

Research article

In vivo involvement of polymorphonuclear neutrophils in *Leishmania infantum* infection

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Abstract

Background: The role of lymphocytes in the specific defence against *L. infantum* has been well established, but the part played by polynuclear neutrophil (PN) cells in controlling visceral leishmaniasis was much less studied. In this report we examine in vivo the participation of PN in early and late phases of infection by *L. infantum*.

Results: Promastigote phagocytosis and killing occurs very early after infection, as demonstrated by electron microscopy analyses which show in BALB/c mouse spleen, but not in liver, numerous PN harbouring ultrastructurally degraded parasites. It is shown, using mAb RB6-8C5 directed against mature mouse granulocytes, that in chronically infected mice, long-term PN depletion did not enhance parasite counts neither in liver nor in spleen, indicating that these cells are not involved in the late phase of *L. infantum* infection. In acute stage of infection, in mouse liver, where *L. infantum* load is initially larger than that in spleen but resolves spontaneously, there was no significant effect of neutrophils depletion. By contrast, early in infection the neutrophil cells crucially contributed to parasite killing in spleen, since PN depletion, performed before and up to 7 days after the parasite inoculation, resulted in a ten-fold increase of parasite burden.

Conclusions: Taken together these data show that neutrophil cells contribute to the early control of the parasite growth in spleen but not in liver and that these cells have no significant effect late in infection in either of these target organs.

Background

The protozoan *Leishmania (L.) infantum* (or *chagasi*) and *L. donovani* are responsible for visceral leishmaniasis (VL) which remains a significant cause of mortality in

many developing countries (OMS). The parasite is inoculated as a flagellated extracellular promastigote by a sandfly vector through the skin of a susceptible host, mainly dog (*L. infantum*) and human (*L. infantum* and

L. donovani). Once in the host tissue, the parasite disseminates throughout the reticulo-endothelial system reaching its target organs – bone marrow, spleen and liver. The main cell target is the macrophage where parasites reside and multiply in parasitophorous vacuoles as obligate intracellular amastigotes. Progressive intracellular infection can be interrupted by generation of T cell dependent specific immunity that induces, mainly through IFN gamma secretion or regulation, macrophage activation, followed by *Leishmania* killing or by control of its growth.

In the liver of experimentally *L. infantum* or *L. donovani* infected BALB/c mice, intracellular amastigotes multiply rapidly during first weeks of infection and the granulomatous tissue response which then occurs, results in a clearing of the parasite [1–9]. By contrast, the spleen becomes a site of chronic infection, where parasites survive for the life of the host [8,10], with progressive destruction of the architecture and loss of follicular dendritic cells and germinal centres [11–13]. The role of cell-mediated immunity and particularly lymphocytes in the specific defence against *L. infantum* has been considerably studied, but the part played by polymorphonuclear cells (PMN) and more particularly the neutrophils (PN) in controlling the disease is much less well established. Previous in vitro or in vivo studies on other parasite species have demonstrated the presence of PMN in areas of *L. amazonensis* destruction during acute and chronic phases of the infection [14,15] and showed that PMN could ingest and kill *L. donovani* promastigotes in vitro [16–19]. Contrasted results have been obtained in studies on dermatotropic *L. major* species. One study showed that susceptible and resistant mice depleted for neutrophils exhibited accelerated parasite spreading, increased footpad swelling and augmented parasite counts in the lesion draining lymph nodes as compared to non-depleted mice [19]. Another study reported that the transient depletion of PMN in BALB/c mice inhibited Th2 cell development and responses and led to a partial resolution of the footpad lesions [20]. In *L. donovani* infected C57BL/6 mice the depletion for neutrophils induced an important enhancement of parasite growth in both liver and spleen [21]. Only one report has been devoted to ex vivo study, showing that PMN from infected dogs can phagocytize and kill *L. infantum* promastigotes [22].

