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Differential impact of metacyclic and blood trypomastigotes on parasitological, serological and phenotypic features triggered during acute *Trypanosoma cruzi* infection in dogs

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Abstract

A detailed follow-up investigation of the major parasitological, serological and phenotypic features in dogs experimentally infected with metacyclic (MT) and blood (BT) trypomastigotes of *Trypanosoma cruzi* strain Berenice-78, typifying vectorial and transfusional transmission of human Chagas disease, has been conducted. Although there were no changes with respect to the window of patent-parasitaemia, significant differences between MT- and BT-infected dogs in both the prepatent period (days 23 and 19, respectively) and the day of maximum parasitaemia (days 26 and 22, respectively) were recorded. A progressive enhancement in the level of *T. cruzi*-specific antibodies accompanied infection by both MT and BT forms, although higher IgG titres developed on days 14 and 21 following infection with MT forms. Higher Thy-1$^+$/CD21$^+$ and lower CD4$^+$/CD8$^+$ cell ratios, occasioned by increased levels of Thy-1$^+$ and CD8$^+$ T-cells and reduced frequencies of CD4$^+$ T-cells and CD21$^+$ B-lymphocytes, were observed in both MT- and BT-infected animals. The reduced frequency of CD14$^+$ leukocytes was revealed as the most relevant phenotypic feature intrinsic to *T. cruzi* infection independent of inoculum source. BT-specific phenotypic features included an early reduction

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in the percentage of circulating $CD21^+$ and $CD14^+$ leukocytes, together with a higher Thy-$1^+/CD21^+$ cell ratio on day 42. On the other hand, higher levels of $CD8^+$ T-cells, together with a lower $CD4^+/CD8^+$ cell ratio on day 28, were characteristic of MT infection. These findings emphasise the importance of inoculum source and suggest that vectorial or transfusional routes of *T. cruzi* infection may trigger distinct parasite–host interactions during acute Chagas disease.

Keywords: Dog; Blood (BT) and metacyclic (MT) trypomastigotes; *Trypanosoma cruzi*; Flow cytometry

1. Introduction

The causative agent of Chagas disease, *Trypanosoma cruzi*, is characterised by a complex biological cycle involving an invertebrate vector and mammalian hosts. Epimastigotes and metacyclic trypomastigotes (MT) are commonly found in the triatomine vectors, whilst amastigotes and blood trypomastigotes (BT) represent, respectively, the proliferative intracellular and the bloodstream forms found in the vertebrate hosts (Andrade and Andrews, 2005).

The major routes of human infection by *T. cruzi* involve either vectorial or transfusional transmission. Vectorial infection with Chagas disease occurs when mucous membranes or abraded skin are exposed to the faeces of triatomine insects infected with MT forms. Transmission of the disease *via* blood transfusion is, however, also an important route of infection since trypomastigotes present in the blood may remain viable in freshly stored haemo-derivates. The transmission of *T. cruzi* resulting from congenital processes, oral infection or laboratory accidents may be mediated by either MT or BT forms (Sandler et al., 2003).

Although it has been demonstrated that both MT and BT forms of *T. cruzi* are fully functional with respect to parasite–host interaction and/or target cell invasion (Ramirez et al., 1993), the two forms differ regarding their surface molecules. Thus, glycoconjugates of BT, but not those of MT, are able to induce the synthesis of cytokines by macrophages during the early stages of *T. cruzi* infection (Bento et al., 1996; Camargo et al., 1997). Moreover, since BT, but not MT, forms are typically associated with membrane-bound anti-*T. cruzi* immunoglobulins, they may infect specific target cells during the early events that take place during acute infection. It is possible, therefore, that the different kinetics of infection brought about by MT and BT forms may lead to dissimilar parasite–host interactions that could influence various biological aspects of Chagas disease.

In experimental infections with *T. cruzi*, BT forms are most commonly employed as inoculum. It has been reported that BT forms derived from different strains of *T. cruzi* may be correlated with distinct tissue tropism and virulence, possibly leading to specific biological associations. However, such forms may not reproduce the scope of the parasite–host interactions that are triggered during vectorial transmission in which MT is the infective form. In this context, the etiopathogenic mechanisms triggered by different sources of trypomastigotes employed as inoculum remain unknown. The development of experimental models that address the impact of inoculum source on the immunoparasitological features associated with acute *T. cruzi* infection would, therefore, be particularly valuable in order to reproduce the features of human infection associated with the two major routes of transmission.

We have previously shown (Lana et al., 1988, 1991, 1992) that the dog represents an appropriate experimental model for Chagas disease since canine infection reproduces several aspects of the human disease, including the major immunoparasitological features of acute infection and the absence of tissue damage that is characteristic of indeterminate forms. Moreover, the important histopathological indicators of cardiac problems, including diffuse fibrosing chronic chagasic cardiopathy, often associated with Chagas disease in man are also manifest in the canine model.

