Rat Models to Investigate Host Macrophage Defense against *Trypanosoma cruzi*

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Key Words

Chagas' disease · *Trypanosoma cruzi* · Inflammation · Monocytes/macrophages · Innate immunity · Heart · Parasitism · Immunopathology · Rats

Abstract

Trypanosoma cruzi is the causal agent of Chagas' disease, an infection with a great impact on public health in Latin America. One of the challenges to understand Chagas' disease lies on the complex host-parasite interaction. The understanding of this interaction requires the use of appropriate experimental models that mimic the human disease. Here, we have used two lineages of rats (Wistar and Holtzman) to comparatively evaluate the course of the acute infection (Y strain). Infection was monitored by parasitemia, cardiac and skeletal muscle parasitism and inflammation, heart ultrastructure, recruitment of monocytes/macrophages and nitric oxide, and arginase production by these cells. Although both rats were able to infect, only Holtzman rats developed a marked infection in the cardiac and skeletal muscles, in parallel to a high recruitment of first-line defense cells. A high number of inflammatory macrophages directed parasite clearance. By the end of the acute phase, Holtzman rats showed consistent disease control. Interestingly, parasite killing was not related to nitric oxide production likely inhibited

by an arginase-dependent mechanism. Our work demonstrates differential responses of Holtzman and Wistar rats to *T. cruzi*, and highlights the use of Holtzman rats as useful models for further studies of cardiac/skeletal muscle tropism and innate immune responses that protect the host against parasite replication. This is important for the development of proper therapeutic interventions.

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Parasitic protozoa infect hundreds of millions of people every year and are collectively some of the most important causes of human misery [1]. The flagellated protozoa *Trypanosoma cruzi* is the causal agent of Chagas' disease (also known as American trypanosomiasis) discovered around a century ago by the Brazilian physician Carlos Chagas [2]. This disease remains a major problem with a great impact on public health in Latin America. Unfortunately, there is no vaccine available to prevent Chagas' disease.

While different tools to manage the infection with regard to detection and treatment have been discussed [3], biological research is still critical to understand basic as-

¹ In memoriam.

pects of the disease. The understanding of the range of interactions between the parasite and its mammalian host requires analyzing every step of the infection process. The parasite has an obligate intracellular, proliferative nonflagellate form, called amastigote. After many division cycles, the amastigote forms convert into a flagellate form, the infective trypomastigote. Due to the high number of parasites into the host cell cytoplasm, the cell membrane disrupts and the infection spreads, affecting different organs [for reviews, see 1, 4, 5].

Acute Chagas' disease elicits a strong inflammatory response. In order to control parasite replication, cells of the monocytic lineage are remarkably recruited and have a pivotal role in the parasite clearance in both human beings and experimental models [for review, see 5]. Activated macrophages are able to produce high levels of pro-inflammatory cytokines and microbicidal products such as hydrogen peroxide (H_2O_2) [6], peroxynitrite [7] and nitric oxide (NO) [8]. However, despite extensive experimental investigations, the mechanisms used by activated macrophages to kill *T. cruzi* are not fully understood. For instance, the production of high amounts of NO through the inducible NO synthase (iNOS) by macrophages has been claimed as one of the main biochemical pathways to kill the parasite during the murine infection [8–12]. On the other hand, there are controversial data about the importance of this molecule in the control of parasite multiplication [6, 13, 14]. In fact, it has also been demonstrated that neither NO production [15] nor iNOS [16] are essential for the *in vivo* control of *T. cruzi* in mice.

Because macrophages are key players in the initial resistance to the infection, a better understanding of their responses to the parasite is hence crucial for the development of appropriate therapeutic interventions and Chagas' disease control. Experimental *in vivo* studies, however, rely on animal models that have to mimic different aspects of the human infection, including the ability to infect the heart, the main *T. cruzi* target organ.

