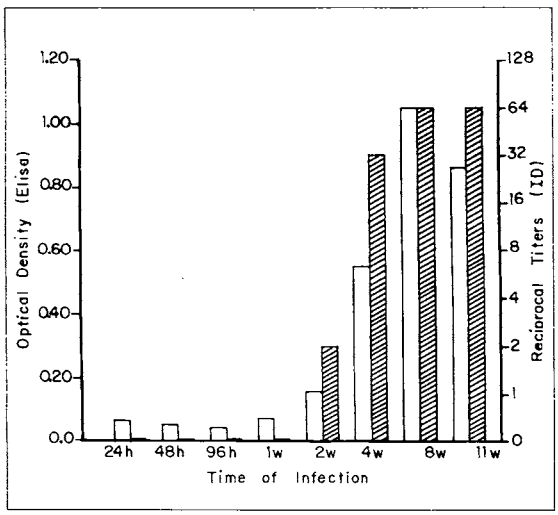

Fig. 3. Antibody titers detected by the immunodiffusion (▨) and Elisa (□) tests in hamsters infected with *Paracoccidioides brasiliensis* according to the time of infection evolution. Immunodiffusion titers were expressed as the reciprocal of the dilution sera and Elisa levels as optical density. The results represent median values for 7 animals sacrificed per period of study. h = hour; w = week.

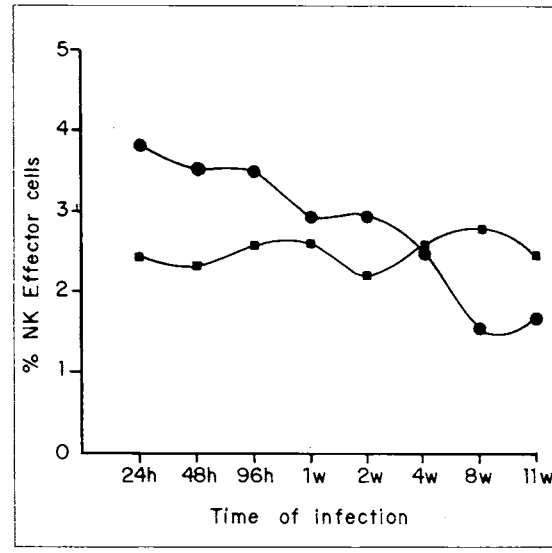

Figure 4. NK activity against K562 target cells is shown for splenic cells of hamsters infected with *Paracoccidioides brasiliensis* (●) according to the time of infection evolution and of control noninfected animals (■) sacrificed at the same time. Results are expressed as mean of the percentage of NK effector cells of 7 animals sacrificed per period of study. h = hour, w = week.

DISCUSSION

Hamsters infected by intratesticular route with *P. brasiliensis* isolated from armadillos developed disseminated infection associated with progressive immunosuppression, and high levels of specific antibodies to *P. brasiliensis,* similar to the pattern of the acute form of human paracoccidioidomycosis.

Cellular immunity was normal until the 4th week of the infection as measured by MIF assay in the presence of PHA. The animals were also capable of maintaining an adequate specific cell-mediated immune response to *P. brasiliensis* as detected by the same test with PbAg.

From the 4th week on there was a progressive decline of the cell-mediated immune response to PHA

and PbAg, followed by dissemination of the infection with progressive involvement of lymph nodes, liver, spleen and other organs. These results were demonstrated by an increase in the extension of the lesions. There is a well-stablished correlation between severity and dissemination of the disease and the level of immunosuppression both in human and experimental paracoccidioidomycosis [19, 23]. The mechanisms involved in this suppression have been attributed to the production of inhibitory plasma factors [8, 20], circulating *P. brasiliensis* antigens and immunecomplexes [5], active suppression by suppressor T cells and/or macrophages and deficiency in interleukin-2 production [11].

In our study significant higher levels of NK cell activity were observed during the first four weeks of the infection. These results suggest that NK cells might be activated during the early phases of the infection being probably involved in the natural resistance of the host. As effector cells against fungi [15] murine NK cells not only inhibit "in vitro" growth of *Cryptococcus neoformans*, but also contribute to the clearance of these organism "in vivo" [14]. However, according to GRANGER & HOBBS [12] the NK cell cytotoxicity against fungi may be attributed to fungistatic effect being possible that the results of growth inhibition may represent the combination of death of some fungal targets and replication of others.

