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Granulocytes in the inflammatory process of BALB/c mice infected by *Leishmania amazonensis*. A quantitative approach

M.L. Pompeu*, L.A. Freitas, M.L.V. Santos, M. Khouri and M. Barral-Netto

Universidade Federal da Bahia (Centro de Pesquisas Gonçalo Moniz-Fiocruz, and Servico de Imunologia, HUPES-FAMED), Bahia, Brazil

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We used previously immunized (partially resistant) and naive (highly susceptible) BALB/c mice infected with *Leishmania amazonensis* for evaluating the role of granulocytes in the course of murine leishmaniasis. The animals were examined at different times post-infection and granulocytes counted in lesion tissues examined ultra-structurally. Polymorphonuclear granulocytes predominated during the early phase of infection and their number decreased with progression of infection; their number was similar in both groups during the early and intermediate phases of infection, though slightly higher in immunized animals during the late phase. Eosinophils represented approximately 10% of cells in the inflammatory infiltrate, being higher during the intermediate phase, and not differing between the groups. Another approach was the evaluation of granulocyte migration to the peritoneal cavity of susceptible BALB/c mice or resistant C57BL/6 mice under several stimuli. There was no statistically significant difference between resistant and susceptible animals in any of the treatments. Despite the influx of granulocytes to the lesion and its possible role in the initial destruction of injected *Leishmania*, this aspect does not seem to have an important effect on the outcome of the leishmanial infection.

Key words: Leishmaniasis; *Leishmania amazonensis*; Granulocytes; Neutrophils; Eosinophils; Tegumentary leishmaniasis; Experimental leishmaniasis

Introduction

Host protective mechanisms in leishmaniasis are not completely known. Cell-mediated immunity is important in this process (Preston and Dumonde, 1976) and more recent work has emphasized the role of lymphocytes in the development of resistance or susceptibility to leishmanial infection in mice (Louis et al., 1979; Handman et al., 1979; Liew et al., 1982, 1984; McElrath et al., 1987). There are also clear hints for the protective action of these cells, acting through macrophage activation (Murray et al., 1982, 1983; Nacy et al., 1983; Titus et al., 1984; Hoover et al., 1986; Panosian, Sypek and Wyler, 1984), or through the destruction of the parasitized macrophages (Pham and Mauel, 1987).

**Present address:* Departamento de Patologia, Universidade Federal do Ceará.
Correspondence address: Manoel Barral-Netto, M.D.; Serviço de Imunologia, Hosp. Universitário Prof. Edgard Santos, R. João das Botas, s/n 40.140 Salvador-Bahia, Brazil.

Much less clear is the role played by the granulocytes in controlling leishmanial infection. The possibility of parasite destruction by human or murine neutrophils has been raised by in vitro and in vivo studies (Deane and Guimarães, 1938; Chang, 1981; Pearson and Steigbigel, 1981; Andrade et al., 1984; Grimaldi et al., 1984; Pearson et al., 1987). The presence of neutrophils and eosinophils has been reported in leishmanial lesions, at the acute and chronic phases of infection (Andrade et al., 1984; Grimaldi et al., 1984; Magalhães et al., 1986; Barral-Netto et al., 1987a). Despite the evidence of the association between eosinophils and neutrophils with leishmanial infection, and of their potential in parasite elimination, no comparison of their importance relative to other cells has been attempted.

In order to evaluate the participation of granulocytes in the tissue reaction against *Leishmania amazonensis* we took advantage of the model of immunized BALB/c mice (Howard et al., 1982). This strain is extremely susceptible to the infection by *Leishmania amazonensis*, but becomes partially resistant upon intravenous immunization with solubilized promastigotes (Barral-Netto et al., 1987b). Histological differences between resistant (immunized) and susceptible animals of the same genetic background cannot be ascribed to genetic dissimilarity; therefore, they may be expected to provide clearer information on protective mechanisms than comparisons between mice from different strains.

Material and Methods

Animals

Ten to 12-week old BALB/c and C57BL/6 mice were obtained from our own colony, and maintained with commercial mouse ration and water ad libitum.

Parasites

Leishmania amazonensis (MHOM/BR/76/Josefa) was used for both infection and antigen preparation. This strain was typed by serodeme and zymodeme analysis (kindly performed by Drs. G. Grimaldi and H. Momem, Fundação Oswaldo Cruz, Rio de Janeiro).