Here, we have used two lineages of rats (Wistar and Holtzman) to comparatively evaluate the course of the acute infection with the Y strain of the parasite. Host responses to *T. cruzi* were investigated by analyzing: (1) parasitemia; (2) mobilization/migration of monocytic cells lineage; (3) parasite burden and pathological changes in the cardiac and skeletal muscles; (4) extent of inflammatory infiltrates in the myocardial and skeletal tissues. In addition, because NO production during the acute *in vivo* infection is still controversial in the literature, we evaluate the ability of recruited blood monocytes as well as peritoneal and splenic macrophages to produce NO.

The release of arginase, an enzyme which interferes with NO production, was also investigated. Although both rats are able to develop a marked parasitemia, only Holtzman rats mimic tissue parasitism and inflammation characteristic of the acute human disease in parallel to a consistent involvement of the innate immune response. The present work sheds light on the use of rat models for *in vivo* studies of the acute *T. cruzi* infection and immune control of this parasite.

Methods

Animals and Infection

Female Holtzman and Wistar rats aged 27–30 days comprised uninfected and infected groups. Rats were inoculated intraperitoneally with a single inoculum of 3×10^5 trypomastigotes of *T. cruzi*, Y strain [17]. Fresh blood samples taken from the tail showed living trypomastigotes in all animals at 6 or 12 days after inoculation. Animals were sacrificed in a CO_2 chamber at days 6, 12 or 20 of infection. Each group contained 4–6 animals, and survival was monitored daily. The Oswaldo Cruz Institute Ethical Committee approved the use of animals (CEUA: P-0069).

Parasitemia

Bloodstream forms of *T. cruzi* were assessed by direct microscopic observation of 5 μ l of fresh tail venous blood during 20 days after infection. Trypomastigotes were counted in a Neubauer hemacytometer. For each rat lineage, 5–8 rats per each time point of infection were examined. Data were expressed as number of parasites $\times 10^4$ mm^3 .

Peripheral Blood Leukocyte Quantification

Samples of blood were taken from the tail of the controls and infected animals at 6, 12 and 20 days after infection. The total number of leukocytes was estimated by hemocytometer counts (20 μ l of blood diluted in 400 μ l of a solution containing 2% acetic acid and 0.01% methylene blue). The leukocyte percentage was determined by counting 200 cells per rat in air-dried smears stained with May-Grünwald/Giemsa solution. Values of the total leukocyte count and the relative percentages and absolute numbers of different types of leukocytes were obtained and compared.

Peripheral Blood Monocyte and Macrophage Isolation and Viability

Peripheral blood was drawn by cardiac puncture (3–5 ml/animal). Monocytes were isolated from heparinized blood (100 units/ml blood) using Histopaque-1077 (Sigma, St. Louis, Mo., USA) by density gradient centrifugation at 500 g for 30 min, at room temperature. Peripheral blood monocytes (PBM) were washed twice in phosphate-buffered saline (PBS) at 280 g for 10 min at 4°C and resuspended in RPMI 1640 medium. The cell viability was assessed by Trypan blue test as before [6], and was higher than 95%.

Peritoneal macrophages were obtained from peritoneal washings with PBS (5 ml/animal) and washed in PBS at 280 g for 10 min at 4°C. Spleens were homogenized individually in 5 ml RPMI using a Teflon-coated tissue homogenizer (Gas-Col Apparatus Co., Terre Haute, Ind., USA) and were washed twice (280 and

175 g for 10 min at 4°C) and resuspended in 5 ml of the same medium. Viability of the cells was assessed by the Trypan blue test. The concentration of all collected cells was adjusted to 2×10^6 cells/ml with fresh RPMI 1640.