Using such a model system, we have recently demonstrated that MT forms of the Be-78 strain of *T. cruzi* (a strain isolated in 1978 from patient Berenice, in whom Chagas disease was first described) are more virulent than the BT forms (Bahia et al., 2002). In the present study, the major parasitological, humoral and cellular immunological features that are triggered during acute infection of dogs with MT and BT forms of *T. cruzi* strain Be-78 have been investigated with the aim of establishing the influence of inoculum source on the applicability of the canine system as a model for the human disease.

2. Materials and methods

All procedures described in the present work were carried out in compliance with current Brazilian regulations relating to Experimental Biology and Medicine as described in the guidelines issued by the Colégio Brasileiro de Experimentação Animal (COBEA, 2006).

All study animals were maintained in the central animal facility at the Universidade Federal de Ouro Preto (UFOP), Minas Gerais, Brazil.

2.1. Parasites, animals and experimental infection

Nymphs of *Triatoma infestans* were allowed to feed on the blood of female Swiss Webster mice (weight range 20–24 g) that had been previously inoculated with *T. cruzi* strain Be-78. Following infection, the triatomine bugs were maintained under starvation conditions for 15 days and then allowed to blood feed on uninfected mice in order to induce the release of MT forms of the protozoan parasite into the faeces. In order to obtain BT forms of the protozoon, female Swiss Webster mice were infected with 1×10^4 blood forms per mouse of *T. cruzi* strain Be-78 and blood samples were collected from the orbital veins at the parasitaemia peak.

Prior to the commencement of the study, 18 mongrel dogs (9 males and 9 females), each 4 months old, were immunised against most common canine infectious pathogens and treated with anti-helminthics. The study animals were maintained in quarantine for 16 weeks, during which time they received drinking water and a balanced commercial feed *ad libitum*. Two groups of six dogs were inoculated intaperitoneally with either MT or BT forms of *T. cruzi* strain Be-78 (2000 per kg body weight), whilst six dogs were maintained uninfected in order to serve as the control group.

2.2. Parasitological parameters

Following inoculation, 5 μL samples of blood were collected daily (from days 1 to 42) by vein-puncture of the ear veins of infected dogs. The numbers of parasites in the blood samples were determined under the optical microscope according to the method of Brener (1962), and parasitaemia curves were plotted using the daily mean numbers of parasites per group of six animals. Five major parasitological parameters were determined from the collected data, namely, the prepatent period, the patent period, the day of maximum parasitaemia, the parasitaemia peak, and the area under the parasitaemia curve.

2.3. Serological profiles

Peripheral blood (5 mL) was collected from the brachial cephalic veins of infected dogs prior to inoculation and weekly thereafter (on days 7, 14, 21, 28, 35 and 42), and placed in vials in the absence of anti-coagulant. All subsequent serological analyses were carried out simultaneously using frozen serum samples that had been stored at −20 °C. Anti-*T. cruzi* IgG reactivity was estimated using a modified form of the ELISA method of Voller et al. (1976) employing 3.0 μg/mL of soluble antigen from *T. cruzi* Y-strain, peroxidase-conjugated goat anti-dog IgG (Sigma, St. Louis, MO, USA) diluted 1:1000 in phosphate buffered saline (PBS) supplemented with 0.05% of Tween-20, and serum samples similarly diluted 1:100. The cut-off point between positive and negative results was taken as $\bar{x} + 2\sigma$, where $\bar{x}$ represents the mean measured absorbance of 10 samples derived from uninfected animals and σ is the determined standard deviation.

2.4. Immunological features

2.4.1. Antibodies

Unlabelled rat monoclonal antibodies (mAbs) recognising canine cell surface markers, defining the major leukocytes subpopulations, were used for indirect immunophenotypic staining. The purified rat mAbs (Serotec, Oxford, UK) included anti-canine Thy-1 (rat-IgG2b; clone YKIX337.217) diluted 1:800, anti-canine CD5 (rat-IgG2a; clone YKIX322.3) diluted 1:800, anti-canine CD4 (rat-IgG2a; clone YKIX302.9) diluted 1:12,500, and anti-canine CD8 (rat-IgG1; clone YCATE55.9) diluted 1:800. Indirect staining was performed using fluorescein isothiocyanate (FITC)-labelled sheep anti-rat IgG (Serotec) diluted 1:200. In direct immunofluorescence procedures, undiluted FITC-labelled mouse anti-human CD21 (mouse-IgG1; clone IOBla; Immunotech, Marseille, France) and PE/Cy-5-conjugated mouse anti-human-CD14 (mouse-IgG2a; clone TÜK4; Serotec) diluted 1:200 were employed. In each case, mAbs were diluted in PBS supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, USA) and 0.1% sodium azide (Sigma), and appropriate isotype control antibodies were obtained from the corresponding suppliers.