In the present report, we investigated PN as *L. infantum* phagocytic cell and its role in controlling the early and late stage of experimental disease using neutrophil-depleted *L. infantum* infected BALB/c mice.

Results

In vivo ingestion of promastigotes by human PMN

When examining bone marrow for diagnosis, observation of *L. infantum* amastigotes localised in human polymorphonuclear cells is an infrequent finding. Figure 1 shows the presence of such intracellular amastigotes in a patient's bone marrow smear, clearly indicating that the ingestion of *Leishmania* by PMN occurs in human VL.



Figure 1

Presence of *L. infantum* in human PMN (arrow) detected at diagnosis in bone marrow of a VL patient.

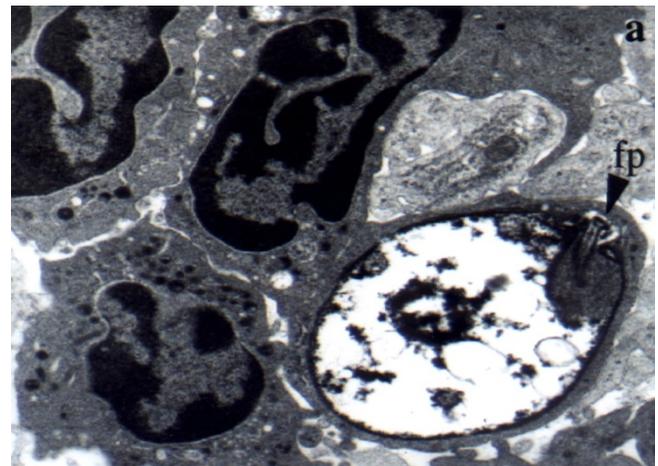


Figure 2

Electron microscopy analysis of early parasite phagocytosis in spleen of infected mice. Micrograph of spleen, 1 h after *L. infantum* inoculation (magnification $\times 6000$).

In mouse, early in vivo involvement of neutrophils occurs in spleen but not in liver

In order to study the early in vivo participation of PN in host defence, spleen and liver of mice were examined by electron microscopy 1 h, 2 h, 5 h and 24 h hours after *L.*

infantum inoculation. In liver, the parasites were readily observed already at 1 h and 2 h after the infection. All parasites were localised close to or inside macrophages and no *Leishmania* were detected inside neutrophil cells, at any time of observation. In contrast, in spleen tissue, parasites were much less abundant and several fields had to be screened in order to find *Leishmania*. A part of spleen neutrophils was found to contain at least one parasite in a more or less degraded state. Figure 2 shows that within one hour post infection, the intraneutrophil protozoan, although recognised by parietal tubules, flagellar pocket and longitudinal-cut flagella, generally lost its ultrastructural integrity. The electron microscopy images do not at all instances allow determining the precise intracellular parasite localisation, so a quantitative evaluation in spleen of the percentage of parasitised macrophages versus parasitised neutrophils would be non-reliable. Twenty-four hours after the infection, neutrophil influx was still observed in the spleen (Figure 3) but no intraneutrophil parasites were detected. In the parasitised monocytes (Figure 4) *Leishmania* appeared relatively intact as compared to that phagocytised by PMN.

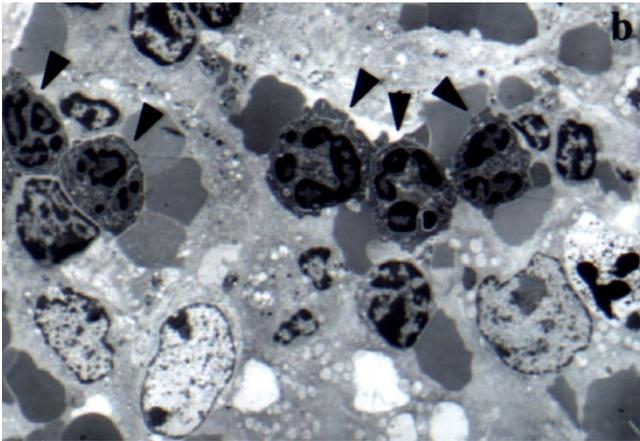


Figure 3
Neutrophil influx to the infected spleen, shown by electron microscopy. Micrograph of spleen, 24 h after *L. infantum* inoculation (magnification $\times 1200$)