NO/NO₂ Measurement

NO generation by PBM and splenic macrophages was measured as accumulated supernatant nitrite (a stable breakdown product of NO) in controls and infected animals at 6, 12 and 20 days of infection by the Griess reaction [18]. The concentration of collected cells was adjusted to 2×10^6 cells/ml with fresh RPMI 1640 medium. Volumes (100 μ l) of cell suspensions were plated onto each well of 96-well flat-bottom tissue culture plates (Costar, Cambridge, Mass., USA). Plates were incubated in humidified atmosphere at 37°C for 48 h. Subsequently, 100 μ l of the supernatant was collected and transferred to new 96-well flat-bottom tissue culture plates. The supernatants were incubated with 100 μ l of Griess reagent [50 μ l of 1% sulfanilamide (Sigma) plus 50 μ l of 0.1% of N-1-naphtylethylenediamine (Sigma) in 5% phosphoric acid solution] at room temperature for 5 min. The absorbance was determined at 540 nm using a Spectra Max 190 spectrophotometer (Molecular Devices, Sunnyvale, Calif., USA). Conversion of absorbance to micromoles was deduced from a standard curve of sodium nitrite in medium. All determinations were performed in triplicate and expressed as micromoles of NO₂/2 $\times 10^6$ cells.

Arginase Assay

Arginase activity was evaluated in cells as before [6]. Briefly, to 10^6 cells previously washed with RPMI (Sigma), 0.5 ml of 0.1% Triton X-100 in buffer containing 0.1 mM/l phenimethylsulfonyl fluoride, 0.01%W/V Leupeptin, 0.2 mg/ml trypsin inhibitor and 1 mM/l of benzamidine in 0.25 M sucrose and 5 M KCl were added. The mixture was then stirred for 30 min at room temperature. After the cells were lysed, 0.5 ml of 25 mM Tris-HCL plus 5 mM of MnCl₂, pH 7.4 were added and the enzyme was activated for 10 min at 56°C. Arginine hydrolysis was initiated by the addition of 25 μ l of 0.5 M L-arginine, pH 9.7 to a 25- μ l aliquot of the previously activated lysate. Incubation was performed at 37°C for 60 min and the reaction was stopped by the addition of 400 μ l of an acid mixture containing H₂SO₄, H₃PO₄ and H₂O (1:3:7). The urea formed was colorimetrically quantified at 540 nm after addition of 25 μ l 9% α -isonitrosopropienophenone (dissolved in 100% ethanol) and heating at 100°C for 45 min. After 10 min in the dark the optical density was determined in a spectrophotometer using 200- μ l aliquots in a nonsterile microculture plate [19]. A calibration curve was prepared with increasing amounts of urea between 1.5 and 30 μ g. All reagents were purchased from Sigma.

Histological Methods and Histoquantitative Analyses

Fragments of the heart (atria and ventricles) and skeletal muscle (diaphragm) from controls and infected animals were fixed in 4% paraformaldehyde in buffered phosphate, pH 7.3, 0.1 M for 24 h, dehydrated and embedded in plastic resin – glycol metacrilate – (Leica, Heidelberg, Germany). Semi-serial 5- μ m-thick sections were cut on a microtome (RM 2155; Leica) stained by hematoxylin and eosin or toluidine blue-basic fuchsin as before [6, 20] and examined for qualitative evaluation of the inflammatory and degenerative processes and quantification of parasitism. The number of amastigote nests in the myocardium and skeletal muscles was evaluated at 70- μ m intervals in order to avoid parasite

recounting [21]. For each group of infected animals, 1,600 fields were analyzed in approximately 145 semi-serial sections of the heart and 1,000 fields in 56 semi-serial sections of the skeletal muscle at a final magnification of $\times 400$.

The area occupied by amastigote nests within cardiomyocytes and inflammatory processes were also examined in the heart at day 12 of infection, using a BX 51 Olympus microscope with the help of an integrating eyepiece with 100 squares at a final magnification of $\times 400$ [22]. A total of 43,200 squares from both atria and ventricles were counted for each group.