Antigen preparation

Two types of leishmanial antigen were used for immunization, a solubilized antigen for intravenous (iv) immunization, and a crude antigen for intraperitoneal (ip) immunization or stimuli. Stationary-phase promastigotes were solubilized as previously reported (Barral-Netto et al., 1987b). Concentration of the solubilized antigen is expressed as parasite equivalents per milliliter (p.e./ml). For preparation of crude antigen promastigotes were subjected to 12 cycles of alternate freezing (liquid nitrogen) and thawing (37°C water bath) and centrifuged at $20000 \times g$ for 30 min, and had their protein content determined.

Immunization and infection

Mice were immunized iv with 3 doses of 5×10^7 p.e. at weekly intervals. One week after the last immunizing dose mice were challenged sc in the right hind foot-pad

with 5×10^6 viable stationary-phase promastigotes. Foot-pad thickness was measured periodically with a dial gauge caliper (C. Starret; Athol, MA, U.S.A.).

Tissue processing

Small fragments of tissue from the lesion were obtained at 6, 12, 24 h and 1, 2, 4, 7, 10 and 13 weeks post-infection, and processed for optical and electron microscopy (Barral-Netto et al., 1987a).

Quantitative analysis of inflammatory cells

For an evaluation of the frequency of cells in the lesion we performed a differential count of 100 to 200 inflammatory cells in ultra-thin sections for each animal. The areas chosen for cell counting were representative of the histological picture for each animal as indicated by examination of semi-thin sections.

Stimulation and obtainment of peritoneal cells

Peritoneal exudate cells were obtained from naive or previously immunized BALB/c or C57BL/6 mice. Different immunization schedules were employed. Groups of BALB/c and C57BL/6 mice (5 per group) were immunized sc with *Leishmania amazonensis* crude antigen (500 μg protein/animal) emulsified in Freund's incomplete adjuvant (FIA) (thus avoiding cross-reactivity between leishmania and mycobacteria). Other groups of mice ($n=5$) received 3 doses at weekly intervals of solubilized leishmanial antigen (5×10^7 p.e./mouse) either iv or sc. The populations of cells collecting in the peritoneal cavity of the animals were examined 7 days after completion of either the iv or sc (with either crude or solubilized leishmanial antigen) immunization protocol. To this effect, animals immunized by the different procedures (see Table 2) were injected ip with 1 mg of crude promastigote antigen; non-immunized mice received ip 50 μg of Concanavalin A (ConA) or 1 mg of bovine serum albumin (BSA) or of crude leishmanial antigen. Peritoneal cells were collected 24 h later (Meltzer, 1981). The peritoneal cavities were washed with 10 ml of cold RPMI 1640 medium, containing 10% FCS and 10 U/ml heparin, and smears of harvested cells were prepared by cytocentrifugation. Smears were stained by Diff-Quick stain and the percentage of different cell types was determined by counting at least 200 cells per mouse. The total number of cells was also determined by counting the cell suspension in a hemacytometer; there was no significant difference in the total number of cells among the different groups studied.

Results

Tissue cell composition

During the first hours of infection there was a marked migration of granulocytes to the lesion, with neutrophils comprising the majority of the cells in the inflammatory infiltrate (Fig. 1). There was no marked difference between mice immunized and infected and those infected without immunization with both groups exhibiting