Effect of mAb RB6-8C5 on cell counts in blood and spleen
To carry out depletion experiments, we used mAb RB6-8C5, which has been shown to bind and lyse neutrophils and eosinophils in vivo and in vitro [19,23,24]. Table 1 shows that in mouse blood, 48 h after a single intra peritoneal injection of the mAb, the neutrophil percentage was reduced 9-fold and lymphocyte percentage was increased two-fold while 6 days after the injection, the counts from depleted and control mice (which received isotype-matched mAb) were comparable. In spleen, flow

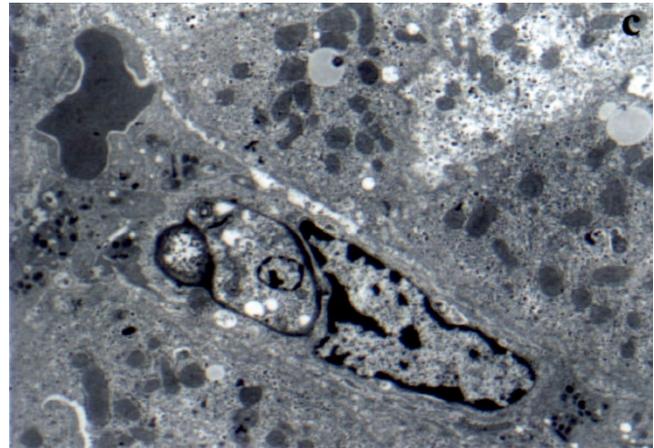


Figure 4
Electron microscopy analysis of early parasite phagocytosis in liver of infected mice. Micrograph of liver, 1 h after *L. infantum* inoculation (magnification $\times 4000$).

Table 1: White cell subpopulations in blood of RB6-8C5 mAb-treated and control mice.

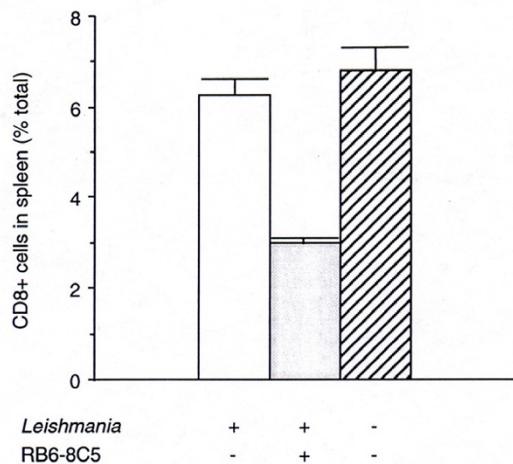
	Neutro Φ	Lympho \subset	Mono \subset	Eosino Φ
48 h after mAb injection				
Control IgG2b	36 \pm 11	51 \pm 8	8 \pm 2	5 \pm 2
RB6-8C5	4 \pm 1	89 \pm 7	5 \pm 5	3 \pm 1
6 days after mAb injection				
Control IgG2b	32 \pm 15	56 \pm 13	7 \pm 2	5 \pm 1
RB6-8C5	55 \pm 5	35 \pm 7	8 \pm 3	2 \pm 1

Mice (4 animals per group) received intraperitoneally RB6-8C5 mAb or irrelevant IgG2b rat mAb (200 μ g per mice). Cells were counted on blood smears and results are expressed as percentage of total number of cells (mean \pm SD). Neutro Φ = Neutrophils, Lympho \subset = Lymphocytes, Mono \subset = Monocytes, Eosino Φ = Eosinophils.

cytometric analyses of cell suspensions were performed 22 days after the RB6-8C5 mAb injection. Although the total number of cells was notably increased [(105 \pm 10) $\times 10^6$, (362 \pm 60) $\times 10^6$, (545 \pm 65) $\times 10^6$ cells per spleen of control, infected RB6-8C5-untreated, infected RB6-8C5-treated mice, respectively], the mAb treatment showed no effect on the percentage of B cells, macrophages, Mac1+ cells and CD4+ cells. The only sub-population affected was that of CD8+ cells, percentage of which was decreased two-fold (Figure 5).