Transmission Electron Microscopy

Heart tissue fragments of controls and infected rats at 12 days after infection were fixed in phosphate-buffered 1% paraformaldehyde and 1% glutaraldehyde, pH 7.3, overnight at 4°C as before [23]. After fixation, tissue fragments were washed in the same buffer and postfixed in a mixture of 1% phosphate-buffered osmium tetroxide and 1.5% potassium ferrocyanide (final concentration) for 1 h prior to dehydration in a graded ethanol series and infiltration and embedding in a propylene oxide-Epon sequence (PolyBed 812; Polysciences, Warrington, Pa., USA). Thin sections were cut using a diamond knife on an ultramicrotome (Sorvall MT2, Newton, Mass., USA) and mounted on uncoated 200-mesh copper grids (Ted Pella, Redding, Calif., USA) before staining with uranyl acetate and lead citrate and viewing with a transmission electron microscope (EM 10; Zeiss) at 60 KV. Electron micrographs were randomly taken at magnifications of $\times 12,000$ and $\times 30,000$. At least 95 electron micrographs were analyzed.

Statistical Analysis

Data from different groups were compared by the Mann Whitney U test ($p < 0.05$).

Results

Holtzman and Wistar Rats Show Similar Parasitemia but Distinct Parasitism

Holtzman and Wistar rats showed prominent parasitemia during the acute phase (fig. 1a). Trypomastigotes, the invasive forms of the parasite, were firstly detected at days 5 and 6 for Wistar and Holtzman rats, respectively. At this time of infection, Wistar rats exhibited a higher amount of parasites compared to Holtzman rats (fig. 1a). The highest peak of circulating parasites for both rat models occurred at day 10 after infection (fig. 1a), as previously described for Holtzman rats [6]. Parasites were completely suppressed as early as 15–16 days after infection for Wistar and Holtzman rats, respectively. Approximately 90–95% of both Holtzman and Wistar rats survived beyond 90 days after infection.

Heart parasitism was extensively studied at days 6, 12 and 20 of infection in both atrium and ventricle (fig. 1b–d). To study heart histopathology we used a histological approach that combines optimal fixation and processing

Fig. 1. Parasitemia (a), heart parasitism (b) and light micrographs of the myocardium from infected Holtzman (c) and Wistar (d) rats at different time points of infection with *T. cruzi* (Y strain). **a** Both rats presented a prominent parasitemia, with peak at day 10 of infection. **b** However, the two rat models showed a dramatic difference in the number of heart parasites at days 6 and 12 of infection. At these points, the number of parasites was significantly higher in Holtzman compared to Wistar rats (* $p < 0.05$). The highest number of parasite nests was observed at day 12 of infection in both rat models (b–d). At this time, amastigote nests (c, d, circles) were frequently seen in the

myocardium, mainly in Holtzman rats (c). Heart parasitism decreased significantly by day 20 in both rats (compared to day 12), but did not differ significantly between the two rat models. Numbers of parasites in the blood are expressed as means $\pm$ SEM of 5–8 animals counted per time point. Tissue parasite numbers were quantitated by analyzing 1,600 fields (800 from atria and 800 from ventricles) on approximately 145 semi-serial sections at $\times 400$ for each group of animals (4–6 rats per group). Results are expressed as means $\pm$ SEM and are representative of 3 independent experiments. Scale bars = 15 μm .

with a plastic resin (glycol methacrylate) embedding. This allows increased tissue resolution and optimal visualization and quantification of the parasite [6, 24]. Multiplying forms of the parasite (amastigotes) within cardiomyocytes were carefully examined and quantified by two different methods (number of nests/animal and area occupied by nests/animal) in semi-serial sections.

Although both Wistar and Holtzman rats had comparable blood parasite levels, the two rat models showed a dramatic difference concerning the ability of the parasite to infect the heart. At day 6, the number of parasites was significantly higher in Holtzman compared to Wistar rats (mean $\pm$ SEM of 112.0 ± 28.6 and 4.0 ± 2.0 per animal for Holtzman and Wistar, respectively, $p < 0.05$; fig. 1b). The highest number of parasite nests was ob-

Fig. 2. Myocarditis induced by acute infection with *T. cruzi*. **a** In Holtzman rats, histopathological analyses at the peak of parasitism (day 12) showed an intense, predominantly mononuclear myocarditis, dissociated myocardial fibers (My) and edematous interstitial tissue. Inset: the boxed area in **a** is shown in higher magnification. Note the presence of macrophage-like cells (aster-

isks) and trypomastigote forms (arrows) close to a disrupted nest. **b** Inflammatory processes in the heart were significantly (* $p < 0.03$) more intense in Holtzman than in Wistar rats. Data are representative of 3 independent experiments. Four to six rats per group. Scale bars = 10 μ M.