2.4.2. Immunophenotyping by flow cytometry

The major circulating subpopulations of leukocytes were quantified prior to inoculation and weekly thereafter (on days 7, 14, 21, 28, 35 and 42), using immunostaining protocols described by Reis et al. (2005). Briefly, 1 mL of EDTA whole blood, collected from the brachial cephalic vein, was submitted to erythrocyte lysis and pre-fixation by the slow addition of 13 mL of FACS Brand Lysing Solution (Becton Dickinson, Mountain View, CA, USA) followed by a 10 min incubation at room temperature. Following centrifugation ($450 \times g$; 10 min; room temperature), the pellet

was resuspended in 500 μL of PBS supplemented with 10% foetal bovine serum in order to obtain a pre-fixed leukocyte suspension. Aliquots (30 μL) of leukocyte suspension were incubated together with 30 μL of previously diluted unlabelled anti-canine cell surface marker mAbs in 96-well U-bottom microtitre plates (Linbro, ICN Biomedicals, Aurora, OH, USA) for 30 min at room temperature in the dark. Leukocytes were additionally incubated under the same conditions in the presence of 60 μL of previously diluted FITC-conjugated sheep anti-rat IgG antibodies. Direct immunostaining procedures were used to quantify the frequency of B-cells and of monocytes using, respectively, 5 μL of undiluted FITC-labelled mouse anti-human-CD21 or 50 μL of previously diluted PE/Cy-5-conjugated mouse anti-human-CD14.

2.4.3. Flow cytometric data acquisition and analysis

Prior to flow cytometric analysis, labelled cells were fixed for 30 min with 200 μL of FACS Fix solution containing 10.0 g/L paraformaldehyde, 10.2 g/L sodium cacodylate and 6.65 g/L sodium chloride adjusted to pH 7.2. Flow cytometry was performed using a FACS Calibur instrument (Becton Dickinson), and a Cell-Quest software package was used in both data acquisition and analysis. A total of 10,000 events were acquired in order to quantify each cell phenotype. Canine whole blood leucocytes were identified from their specific forward- and side-scatter properties, the lymphocytes being first selected on the basis of their size *versus* granularity distribution. The fluorescent properties of gated lymphocytes were determined from their FITC-FL1 spectra using single-histogram representations. The frequency of circulating monocytes was estimated from Cy5-PE-FL3 single histograms obtained directly from ungated leucocytes. The percentage of positive cells was determined for each cell phenotype by setting a marker on the respective isotypic control that encompassed > 98% of the unlabelled cells. The results were expressed in terms of the percentage of positive cells (Thy-1$^+$, CD5$^+$, CD4$^+$, CD8$^+$, CD21$^+$) within the gated lymphocytes, from which the ratios Thy-1$^+$/CD21$^+$ and CD4$^+$/CD8$^+$ could be readily calculated. The relative counts for monocytes were determined as the percentage of CD14$^+$ positive cells within ungated leukocytes.

2.5. Statistical analysis

Statistical analyses were performed using SPSS version 8.0 software (SPSS, Chicago, IL, USA) (Norussis, 1990). The differences in the prepatent and patent periods between the groups were analysed using the Schéffe hypothesis test (Snedecor and Cochran, 1989). Analyses of the differences in IgG reactivities and phenotypic features between groups were performed by variance analysis followed by the application of unpaired Student's *t*-test (Snedecor and Cochran, 1989). Since the area beneath the parasitaemia curve was non-parametric in nature, comparative analyses between groups were performed using the Kolmogorov–Smirnov test (Conover, 1980). In all cases, the differences determined were considered significant when the probabilities of equality, *P* values, were ≤ 0.05.

3. Results

3.1. Parasitological parameters

Infection was confirmed in all 12 dogs that had been inoculated with MT or BT forms of *T. cruzi* strain Be-78, although mortality was not observed within the 42-day experimental period. The kinetics of the five major parasitological parameters evaluated, namely, the prepatent period, the patent period, the day of maximum parasitaemia, the parasitaemia peak, and the area under the parasitaemia curve, are shown in Fig. 1. There were no statistically significant differences between MT- and BT-infected dogs in respect of patent period or area under the parasitaemia curve. Furthermore the parasitaemia peak of 6250 trypomastigotes/5 μL of blood attained on the 26th day after inoculation with MT forms was similar to that produced by BT forms, namely, 5000 trypomastigotes/5 μL of blood on the 22nd day. Although