In our experiment, after an initial activation, NK cells have not restricted fungal growth which disseminated to different organs of the infected animals. Impairment of NK cell activity was observed from the 4th week on, reaching the lowest levels at 8th week when cell-mediated immunity was also depressed and the lesions were more extensive. There is a positive correlation among low levels of NK cytotoxicity and deficient MIF production.

The maintenance of high antibody levels at the 8th and 11th weeks of the infection are probably related to the antigenic stimulation due to the increase of lesions containing large numbers of fungi [23]. These antibody levels correlated negatively with cell-mediated immune response and with NK cell activity. Correlation among specific antibody levels, impairment of cell-mediated immunity and fungal load were observed in experimental models of paracoccidioidomycosis and in human disease mainly in the more severe cases [2, 11].

Impaired NK cell activity has been reported in other infections such as leprosy [7] and infectious mononucleosis [34]. In schistosomiasis the impairment of NK cell cytotoxicity showed a direct relationship to the patient's parasite load and depressed lymphoproliferative responses [10].

NK cell deficiency in the late phase of our model might be related to control of activation by cytokines. Gamma-interferon, interleukin-2 and other biological modifiers can activate NK cells [30]. Thus, the diminished NK cell activity in the late stages of the infection might be associated to the immunoregulatory disturbances associated with paracoccidioidomycosis.

In this mycosis deficiency in interleukin-2 [27] and gamma-interferon [1] production might lead to a reduced NK fungicidal activity and depressed T-cell response, interfering with the control of immunoglobulin synthesis. As a consequence, high levels of specific antibodies might mediate the formation of circulating immune-complexes that play inhibitory effect on NK effector/target cell conjugate formation as proposed by PEDERSEN et al. [22]. Immunecomplexes have been detected in sera of patients with paracoccidioidomycosis with a positive correlation between levels of complexes, activity of the disease and depression of cell-mediated immunity [5]. Immunecomplexes could also inhibit NK cell activity by stimulating suppressor T-cells and/or macrophages to produce immunosuppressive substances [22].

Circulating *P. brasiliensis* antigens containing mannose [25] may function as general metabolic inhibitors of NK-target cell binding and lytic steps. The possible importance of certain carbohidrate moieties, particularly D-mannose, in the recognition, and in the subsequent lytic steps of the NK cytolytic pathway in the human and mouse systems has been suggested [32].

Considering the role of NK cells as regulatory cells of different immune functions, the elucidation of the mechanisms involved in depressed NK cell activity will permit a better understanding of immunoregulatory disturbances associated to paracoccidioidomycosis.

RESUMO

Atividade de células natural killer na paracoccidioidomicose experimental do hamster

Com o objetivo de avaliar a atividade de células

natural killer na paracoccidioidomicose experimental do hamster, 80 hamsters foram infectados por via intratesticular com *Paracoccidioides brasiliensis* e sacrificados após 24h, 48h, 96h, 1, 2, 4, 8 e 11 semanas de infecção. Como controle foram avaliados 40 hamsters normais, não infectados. Os animais foram submetidos ao estudo da atividade citotóxica de células NK pela técnica de "single-cell assay" e da resposta imune humoral pelas técnicas de imunodifusão dupla e Elisa. A produção do fator inibidor da migração de macrófagos em presença de Phytohemaglutinina e antígeno de *P. brasiliensis* e a histopatologia das lesões foram estudadas após 1, 4, 8 e 11 semanas de infecção. Os animais infectados, quando comparados aos controles, apresentaram níveis de atividade NK significativamente elevados durante as 4 primeiras semanas de infecção, havendo diminuição dessa atividade a partir da 8ª semana. Foi observada correlação inversa entre atividade NK e níveis de anticorpos específicos e, associação entre diminuição da atividade NK, depressão de resposta imune celular e aumento da extensão das lesões histopatológicas. Os resultados sugerem que após ativação inicial, as células NK são incapazes de controlar a disseminação do fungo pelos tecidos. A depressão da atividade NK na fase tardia da infecção parece estar relacionada com os distúrbios imunorregulatórios associados à paracoccidioidomicose.

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