served at day 12 of infection in both rat models. However, Holtzman rats had a 7.5-fold higher amount of nests than Wistar rats at this time (mean $\pm$ SEM of $1,987.8 \pm 570.7$ and 264.7 ± 151.5 per animal for Holtzman and Wistar rats, respectively, $p < 0.05$; fig. 1b–d). Because the size of the parasite nests apparently differed between the two rat models at day 12 of infection, we next evaluated the area occupied by parasite nests in the heart. Amastigote nests represented 2.2% of the heart in Holtzman rats while in Wistar rats this proportion was only 0.56%. Heart parasitism decreased significantly at day 20 in both rats, compared to day 12. This decrease was dramatically observed in Holtzman rats. At day 20 of infection, the number of parasite nests did not differ significantly between Wistar and Holtzman rats (mean $\pm$ SEM of 3.2 ± 2.0 and 1.1 ± 2.0 , respectively; fig. 1b). Of note, our histological approach enabled us to identify not only amastigote nests, but also breaching nests in the exact moment of liberation of trypomastigote forms on the way to reach nearby cardiomyocytes (fig. 2a, inset).

We next studied the parasite burden and inflammation within the diaphragm, a skeletal muscle that is severely affected by the parasite [25]. The parasite numbers, analyzed at the peak of the parasitism (day 12 of infection), was 5 times higher in Holtzman compared to Wistar rats (mean $\pm$ SEM of 35.3 ± 6.8 and 7.0 ± 5.0 , respectively, $p < 0.05$) at this time point (fig. 3). Our findings show that Holtzman and Wistar rats greatly differ

regarding the tissue colonization pattern by the Y strain of the parasite.

Inflammation Is More Intense in Holtzman than in Wistar Rats

The rupture of amastigote nests during the acute phase of Chagas' disease provokes cell destruction and a potent inflammatory process, especially in the heart [26]. Because a high number of parasites were observed within cardiomyocytes, we next evaluated the occurrence of inflammatory processes in both rat models. In addition to qualitative analysis, inflammation was quantitatively evaluated.

The presence of a diffuse mononuclear myocarditis was always observed in the heart of both rats, at all infection times, but the inflammation intensity varied greatly during the acute phase. At day 6, the inflammatory process was discrete in both rat models, but became very intense at day 12 only in Holtzman rats (fig. 2a). Wistar rats showed a moderate inflammatory process at this time (fig. 1d). When we quantitated the area occupied by the inflammatory process, Holtzman rats had a significantly higher proportion of inflammatory cells compared to Wistar rats ($p < 0.05$; fig. 2b).

Scattered, large macrophage-like cells were frequently observed in the myocardium of the infected animals, often invading amastigote nests at days 6 and 12 after infection, especially in the heart of Holtzman rats (fig. 2a,

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Fig. 4. Ultrastructural view of a rat heart infected with *T. cruzi*. Electron micrographs show a normal (a) and an infected myocardium (b) from a Holtzman rat. TEM revealed a large number of inflammatory macrophages (outlined in red, color only in online version) infiltrated in the myocardium (My). Degenerating parasite amastigote forms (asterisks) are seen within these cells. Samples were processed at day 12 of infection with *T. cruzi*. Electron microscopy results are based on the analysis of at least 95 electron micrographs. N = Nucleus; V = blood vessel. Scale bars = 1.5 μm (a); 2 μm (b).

Fig. 3. Skeletal muscle parasitism and inflammation in rats infected with *T. cruzi* (Y strain) at day 12 of infection. Holtzman (a, c, e, h) and Wistar (b, d, f) rats showed mononuclear cell infiltration and parasites (encircled) in the diaphragm. g Parasitism was significantly higher in Holtzman rats (* $p < 0.05$). Note the prominent myositis in these animals (c, e). In h, mononu-

clear cells are indicated (arrowheads). Tissue parasite numbers were quantitated by analyzing 1,000 fields on approximately 46 semi-serial sections at $\times 400$ for each group of animals (3 rats per group). Results are expressed as means $\pm$ SEM. Scale bars = 15 μm (a, b, e, f, h); 20 μm (c, d).