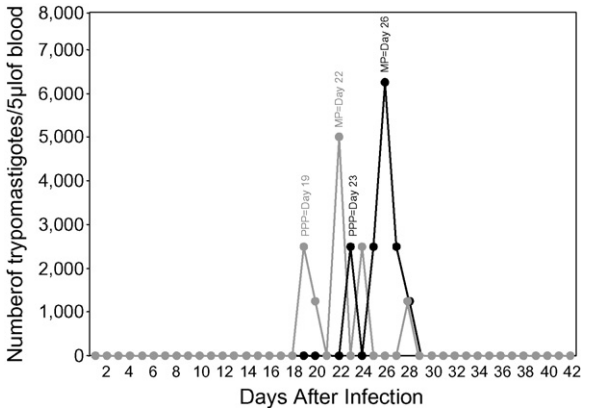

Fig. 1. Kinetics of parasitological parameters in canine peripheral blood during acute infection with metacyclic (●) or blood (●) trypomastigotes of Be-78 *T. cruzi* strain. Data are expressed as mean daily values of parasitemia for each experimental group ($n = 6$), according to Brener (1962). Outstanding parasitological parameters are highlighted at specific timing point in the figure.

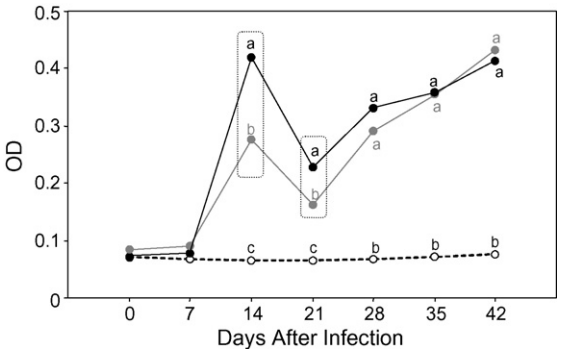

Fig. 2. Kinetics of serological features during acute canine infection with metacyclic (●) or blood (●) trypomastigotes of Be-78 *T. cruzi* strain in comparison to uninfected controls (○). Data are expressed as mean optical density (OD) of anti-*T. cruzi* IgG reactivity estimated by *T. cruzi* soluble antigen ELISA. Different letters represent significantly differences between groups at $P<0.05$. Doted rectangles highlight significantly differences between MT and BT at $P<0.05$.

there were no differences with respect to the window of patent-parasitaemia, the data revealed a statistically significant divergence ($P<0.05$) between MT- and BT-infected dogs in both the prepatent period (days 23 and 19, respectively) and the day of maximum parasitaemia (days 26 and 22, respectively).

3.2. Serological profiles

The kinetics of anti-*T. cruzi* IgG reactivity, as determined by ELISA assay, in dogs inoculated with MT and BT forms of *T. cruzi* strain Be-78 are presented in Fig. 2 (data expressed in terms of mean optical densities). Whilst no IgG reactivity was observed in any of the uninfected control animals during the study, the presence of anti-*T. cruzi* specific antibodies could be clearly detected in inoculated dogs from day 14 and throughout the remainder of the experimental period. An early peak of IgG reactivity was observed on day 14 in dogs infected with either MT or BT forms, and this was followed by a slight reduction in reactivity on day 21 and progressive enhancement of of *T. cruzi* specific antibodies up to day 42. Although the IgG reactivity profiles for MT and BT infections were similar, significantly higher titres ($P<0.05$) were observed with MT forms on days14 and 21 following infection.

3.3. Immunological features

The dynamics of the major circulating leukocyte subpopulations, including T-cells (Thy-1$^+$, CD5$^+$, CD4$^+$ and CD8$^+$), B-lymphocytes (CD21$^+$) and monocytes (CD14$^+$), within the period commencing immediately before inoculation and for the following 42 days are presented in Fig. 3. Analysis of the data derived from uninfected animals revealed homogeneous frequencies of circulating leukocytes throughout the 42-day period with respect to each of the cell phenotypes evaluated. These findings were confirmed by linear regression analysis (Thy-1$^+$ cells $\rightarrow y=-0.054x+74.158$, $R^2=0.0499$; CD5$^+$ cells $\rightarrow y=-0.2274x+72.824$, $R^2=0.4044$; CD4$^+$ cells $\rightarrow y=-0.0548x+48.554$, $R^2=0.0535$; CD8$^+$ cells $\rightarrow y=-0.045x+21.674$, $R^2=0.131$; CD21$^+$ cells $\rightarrow y=-0.0817x+22.416$, $R^2=0.4448$; CD14$^+$ cells $\rightarrow y=0.0105x+7.4622$, $R^2=0.0428$).

Fig. 3A displays the frequencies of Thy-1$^+$ T-cells during the 42-day period following inoculation of dogs with *T. cruzi* strain Be-78. Infection with either MT or BT forms gave rise to significant increases ($P<0.05$) in the levels of Thy-1$^+$ cells compared with uninfected controls, specifically on days 28 and 35 for MT and on day 14 for BT. No significant differences were observed between animals inoculated with MT and BT forms with respect to the frequencies of Thy-1$^+$ cells, although levels in MT-infected dogs increased significantly throughout the 42 day study period, a finding that was supported by linear regression analysis (MT $\rightarrow y=0.3375x+71.324$, $R^2=0.6149$; BT $\rightarrow y=0.1534x+74.491$, $R^2=0.5973$).