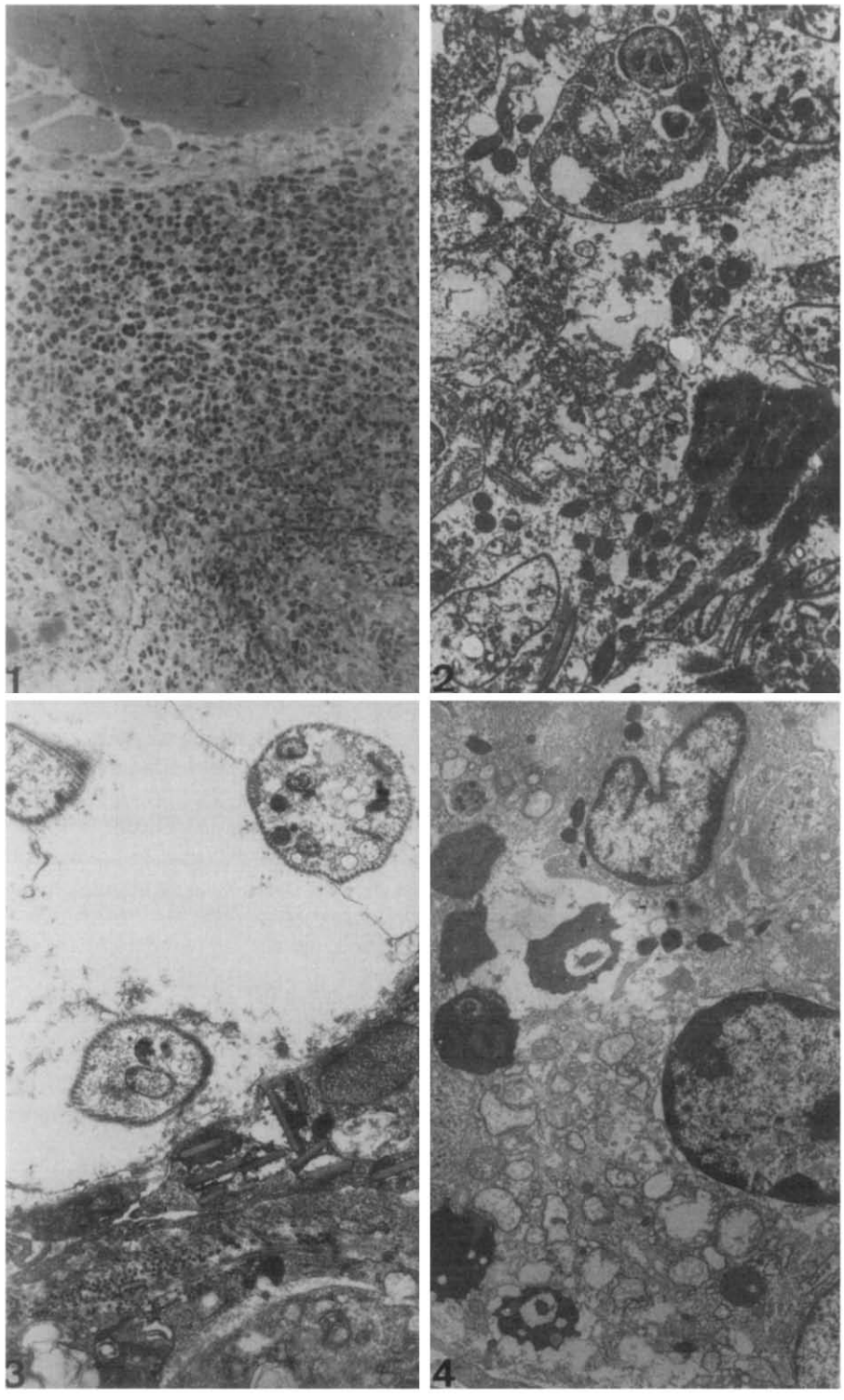
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TABLE 1

Percentage of granulocytes in the inflammatory infiltrate in the Lesion of BALB/c mice infected by *Leishmania amazonensis* with or without previous immunization with solubilized leishmanial antigen

Stage	Neutrophils		Eosinophils	
	Unimmunized	Immunized	Unimmunized	Immunized
Early				
03 h[b]	83.4[a]	80.8	7.6	5.9
06 h	86.7	81.8	4.5	9.9
12 h	87.1	80.2	3.2	5.1
24 h	60.6	49.3	4.8	3.7
Intermediate				
1 w[c]	7.1	17.6	13.3	7.1
2 w	18.0	12.2	21.5	20.1
4 w	14.1	12.1	17.2	6.2
Late				
10 w	5.8	18.5	10.0	14.1
13 w	7.1	17.3	2.5	1.4

[a]Percentage of neutrophils among all cell types present in the inflammatory infiltrate. Other cell types as macrophages, lymphocytes and plasma cells were also present at each time point.
[b]Hours post-infection.
[c]Weeks post-infection.

approximately 80% of neutrophils and 5% of eosinophils in the first 12 h post-infection (Table 1). At 6 h post-infection approximately 18.5% of the neutrophils contained intracellular parasites, and at 24 h this figure had decreased to 3.6%, suggesting a massive destruction of phagocytosed parasites. There was also a large number of lysed parasites in the extracellular space, in the vicinity of eosinophil granules and leukocyte debris (Fig. 2).