Fig. 5. PBM are highly mobilized during the acute infection with *T. cruzi* in Holtzman rats. PBM numbers increased (* $p < 0.05$) at day 6 compared to control values. At day 12, these numbers dramatically increased (* $p < 0.05$) in comparison to numbers found at day 6 (* $p < 0.05$) while at day 20, they decreased (* $p < 0.05$) compared to day 12 (* $p < 0.05$). In Wistar rats, there was no significant difference between PBM numbers in control and infected groups. Holtzman rats showed significant increase of PBM compared to Wistar rats at days 6 and 12 (** $p < 0.01$). At day 20, the numbers of PBM did not differ between Holtzman and Wistar rats. Data are expressed as means $\pm$ SEM and are representative of 3 independent experiments. Four to six rats per group.

Fig. 6. NO production by peritoneal macrophages during the acute *T. cruzi* infection in rats (Y strain). In both Wistar and Holtzman rats the infection did not elicit NO production during different time points of the acute phase. Data are expressed

as means $\pm$ SEM and are representative of 3 independent experiments. NO generation was measured as accumulated supernatant nitrite by the Griess reaction [18]. Four to six rats per group.

inset, asterisks). Degenerating cardiomyocytes showing vacuolated cytoplasm and condensed nucleus were also observed in the heart associated with the presence of edema. These alterations were more pronounced in Holtzman rats at day 12 of infection. At day 20, the extent of myocarditis consistently dropped in both rats, being virtually absent. At this time, cardiomyocytes had a normal appearance and nests were rarely observed.

Because a high number of infiltrating macrophage-like cells were observed in the heart of infected Holtzman rats by light microscopy, we next evaluated the heart of these

animals by transmission electron microscopy (TEM; fig. 4). Our electron microscopy analyses confirmed the presence of a high number of inflammatory macrophages exhibiting many phagocytic vacuoles in the cytoplasm (fig. 4a). Degenerating amastigotes were frequently observed within phagolysosomes (fig. 4b, asterisks) as previously demonstrated by our group [6, 23, 27, 28].

Inflammation was also studied within the skeletal muscle at day 12 of infection. During this time point, predominantly focal inflammatory processes with a great number of mononuclear cells were observed in the dia-

phragm of all infected Holtzman and Wistar rats. However, myositis was clearly more severe in Holtzman compared to Wistar rats (fig. 3).

Mobilization/Recruitment of Monocytic Lineage Cells Is High in Holtzman Rats and Deficient in Wistar Rats

Because a prominent influx of macrophages was observed into both cardiac and skeletal muscles during the acute infection, we next compared the recruitment of PBM between the two rat models during different time points of the acute infection (fig. 5).

Acute infection induced prominent mobilization of cells from the monocytic lineage in Holtzman rats as previously observed [6, 20]. In this rat model, PBM numbers showed a significant increase at all time points of infection when compared to values from uninfected controls (fig. 5). In addition, PBM values significantly differed during the acute phase. At day 6 after infection, PBM numbers increased 170% compared to the control group. At day 12, the highest mobilization of PBM was observed: an increase of 5.7 and 9.9 times compared to day 6 and controls, respectively. At day 20 after infection, PBM numbers decreased significantly (59%) compared to day 12, but remained significantly higher (135%) than the value found at day 6 (fig. 5). In sharp contrast to Holtzman rats, Wistar rats did not show a significant increase in PBM in infected rats compared with uninfected controls (fig. 5). This means that the acute *T. cruzi* infection did not induce significant PBM mobilization in Wistar rats. Interestingly, at day 20 of infection the numbers of PBM were not significantly different between Holtzman and Wistar rats (fig. 5). Of note, the control values of PBM as well as total number of leukocytes did not differ between Wistar and Holtzman rats (data not shown).