Effect of neutrophil depletion on parasite burden

In the first series of experiments, we studied the role of neutrophils in the early stage of infection. RB6-8C5 mAb was administered as a single injection to four groups of

**Figure 5**

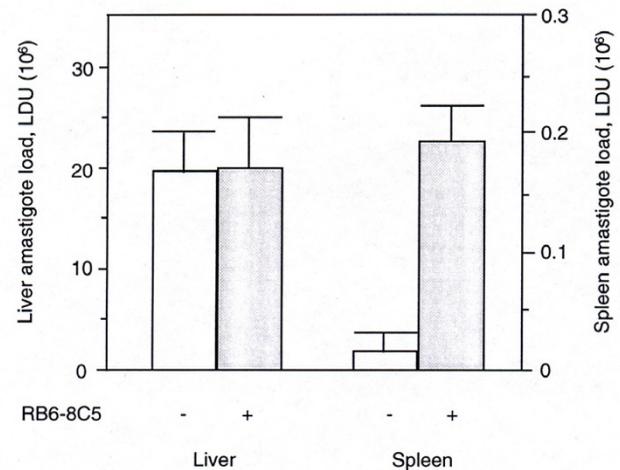
Influence of RB6-8C5 mAb treatment on CD8+ cell sub-population in spleen, assessed by flow cytometry 22 days after *Leishmania* and antibody injection, as described in Methods. Results are expressed as percentage of total number of cells in the spleen for each group [mean \pm sem (4 mice per group)]. The total number of cells is indicated in the text.

mice (5 mice per group), 48 h and 5 h before and 72 h and 168 h after *L. infantum* inoculation. Amastigote burden in spleen and liver was examined 22 days post infection. Figure 6 shows that whereas hepatic amastigote loads in depleted and control groups were similar, in the spleen the parasite load increased 12-fold in neutrophil depleted mice as compared to controls. The data shown in Fig. 6 relate to mice that were RB6-8C5-treated 5 h before *Leishmania* infection; the same enhancing effect of neutrophil depletion on parasite multiplication was observed in all four groups of mice treated with anti-neutrophil mAb at different times. These results indicate that early in infection, in the spleen but not in liver, the neutrophil cells play a role in controlling *L. infantum* burden, and this effect is well detectable even after the state of depletion wears off.

Next, to examine the effect of neutrophil depletion in the late stage of infection, mice infected 3 months earlier were treated with RB6-8C5 mAb twice a week, for 4 weeks (4 RB6-8C5-treated mice and 4 controls). No significant increase of amastigote load was found either in the spleen, or in the liver (not shown), indicating that neutrophils are not significantly involved in the host protection against *L. infantum* in chronically infected mice.

Discussion

Obligate intracellular micro-organisms such as *Leishmania*, have adopted many diverse strategies for their replication inside the host (reviewed in [25]) and the

**Figure 6**

Liver and spleen amastigote load in neutrophil-depleted and control mice. Animals received intraperitoneally 200 μ g of RB6-8C5 or of irrelevant IgG2b rat mAb 5 h prior to *L. infantum* inoculation (10^8 stationary-phase promastigote per mouse). The parasite counts were determined 22 days after the infection from Giemsa-stained spleen and liver touch prints, and are expressed (mean \pm SEM) in Leishman Donovan units (LDU = number of amastigotes per 1000 nucleated cells \times organ weight (in grams) $\times 2 \times 10^8$). The experiment was repeated twice.