At intermediate stages of infection (1 to 4 weeks post-infection) the inflammatory infiltrate contained a mixed cell population. At the second week post-infection it contained up to 18.0% of neutrophils and 21.5% of eosinophils, without major differences between unimmunized and immunized and infected animals (Table 1). At this stage eosinophils were frequently observed nearby parasitized macrophages, and sometimes exhibited contact with these cells. At the areas of contact between eosinophils and macrophages a polarization of eosinophil granules was observed (Fig. 3); such granules were also observed in the extracellular space close to, or even inside the cytoplasm of, parasitized cells (Fig. 4). Generally such macrophages were vacuolated, or lysed with rupture of the cytoplasmic membrane and parasite exterioriza-

Fig. 1. Extensive collection of inflammatory granulocytes with focal areas of purulent necrosis. (Semi-thin section. Toluidine blue × 200).

Fig. 2. Electron micrograph showing lysed parasites in the extracellular space, cellular debris and free eosinophil granules (× 7200).

Fig. 3. Close contact between eosinophil granulocyte and vacuolated macrophages containing amastigote parasites. Polarization of eosinophil granules. (× 7000).

Fig. 4. Eosinophil granulocyte in the vicinity of a parasitized macrophage. Eosinophil granules are seen in the extracellular space, close to the macrophage membrane. (× 7000).

tion. Areas with extracellular parasites frequently exhibited neutrophils or eosinophils. Such cells were mostly preserved but sometimes degenerated, however most of the parasites were severely damaged or lysed. Approximately 13% of neutrophils and 2.8% of the eosinophils present at 7 weeks post-infection contained phagocytized parasites, most of them degenerated.

At the late stages of infection (10 and 13 weeks post-infection) the percentage of granulocytes tended to decrease, except for the presence of neutrophils in the immunized and infected animals (Table 1). Ultra-structural aspects were not different from those observed during the intermediate stages of infection.

Peritoneal cavity cell migration

Table 2 shows the percentage of cells migrating to the peritoneal cavity of different mouse groups, with or without previous treatment, and stimulated by leishmanial antigen, unrelated antigen (BSA) or a mitogenic non-specific stimulant (Con A). Although leishmanial antigen was responsible for a higher neutrophil migration than Con A or BSA there was no difference between the susceptible BALB/c strain and the resistant C57BL/6 mice. Additionally, previous sensitization of both mouse strains with subcutaneously inoculated leishmanial antigen emulsified in FIA abrogated the increased neutrophil migration to the peritoneal cavity following antigen injection. Also indicative of the lack of relation between migration of neutrophils or eosinophils and protection are data obtained with BALB/c after intravenous immunization (a treatment which leads to resistance), and mice immunized subcutaneously (leading to enhancement of the infection). Both groups of animals exhibited similar patterns of cell migration to the peritoneal cavity following parasite antigen injection.

TABLE 2

Peritoneal cavity cell populations of C57BL/6 or BALB/c mice under different treatments

Animals	Immunization	Stimulus	Percentage in the peritoneal exudate cells			
			Neutrophils	Eosinophils	Macrophages	Lymphocytes
C57BL/6	None	Con A	11.7±3.0[a]	0.5±0.6	59.4±13.0	28.7±15.2
	None	BSA	21.4±6.8	11.8±7.2	65.3±12.4	1.4±1.4
	None	*La*[c]	25.1±8.7	2.5±2.1	61.2±9.9	10.9±6.2
	La+FIA[d]SC	*La*	4.4±0.3	7.7±3.3	57.4±3.3	30.5±4.1
BALB/c	None	Con A	13.0±7.5	–	52.7±3.9	34.4±10.2
	None	BSA	16.9±9.6	7.8±2.7	60.1±11.1	8.8±10.5
	None	*La*	31.6±9.6	4.0±2.8	52.9±9.1	8.8±2.5
	La+FIA SC	*La*	10.2±5.4	7.3±2.6	55.3±6.3	26.7±8.5
	Sol. Ag[b] iv	*La*	28.2±15.3	5.9±3.0	49.7±9.6	14.8±5.7
	Sol. Ag SC	*La*	38.2±12.7	5.9±2.4	48.0±9.5	7.1±2.6

[a]Mean±standard deviation ($n=5$).
[b]Solubilized promastigote antigen.
[c]Crude *Leishmania amazonensis* antigen.
[d]Freund's Incomplete Adjuvant.