No significant differences were detected in the frequencies of circulating CD5$^+$ T-cells in MT- and BT-infected dogs, compared with uninfected controls, during the experimental period (Fig. 3B). Linear regression analysis confirmed the homogeneous distribution of CD5$^+$ cells in inoculated animals (MT $\rightarrow y=-0.2174x+66.243$, $R^2=0.402$; BT $\rightarrow y=-0.0689x+62.543$, $R^2=0.0551$). In contrast, a significant decrease ($P<0.05$) in the percentage of circulating CD4$^+$ T-cells was observed on days 28, 35 and 42 in both MT- and BT-infected animals compared with the uninfected control group (Fig. 3C). Moreover, on day 28 a remarkable decrease ($P<0.05$) in the percentage of CD4$^+$ T-cells was observed in MT-infected dogs compared with their BT-infected counterparts. Linear regression analysis supported the observed tendency of the CD4$^+$ cell level to diminish throughout the study period (MT $\rightarrow y=-0.2991x+41.449$, $R^2=0.5802$; BT $\rightarrow y=-0.4751x+47.517$, $R^2=0.8804$).

In comparison with uninfected animals, significant increases ($P<0.05$) in the percentages of circulating CD8$^+$ T-cells, as recorded on days 14, 21, 28, 35 and 42, were an intrinsic feature of infection by *T. cruzi* strain Be-78 (Fig. 3D). Although the tendency towards enhanced levels of circulating CD8$^+$ cells was observed with both MT and BT forms (MT $\rightarrow y=1.0996x+20.892$,

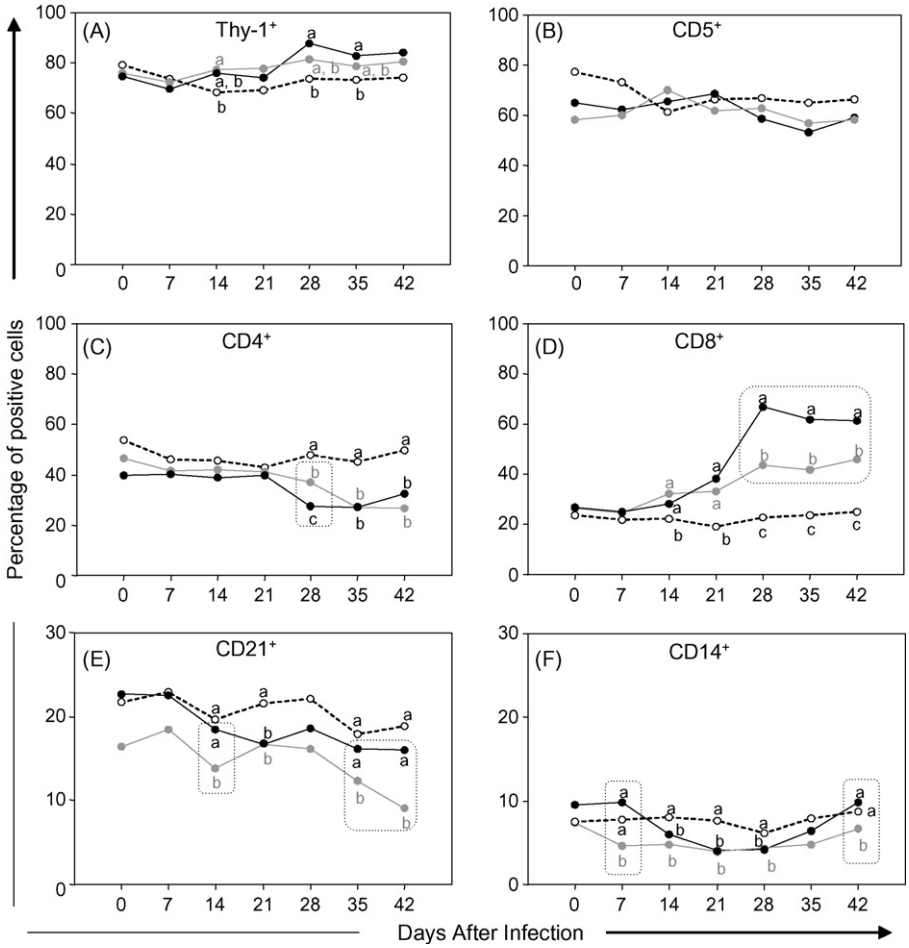

Fig. 3. Kinetics of circulating T-lymphocytes – Thy-1$^+$ (A), CD5$^+$ (B), CD4$^+$ (C) and CD8$^+$ (D), B-cells – CD21$^+$ (E) and monocytes – CD14$^+$ (F) in canine peripheral blood following infection with metacyclic (●) or blood (●) trypomastigotes of Be-78 *T. cruzi* strain in comparison to uninfected controls (◯). Flow cytometry immunophenotyping data are expressed as mean percentage of positive cells within gated lymphocytes, except for monocytes estimated as the percentage of CD14+ cells within ungated leukocytes. Different letters represent significantly difference at $P < 0.05$. Doted rectangles highlight significantly differences between MT and BT at $P < 0.05$.