host resistance is thought to depend on the development of specific cell-mediated immunity acting through activation and expansion of CD4+ T lymphocytes of the Th1 subset [26,27]. These activated T cells secrete cytokines such as gamma interferon that activate the mononuclear phagocytes turning them into cells with potent leishmanicidal activity (reviewed in [2]). This activity of macrophages is non-specific in nature since it is also effective against heterologous microorganisms with unrelated antigenic specificities [28]. Among other immune defence mechanisms, polymorphonuclear neutrophils, which have been shown to play a crucial role in the early stage of innate resistance to listeriosis [24,29], have been reported to participate to the control of *Leishmania* infection. Indeed, studies showing that PN are present in vivo in areas of *Leishmania* destruction [14,15,30] or can kill the parasites in vitro [16-18] have suggested that these cells might be involved in the inhibition of the parasite multiplication. However, only very few studies using neutrophil-depleted mice infected with *L. major* [19] or *L. donovani* [21] have provided direct evidence for the participation of PN in non-specific resistance to these infections and only one report [22], studying the ex vivo phagocytosis by PN from infected dogs, has suggested a possible role for these cells in the infection with *L. infantum*.

In this report we show *in vivo* that neutrophil granulocytes are effectively capable to ingest and kill *L. infantum* promastigotes. The *in vivo* implication of PN, occasionally observed in human bone marrow as illustrated in Fig 1, was examined in mouse spleen and liver. The very early engagement of neutrophils and their ability to efficiently eliminate *L. infantum* were confirmed in the spleen using electron microscopy analyses. Indeed, as early as one hour after the infection, many PN were found to harbour 1 or more parasites presenting a marked loss of ultrastructural integrity. By contrast, in the liver more numerous parasites and in a less degraded state were observable. In both organs, parasites appeared undegraded in monocytic cells.

To further characterise the role of PN, we next performed depletion experiments in early and late phases of *L. infantum* infection, using the mAb RB6-8C5 directed against mature mouse granulocytes. The apparent health status of mice was unaffected neither by a single mAb administration (which efficiently reduced the number of circulating PN for at least 48 h) nor by repeated injections. We showed that in the early phase of *L. infantum* infection, the neutrophil cells play a protective role in spleen, since early PN depletion resulted in marked increase of the parasite growth. At the time of the parasite load assessment, the subpopulation of CD8+ cells was significantly decreased in the spleen of PN depleted animals. This reduction of CD8+ cells could be due to their neutrophil-dependent recruitment as observed in some studies [31] but also may be related to a possible recognition of this cell subset by RB6-8C5 [32]. No significant effect of neutrophils depletion on the parasite load was evidenced in liver.

Very interesting results published recently [21], reported that the resistance of B cell-deficient C57/BL6 mice to *L. donovani* infection was accompanied by neutrophil dependent destructive pathology in liver. In C57/BL6 mice neutrophil depletion led to enhancement of parasite growth both in liver and in spleen of B-cell deficient and wild type animals. It is noteworthy that in this study [21], neutrophil depletion of BALB/c mouse caused in spleen the enhancement of parasite growth close to that reported here, and a much lower, but significant, increase of liver burden. Therefore, the effect of neutrophil depletion on liver parasite multiplication might depend both on the mouse strain as well as the infecting parasite species. The lack of effect on liver parasite load observed in our study might be related to the relatively weak neutrophil influx elicited by *L. infantum* infection to this organ, as compared to spleen. It is noteworthy that in mouse liver amastigote load is initially larger than that in spleen but it resolves spontaneously, while the spleen supports a sustained parasite load [33].