Discussion

The presence of granulocytes in areas of leishmania destruction was observed in this study in both the acute and chronic phases of murine infection. Our data suggest an important parasiticidal role of granulocytes during the early phase of leishmanial infection in BALB/c mice, but their participation in parasite killing at later stages of infection is less clear. During the acute phase of infection the leishmanicidal activity is related mainly to neutrophils, leading to a massive destruction of the injected parasite burden. During the chronic phase there is the possibility of a cytotoxic role exerted by eosinophils over parasitized macrophages suggested by ultrastructural findings of parasitized macrophages surrounded by eosinophils. At the late stage of infection eosinophils and neutrophils may also exert a leishmanicidal action, both intra- and extra-cellularly. Such findings are, however, less frequent than in the acute phase considering the reduced frequency of these cell types at the late stages of infection.

Cell migration patterns to the peritoneal cavity did not differ significantly among groups of animals susceptible (naive or sc immunized BALB/c mice) or partially resistant to leishmanial infection (C57BL/6 or iv immunized BALB/c mice) when submitted to the same stimuli. The use of FIA in the immunization of mice resulted in decreased granulocyte accumulation and led to higher lymphocyte numbers following leishmanial antigen peritoneal stimulation; it should be pointed out, however, that such changes were very similar in C57BL/6 and BALB/c mice suggesting that such phenomenon does not correlate with resistance. It is also worth noting that neutrophil accumulation following leishmanial antigen stimulation in mice immunized by solubilized promastigote antigen (either by sc or iv routes) was similar to the response obtained with similar stimulus in naive BALB/c mice.

The importance of granulocytes in the phagocytosis and destruction of *Leishmania* had been demonstrated long ago by Deane and Guimarães (1938) by the examination of peripheral blood smears of patients with visceral leishmaniasis. Studying human phagocytes in vitro, Chang (1981) and Pearson et al. (1987) observed that both neutrophils and eosinophils are able to kill *Leishmania*. Chang's results (1981) showed that neutrophils are more efficient than monocytes, and these more efficient than eosinophils, in the phagocytosis of *Leishmania donovani*; additionally it has been pointed out that inside monocytes the amastigotes remained intact while they are degraded inside the granulocytes. Ultra-structural studies in vivo in experimental cutaneous leishmaniasis have also corroborated these findings, demonstrating the presence of degraded parasites inside granulocytes and the preservation of amastigotes in the interior of the macrophages (Grimaldi et al., 1984; Andrade et al., 1984). The results presented in this paper indicate that the presence of granulocytes is important in the initial steps of the infection, and that a large portion of injected parasites are taken up by these cells. The rapid clearance of the ingested parasites is indicative of their efficiency in eliminating the amastigotes. In the host defense against *Candida albicans* it has been postulated that both neutrophils and delayed hypersensitivity are active and that their actions seem to be complementary with cell-mediated mechanisms being responsible for the clearance of organisms surviving the initial contact with neutrophils (Wilson and Sohnle, 1986). It is possible that a similar pattern is relevant in leishmaniasis.

There are several possibilities for the anti-leishmanial activities of the granulocytes.

Neutrophils, most likely, exert their parasiticidal role by phagocytosis and intracellular destruction. The role in the initial response to *Leishmania* of several inflammatory cytokines that stimulate and enhance neutrophil degranulation and superoxide production, such as IL-1 (Ozaki et al., 1987), TNF (Figari et al., 1987) or interferons (Lappegard et al., 1988), remains to be explored.

Our observation of fewer eosinophils in comparison to neutrophils during the early stages of the infection, and their reported smaller phagocytic ability (Chang, 1981) point to a less important role of eosinophils in intra-cellular *Leishmania* destruction. The increased number of eosinophils at later stages of infection, the rare observation of phagocytized amastigotes during these stages, and the close contact of these cells with parasitized macrophages suggest other defensive role(s) for these cells. The activation of the helmintotoxicity capacity of eosinophils by macrophages has been shown (Elsas et al., 1987), and it is possible that eosinophils exert a cytotoxic activity on parasitized macrophages in leishmaniasis.

Despite the demonstrated influx of granulocytes to the lesion and its possible major role in the initial destruction of injected *Leishmania* promastigotes, this aspect does not seem to have a relevant effect on the outcome of the leishmanial infection. Immunized or naive mice from both resistant or susceptible strains had a similar influx of granulocytes to the site of leishmanial antigen stimulation. Additionally, mice which exhibited an extremely different course of disease had a similar pattern of inflammatory infiltrate in the lesion, with similar frequency of granulocytes during all the phases of the infection.

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