$R^2 = 0.8016$; BT $\rightarrow y = 0.5293x + 24.277$, $R^2 = 0.9025$), specific changes relating to MT infection were implied by the higher percentages of CD8$^+$ cells observed during the period from days 28 to 42 in comparison with BT infection.

A further inoculum-independent intrinsic feature of infection by *T. cruzi* strain Be-78 was the significant decrease in the level of CD21$^+$ B-cells, in comparison with uninfected controls, recorded on day 21 in both MT- and BT-infected animals (Fig. 3E). This observation was also confirmed by linear regression analysis (MT $\rightarrow y = -0.1657x + 22.2$, $R^2 = 0.784$; BT $\rightarrow y = -0.163x + 18.122$, $R^2 = 0.5972$). In this case, however, analysis of the B-cell compartment revealed BT-specific immunological features as exemplified by the lower percentages of CD21$^+$ cells in BT-infected animals on days 14, 35 and 42 compared with MT-infected counterparts.

Lower levels of CD14$^+$ monocytes cells were observed in MT- and BT-infected dogs on days 14, 21 and 28 in comparison with uninfected controls (Fig. 3F). Moreover, a further BT-specific phenotypic feature was revealed by the lower frequency of monocytes recorded on days 7 and 42 following infection with BT compared with MT. Linear regression analysis did not, however, offer any further support for this finding (MT $\rightarrow y = -0.0386x + 7.9346$, $R^2 = 0.0507$; BT $\rightarrow y = -0.0096x + 5.3952$, $R^2 = 0.0124$).

Consistent with the observed increased levels of Thy-1$^+$ T-cells and the reduced frequencies of B-lymphocytes observed following infection with *T. cruzi* strain Be-78, the data revealed an increase in the Thy-1$^+$/CD21$^+$

Table 1
Kinetics of Thy-1$^+$/CD21$^+$ and CD4$^+$/CD8$^+$ cell ratios in canine peripheral blood during acute infection with metacyclic or blood trypomastigotes of *T. cruzi* strain Be-78

Phenotype	Groups	Days after infection					
		7	14	21	28	35	42
THY1$^+$/CD21$^+$ cell ratio	C	3.6 ± 1.0	3.8 ± 1.5	3.3 ± 1.5	3.7 ± 15	4.4 ± 1.1	4.9 ± 1.3
	MT	3.5 ± 0.8	5.0 ± 2.9*	4.6 ± 2.1*	4.9 ± 1.3*	5.3 ± 1.6*	5.6 ± 1.6*
	BT	4.1 ± 1.3	6.2 ± 2.0*	5.4 ± 1.9*	5.6 ± 1.4*	6.4 ± 1.2*	8.9 ± 1.0*,#
CD4$^+$/CD8$^+$ cell ratio	C	2.2 ± 2.1	2.1 ± 3.0	2.3 ± 1.3	2.3 ± 0.6	2.0 ± 1.4	2.0 ± 1.0
	MT	1.7 ± 0.8	1.4 ± 1.0	1.1 ± 0.4*	0.4 ± 1.3*,#	0.4 ± 0.4*	0.5 ± 0.9*
	BT	1.8 ± 0.9	1.4 ± 1.0	1.3 ± 1.0*	0.8 ± 0.9*	0.6 ± 1.2*	0.6 ± 0.7*

C: uninfected control group; MT: group infected with metacyclic trypomastigotes; BT: group infected with blood trypomastigotes. Data are expressed as mean cell ratio ± standard deviation. *Significant differences ($P < 0.05$) between infected (MT and BT) groups and control groups; #Significant differences ($P < 0.05$) between MT and BT groups.

cell ratio in MT- and BT-infected animals, compared with uninfected controls, starting on day 14 (Table 1). Moreover, a pronounced BT-specific increase, compared with the MT group, in the Thy-1$^+$/CD21$^+$ cell ratio was observed on day 42, and this is consistent with the significant reduction in B-lymphocytes recorded exclusively in BT-infected dogs.

The progressive decrease of CD4$^+$ T-cells together with the continuous increase of CD8$^+$ T-cells observed following infection with *T. cruzi* strain Be-78, gave rise to an inverted CD4$^+$/CD8$^+$ cell ratio starting on day 21 that could be recognised as a further intrinsic immunological feature of the infection (Table 1). However, the significant MT-specific increase of CD8$^+$ T-cells observed on day 28, together with the remarkable reduction in frequency of CD4 + T-cells, produced a considerably lower CD4$^+$/CD8$^+$ cell ratio compared with BT-infected animals, which served as a characteristic immunological feature of MT infection.