Acute Infection Does Not Induce NO Release by PBM and Macrophages in Both Rat Models

NO production was evaluated in both rat models. First, we studied the ability of peritoneal macrophages to produce NO, measured as NO_2^- release, during the initial stages of the acute infection (48 h, 6 and 12 days; fig. 6). At these time points, the levels of NO were not significantly different from the controls in both rat models (fig. 6). Next, we studied the ability of PBM and splenic macrophages to release NO during different times of the acute infection (fig. 7). As previously described by us in Holtzman rats [6], the infection did not trigger NO release by PBM. On the contrary, the levels of NO were sig-

nificantly reduced at days 6 and 20 when compared to the control group (fig. 7a). Interestingly, the acute infection did not elicit NO release by PBM in Wistar rats as well, at any time of infection (fig. 7a). In controls, there is no significant difference between the basal levels of NO in PBM from Holtzman and Wistar rats (fig. 5a).

As demonstrated for peritoneal macrophages and PBM, splenic macrophages from both rat models did not produce NO in response to the acute infection. On the other hand, splenic macrophages from infected Holtzman rats had the NO levels significantly reduced in all time points analyzed when compared to control group (fig. 7b). Because NO in Holtzman rats was inhibited during the acute infection, we next evaluated the production of arginase (as urea releasing), by splenic macrophages. Arginase is known to compete with NOS by L-arginine as substrate [29]. Levels of urea were significantly higher at day 20 after infection (fig. 7c) when compared with the control group (11.0 ± 0.7 and $9.0 \pm 0.6 \mu\text{U}$, respectively). This increase occurred in parallel with the lowest rate of NO production by splenic macrophages (fig. 7c).

Discussion

The most intriguing challenge to understand the mechanisms of Chagas' disease still lies on the complex host-parasite interaction. In this work, host responses to the acute *T. cruzi* infection were investigated by evaluating the course of infection in two lineages of rats (Wistar and Holtzman). Studies were performed with the Y strain of the parasite. This virulent strain is characterized by fast in vitro growth, regular parasitemia and high tropism for cardiac as well as skeletal muscles, being used in numerous studies focused on host defense [5, 25, 30]. The infection was monitored by parasitemia, myocardium and skeletal muscle parasitism, evaluation of inflammatory processes by light and electron microscopy, recruitment of monocytic lineage cells and NO and arginase production by these cells. The characterization of these parameters is central to undertake in vivo studies on Chagas' disease.

A good experimental model should mimic the human infection being able to support a subclinical parasitemia, develop a consistent cellular and/or humoral response, display lesions in a relatively short period of time and survive to the acute phase of the infection [31]. The present work sheds light on the use of rat models for in vivo studies of the acute *T. cruzi* infection. In contrast to mice models, both rat models presented, as general feature, a high survival rate during the acute disease. This aspect is

Fig. 7. NO production by PBM and splenic macrophages in response to the acute *T. cruzi* infection in rats (Y strain). **a, b** In both Wistar and Holtzman rats the infection did not elicit NO production during the acute phase. On the contrary, PBM (**a**) and splenic macrophages (**b**) from infected Holtzman rats had the NO levels significantly reduced (* $p < 0.05$) when compared to the control group. **c** Arginase levels produced by spleen macrophages in this model (line) at day 20 after infection were significantly higher (* $p < 0.05$) during the acute infection when compared to the control group. This increase occurred in parallel with the lowest rate of NO production (bars) by the same cells. Data are expressed as means $\pm$ SEM and are representative of 3 independent experiments. NO generation was measured as accumulated supernatant nitrite by the Griess reaction [18]. Arginase activity was measured as urea accumulated on supernatants [19]. Four to six rats per group.

important because the human disease runs unnoticed in most of the infected individuals and less than 5% of the patients with symptoms die during the disease's acute phase [4, 32].