In chronically infected mice, long term administration of anti-neutrophil antibody, did not significantly enhance parasite counts in either liver or spleen. Therefore, neutrophil cells do not appear to be involved in the late phase of *L. infantum* infection, in contrast to the early phase where they crucially contribute to parasite killing. The precise mechanisms of *Leishmania* destruction remain to be established. Some reports [34–36] suggest that PN could lyse infected host cells. A second hypothesis is that PN kill extracellular parasite before they enter macrophage or even after they have been released by necrotic cells. In this case, as already hypothesised [21], reactive oxygen intermediates, which play an important role in the early regulation of parasite growth and which are among others produced by PN under the control of TNF α , might be responsible for the parasite destruction. Finally, PN could function as immunoregulatory cells by releasing soluble mediators such as TNF α [37] that regulate the entry and intracellular multiplication of parasite in host cells.

We hypothesise, that the neutrophil-generated early destruction of *Leishmania*, although beneficial for the host in terms of the early control of parasite growth, might be also responsible for induction of inflammatory pathology in the spleen which subsequently contributes to the lack of parasite clearance and chronicity [33].

Materials and methods

Parasites

L. infantum MON1 (MHOM/FR/94/LPN101), isolated from a patient with visceral leishmaniasis, was maintained by serial passages in Syrian hamsters. The promastigote form was cultured and prepared for infection experiments as described previously [8–10].

Infections of mice

Six-week-old female BALB/c mice (Iffa Credo, L'Arbresle, France) were inoculated in the caudal vein with 10^8 stationary-phase promastigotes/mouse or left uninfected, split randomly into groups of 2 to 5 animals, and maintained in positive pressure chamber (Esi-Flufrance, Wissous, France). For electron microscopy (EM) analyses, mice were examined 1 h, 2 h, 5 h, and 24 h after infection. For neutrophil depletion experiments, rat anti-mouse PMN cells mAb RB6-8C5 was used [23]. The hybridoma producing RB6-8C5 mAb was kindly provided by Dr Appelberg (Centro di Citologia Experimental, Porto, Portugal); the antibody was purified from ascitic fluids produced in nude mice, using protein G Sepharose affinity chromatography (Pharmacia). Purity of RB6-8C5 mAb was controlled by SDS-PAGE and specificity was verified by cell sorting on whole mouse blood. Mice were injected intraperitoneally with 200 μ g mAb RB6-8C5 or isotype-matched rat IgG (IgG2b, CliniSciences,

France) 48 h and 5 h before or 72 h and 168 h after *L. infantum* inoculation and sacrificed 22 days post infection. Alternatively, animals previously infected for three months were injected as above twice a week during four weeks and analysed 2 days after the last injection. The assessment of the amastigote burden in livers and spleens was carried as described previously [8–10]. The neutrophil depletion was assessed by microscopic examination of Giemsa-stained blood smears, or cell sorting analyses of mouse blood. Neutrophil depletion did not lead to any deterioration in apparent health status of mice.

Tissue processing for electron microscopy

Samples of livers and spleens were fixed for 1 h with 2.5 % glutaraldehyde (Merck) in PBS buffer and cut into pieces of approximately 1 mm³. The material was fixed for another 24 h washed in the buffer, postfixed in 1% osmium tetroxide in buffer for 2 h, washed in buffer, dehydrated with graded ethanol, and embedded in Epon (TAAB). Ultrathin sections were poststained with uranyl acetate and lead citrate, examined, and photographed using a Zeiss 1200EXII transmission electron microscope.

Flow cytometry

Cell suspensions from spleen (20 mg tissue per ml) were counted in a Malassez haemocytometer after erythrocytes lysis and two washes. Phenotypic analyses of spleen cells subpopulations were performed after triple staining with FITC-conjugated anti-B220 (clone RA3-6B2, Caltag), phycoerythrin-conjugated anti-CD8 (clone 53-6.72, Pharmingen) and anti-CD4 (clone CT-CD4, Caltag), and biotin-conjugated anti-CD11b (clone Mac1, Caltag) or F4/80 (clone C1-A31, Caltag), followed by streptavidin-tricolor (TC, Caltag). All the staining steps were carried out at 4°C in PBS containing 5 mM EDTA and 3% (v/v) FCS. Analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), using Cellquest software (Becton Dickinson).

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