4. Discussion

This paper reports a follow-up investigation of the major parasitological, serological and phenotypic features in dogs experimentally infected with MT and BT forms of *T. cruzi* strain Be-78. Previous results from our laboratories have indicated that infection, *via* the intraperitoneal route, with MT forms of the Be-78 strain leads to a higher parasitaemia peak compared with animals infected with BT forms (Bahia et al., 2002). This apparently controversial data may represent an age-related phenomenon considering that the dogs included in the previous investigation were much younger (average age 60 days) than those presently evaluated (average age 120 days). Major changes in the immunological status of young puppies, involving maturation of the immune response, could be the putative key element contributing to the control of parasitaemia levels in older animals. Age-related increases in resistance to acute *T. cruzi* infection have been recently described in experimental infection in rats (Pascutti et al., 2003).

Shifts of both the prepatent period and the day of maximum parasitaemia, respectively, from day 19 and 22 in BT and from days 23 and 26 in MT, were observed. The longer prepatent period, together with the later parasitaemia peak, in MT-infected animals suggests that parasite/host cell interaction may differ depending on the inoculum source. Similar results, suggesting a differential impact of the inoculum source on the development of the acute phase of Chagas disease, have been previously reported (Lopes et al., 1995; Villalta and Kierszenbaum, 1986). This typical parasitemia profile have been frequently observed for Be-78 as well as other T. cruzi strains in the dog model for Chagas disease, despite the inoculum source and the inoculation route (Lana et al., 1992; Bahia et al., 2002). Therefore, it is important to mention that herein we have consider the terminology of acute disease more related to the chronological aspects of early infection than particularly the patent parasitemia period.

The various infective forms of *T. cruzi* have been shown to display distinct invasion mechanisms occasioned by differential expression of their surface molecules that are also dependent on the phenotypic features of the host target cells (Mortara et al., 1999). It has been demonstrated that both MT and BT forms possess the surface molecules required for parasite–host cell interaction and invasion (Ramirez et al., 1993). However, since BT, but not MT, forms are typically associated with membrane-bound anti-*T. cruzi* immunoglobulins, these forms may interact rapidly with a broader range of target cells thus leading to earlier parasite–host

cell interaction events favouring their faster release into the circulation system of the host. Moreover, it has been reported that MT and BT forms differ with respect to a number of other surface molecules, in particular the glycosylphosphatidylinositol-anchored mucin-like glycoproteins. It has been observed that BT-derived glycoconjugates are more effective in activating macrophages during the early stages of *T. cruzi* infection and in initiating the synthesis of pro-inflammatory cytokines (Bento et al., 1996; Camargo et al., 1997).

Since BT and amastigotes, but not MT and epimastigotes, are able to promote the synthesis of cytokines in macrophages and to activate different compartments of the immune system (Camargo et al., 1997; Dos Reis et al., 2002), we hypothesise that, initially, MT may infect host cells in a silent manner. However, parasite clearance in peripheral blood was observed simultaneously in MT- and BT-infected animals on day 28 following inoculation, and would account for the observed similarities between the MT and BT infection profiles. This suggests, therefore, that the major inoculum-dependent immunological events take place early in the infection process. It is possible that the intrinsic pattern of parasite–host cell interactions triggered during the acute phase by different inoculum sources may influence various immunological aspects of Chagas disease. For this purpose we have further addressed relevant aspects of the immune response elicited during the acute phase of *T. cruzi* infection, focusing attention particularly on the profile of anti-*T. cruzi* IgG reactivity as well as on a range of cell phenotypes related to both innate and adaptive immunity.

Although a progressive enhancement in the level of *T. cruzi*-specific antibodies followed infection by both MT and BT forms, higher IgG titres appeared as an early intrinsic immunological feature of MT infection (days 14 and 21 after infection), and equalisation occurred only during the later stages of infection. This early distinct profile of humoral immune response may result from different events that take place during the first parasite–host interaction. The differentiated immune response against a given infective form, here characterised by a modulated pattern with respect to BT, may represent additional interactions of opsonised trypomastigotes with the Fc-gamma receptors on B-cell surfaces leading to a down-regulation of immunoglobulin synthesis. Indeed, some investigations have focussed specifically on the role of immunoglobulin receptors during *T. cruzi* infection (Araujo-Jorge et al., 1993; Henriques-Pons et al., 2005). Moreover, the ability of MT preferentially to activate IFN-gamma synthesis by macrophages (Camargo et al., 1997) may also account for the establishment of effective cell-cell networks that favour the higher antibody response observed during MT infection.