We showed that Wistar and Holtzman rats present a similar pattern of parasitemia, but greatly differ regarding tissue colonization pattern and inflammatory reaction. Cardiac and skeletal muscle parasitism was dramatically higher in Holtzman compared to Wistar rats. In addition, a consistent parasitism was detected earlier in Holtzman rats as observed in the heart at day 6 of infec-

tion. The infection in Holtzman, but not in Wistar rats, also elicited a strong recruitment of PBM in parallel to the increase in parasite numbers in the target tissues. These data show that Holtzman rats are more susceptible to the acute infection compared to Wistar rats, but are able to respond to parasite with an accentuated mobilization and recruitment of monocytes/macrophages. Variation between inbred mice of susceptibility to experimental *T. cruzi* infection has frequently been described [33–36] but to our knowledge, this is the first work showing such variation in rat lineages.

As the acute phase progresses, a reduction in myocardial parasite burdens occurred in both rat lineages. Interestingly, in spite of being more susceptible to the early infection, Holtzman rats did not show a retarded decrease in parasitism in comparison to Wistar rats. In fact, our analyses demonstrated that by day 20 of infection there is no significant difference between the numbers of parasites in the heart of these 2 models. This means that Holtzman rats had an efficient control of the acute infection, likely through the involvement of the monocyte/macrophage system remarkably mobilized in these animals.

Indeed, activated macrophages exert critical activities in immunity to parasites and both mobilization of PBM and increase in heart parasitism in Holtzman rats were paralleled by a consistent influx of macrophages in the myocardium as evidenced here by TEM. Heart inflammatory macrophages exhibited a morphological pattern of activation and were involved in the parasite phagocytosis as previously demonstrated [20, 23, 27]. Of note, Holtzman rats are also able to develop nerve lesions [22, 37] classically identified in patients with the acute disease [for review, see 38].

The immune control of many intracellular pathogens, including *T. cruzi*, is reported to be dependent on the production of NO by IFN- γ -activated macrophages [8–11]. Here, we have performed a detailed ex vivo study on the production of NO by PBM as well as peritoneal and splenic macrophages during the acute infection. We show that production of NO is not the decisive factor determining resistance or susceptibility to *T. cruzi*. Our data, demonstrating consistent parasite clearance in both blood and heart in the absence of NO production, support a study with a low virulent strain of the parasite and iNOS-KO mice [15]. By analyzing parasitemia and tissue pathology, the authors have shown that NO is not essential for the in vivo control of the parasite [15]. Accordingly, iNOS-deficient mouse lineages, infected with the virulent Brazil or Tulahuen strains of *T. cruzi* had parasitemia, tissue parasite load and survival rates similar to wild-type mice [16]. Our results, using another virulent strain of *T. cruzi* (Y strain) and a different experimental model, strongly indicate that NO is not of primary relevance to the resistance of acute *T. cruzi* infection.

Interestingly, NO levels produced by PBM and spleen macrophages in infected Holtzman but not in Wistar rats were significantly lower than the basal level found in control noninfected animals, as also observed in other experimental models of Chagas' disease [13, 39, 40]. This may be related to the ability of the parasites to activate arginase, which in turn leads to a depletion of L-arginine,

the iNOS substrate, resulting in lower levels of NO and increased production of polyamines. These substances are involved in parasite growth and differentiation [29]. Therefore, we can speculate that the high parasite burden in Holtzman rats might be responsible for the in vivo NO downregulation found in these models. Our present results showing significant increase in the arginase levels at day 20 provide evidence for the occurrence of this pathway as previously indicated [6]. On the other hand, production of other parasite antigen-induced cytokines including IFN- γ , TNF- α and GM-CSF may help compensate for the absence of NO [16, 41].

In summary, our findings demonstrate differential responses of Holtzman and Wistar rats to *T. cruzi*, and highlight the use of Holtzman rats as useful models to study pathological aspects of the acute disease, including parasite tropism for the cardiac and skeletal muscles as well as mobilization/activation of the monocyte/macrophage system. A better understanding of the functional capabilities of these cells, including a better characterization of monocyte and macrophage subsets may be instrumental for the development of appropriate therapeutic interventions and Chagas' disease control.

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