Since infection with *T. cruzi* induces a strong activation of the immune system during the acute phase of the disease, several investigations have focused on the major features that are triggered by the host immune system early in the infection, with the aim of correlating them with different mechanisms observed during the chronic phase (Marinho et al., 1999). Innate immunity has been described as the key element responsible for the mechanisms linked to disease onset as well as to parasitism control. Despite the relevance of the immunological findings during early experimental murine infection with *T. cruzi*, the mechanisms underlying the early activation of the immune system following *T. cruzi* infection in dogs is poorly understood.

In the present study we have performed a phenotypic analysis of peripheral blood monocytes following the acute infection of young dogs with *T. cruzi*. Our major findings revealed the presence of decreased levels of circulating $CD14^+$ leukocytes that are intrinsic to *T. cruzi* infection independent of the inoculum source. A massive activation of macrophages during the early stages of experimental infection has been previously recorded (Brener and Gazzinelli, 1997). We hypothesise that this decrease in the monocyte count in peripheral blood may reflect macrophage recruitment for target tissues, and this possibility is currently under investigation in our laboratory.

It has been reported that *T. cruzi* infection induces, in both humans and in experimental models, important alterations in the host cellular immune response that reflect major phenotypic changes not only in the innate compartment but also in the adaptive immunity context (Brener and Gazzinelli, 1997; Sathler-Avelar et al., 2003). Moreover, during the early stages of infection, a major activation of B-cells with the minor involvement of T-lymphocytes has been observed (Minoprio et al., 1986; Sathler-Avelar et al., 2003).

In the present study, the phenotypic data relating to adaptive immunity indicated a higher $Thy\text{-}1^+/CD21^+$ and a lower $CD4^+/CD8^+$ cell ratio in both MT- and BT-infected animals occasioned by increased levels of $Thy\text{-}1^+$ and $CD8^+$ T-cells and reduced frequencies of $CD4^+$ T-cells and $CD21^+$ B-lymphocytes. Furthermore, the reduced frequency of $CD14^+$ leukocytes was revealed as the most relevant phenotypic feature intrinsic to *T. cruzi* infection regardless of the inoculum source. BT-specific phenotypic features included an early reduction in the percentage of circulating $CD21^+$ and $CD14^+$ leukocytes together with a higher $Thy\text{-}1^+/CD21^+$ cell ratio on day 42. On the other hand, higher levels of $CD8^+$

T-cells together with a lower $CD4^+/CD8^+$ cell ratio on day 28 were characteristic of MT infection.

Both $CD4^+$ and $CD8^+$ T lymphocyte subsets appear to be involved in the complex events associated with host immunopathology and protection against *T. cruzi* infection. The increase of $CD8^+$ T-cells suggests that these cells participate in protective events during the acute phase of the infection. Indeed, it may be that $CD8^+$ T-cells play a dual role in controlling parasite spreading as well as inducing immunopathology depending on the phase of the disease (Fuenmayor-Meza, 2000; Vitelli-Avelar et al., 2005).

We have been able to perform a comparative analysis of the parasitological and immunological alterations that occur during the acute infection of dogs with *T. cruzi*. Our major observation highlighted that, as parasitaemia is controlled by day 28 following infection, an increase in monocyte count together with an expressive increase in the levels of $CD8^+$ T-cells in the peripheral blood is observed. These findings are in agreement with previous reports that describe the monocytes and the $CD8^+$ cells as important elements underlying protective immunity during acute Chagas disease (Brener and Gazzinelli, 1997; Sun and Tarleton, 1993; Tarleton, 1990, 1991; Tarleton et al., 1992). The prevalence of $CD8^+$ T-cells has also been demonstrated in inflamed infiltrated hearts in both experimental models (Sato et al., 1992; Sun and Tarleton, 1993) and in humans (Fuenmayor-Meza, 2000), as well as in the cardiac tissue of dogs infected with *T. cruzi* strain Be-78 (Caliari et al., 2002).

It is important to mention that during acute *T. cruzi* infection the absolute counts of lymphocytes recruited in the mammalian host are known to increase significantly, besides a typical hyperplasia documented in the spleen and lymph node. Therefore it is relevant to consider these findings in the frame of absolute cell counts. Once established that the changes in percentages of the immune cell types are relevant, as valuable information, we have performed a detailed analysis the absolute counts of the major circulating T-cell subsets as well as B-cells and monocytes. Besides confirming picture described by the analysis of percent values, our findings expressed as absolute cell counts further emphasize the outstanding increase of $CD8^+$ T-cells during acute *T. cruzi* infection in dogs (data not shown).

Our results reveal that MT and BT infections are associated with distinct parasitological and serological findings together with intrinsic and inoculum source-specific changes in the circulating leukocytes. In summary, our findings emphasise the importance of taking into account the inoculum source of *T. cruzi* when attempting to transfer information from experimental models to human infection, since vectorial or transfusional routes of *T. cruzi* infection may trigger distinct parasite–host interactions during the acute phase that may influence relevant biological aspects of chronic Chagas disease.

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