Local and systemic cytokine expression during experimental chronic Trypanosoma cruzi infection in a Cebus monkey model

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SUMMARY

Cebus apella is an acceptable model for chronic chagasic cardiomyopathy (CCC), since it is possible to experimentally induce cardiac lesions after 1 year of Trypanosoma cruzi infection. The T. cruzi Y strain, shown previously to produce CCC in C. apella monkeys, was used to experimentally infect 10 monkeys. Parasitological, serological and clinical parameters were monitored during a 19-month follow-up, and systemic cytokine responses were assessed sequentially in five monkeys selected according to the differential parasitemia pattern exhibited. Ten additional monkeys, infected with the same strain for 5, 10 and 12 years, were analysed cross-sectionally. Three monkeys/time point and one uninfected control animal were sacrificed for gross pathology, histology, presence of parasites, and local cytokine gene expression. Elevated expression of interleukin (IL)-4 was observed throughout the study in monkeys that had persistent, high parasitemias, whereas a high level of interferon (IFN)-γ was seen in monkeys that controlled parasitemias soon after infection. Chronically infected monkeys expressed a nonpolarized, Th0-type response. Cardiac tissue collected from a monkey that succumbed to acute infection had elevated levels of proinflammatory cytokine (IL-1β, IL-6, tumour necrosis factor-α) and interstitial cell adhesion molecule (ICAM)-1, platelet-derived growth factor (PDGF)-α, transforming growth factor (TGF)-β and IL-10 transcripts. Cytokine production in cardiac tissue of chronically infected monkeys was also characterized by elevated expression of ICAM-1, PDGF-α and TGF-β, which correlated with the detection of T. cruzi DNA by polymerase chain reaction.

Keywords Trypanosoma cruzi, Chagas’ disease, Cebus apella, cytokines, RT-PCR

INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas’ disease, is an important cause of heart disease in Latin America (Hontebeyrie-Joskowicz 1992). The mechanisms which are responsible for the development of pathology in Chagas’ disease are not fully understood, however, there is considerable experimental evidence for an immunopathogenic component of chronic disease (DeTito et al. 1985). Over the past decade, it has become clear that cytokines are integral to the complex interactions which are required for induction and regulation of immunological effector function. The failure to control or resolve clinical manifestations often results from inappropriate or misdirected immune responses rather than a lack of effective antiparasite immunity. Characterization of cytokines that are expressed at various stages of disease could provide insights into the processes that culminate in chronic chagasic cardiomyopathy. Although the murine T. cruzi model is particularly suited for studies on acute infections, a model which better resembles the chronic chagasic condition in humans is needed.

To this end, the New World nonhuman primate, Cebus apella, has been developed for use as an experimental model for chronic chagasic pathology (Falasca et al. 1986, Rosner et al. 1989, Malchiodi et al. 1993). This model is advantageous in that it displays the following characteristics: (1) supports a long-lasting parasitemia, detectable by conventional methods; (2) infections evolve through clinical stages typical of those seen in humans: acute, indeterminate and chronic phases, with electrocardiographic alterations, congestive heart failure and gastrointestinal complications; and (3) lesions develop in a relatively short period of time. The present study was designed to investigate the cellular cytokine responses that are induced during the course of T. cruzi infections in C. apella monkeys and to analyse the types of cytokines which are produced locally in chronically affected, cardiac tissue.

MATERIALS AND METHODS

Monkeys

Twenty clinically healthy, feral adult C. apella monkeys
were used in this study. These animals were part of a colony maintained at the Instituto de Investigaciones en Ciencias de la Salud in Paraguay. Monkeys were fed a standard commercial chow containing essential vitamins and minerals. Diet was supplemented with fresh fruit twice weekly; water was supplied ad libitum. Monkeys were treated for standard gastrointestinal parasites and were determined to be free of T. cruzi by parasitologic (microscopy) and serologic (indirect immunofluorescence) methods.

Experimental T. cruzi infections
Ten monkeys (five animals of each sex; average weight = 1.9 kg) were each inoculated subcutaneously with 3.5 × 10^5 T. cruzi Y strain trypomastigotes. The virulence and pathogenicity of the Y strain have been well characterized (Andrade 1974); these parasites have been shown to consistently produce cardiac lesions in experimentally infected C. apella monkeys (Rosner et al. 1989). Experimental animals were sampled at weekly intervals throughout a 19-month follow-up period for parasitemia (direct microscopy), anti-T. cruzi antibody responses [immunoglobulin (Ig)M and IgG enzyme-linked immunosorbent assay, Kaspar et al. 1988], and electrocardiographic (EKG) determinations. Systemic cytokine expression profiles in peripheral blood mononuclear cells (PBMC) from infected monkeys (n = 5) were analysed longitudinally in animals that showed defined parasitemia patterns: high (Monkey ID M120, M125, M143) and low (M136, M140) parasitemias. Blood samples were collected at 7-day intervals for the first month, every 14 days for the next 3 months, and then monthly until the end of the 19-month experimental period. Ten other monkeys, previously inoculated with the identical strain and numbers of parasites, remained chronically infected. These animals had remained infected for five (M114, M131, M132), 10 (M20, M84, M100) and 12 (M3, M11, M43, M45) years, respectively. A subset of infected monkeys, plus an uninfected control, were sacrificed for an analysis of gross pathology, histological abnormalities, and local cytokine expression in cardiac tissue. Monkeys in this group (with corresponding length of infection) were as follows: M136, M143 = 19 month; M114 = 5 years; M20, M100 = 10 years.

Semi-quantitative reverse transcriptase-polymerase chain reaction
An analysis of cytokine mRNA transcripts using reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out according to Samudio et al. (1998). Briefly, PBMC were isolated and cultivated for 48 h at 37°C in 5% CO2 in humidified air in the presence of T. cruzi epimastigote lysate antigen (20 μg/ml) prepared from sonicated parasites or PHA mitogen (10 μg/ml). Cardiac tissue specimens were prepared during necropsy of chronically infected monkeys for RNA (RT-PCR) isolation and frozen at – 80°C until use. Total RNA was extracted from PBMC cultures and cardiac tissue using RNA STAT-60™ reagent (Tel-Test, Inc., Friendswood, TX, USA) and the RNAgents Total RNA Isolation System (Promega Corp., Madison, WI, USA), respectively. First-strand cDNA synthesis was primed with random hexamers.
and murine leukaemia virus reverse transcriptase. The RT template was then PCR amplified using the following primers and probes: \( \beta \)-actin internal control, interleukin (IL)-1\( \beta \), IL-2, IL-4, IL-6, IL-10, interferon (IFN)-\( \gamma \), tumour necrosis factor (TNF)-\( \alpha \), transforming growth factor (TGF)-\( \beta \), platelet-derived growth factor (PDGF)-\( \alpha \) and interstitial cell adhesion molecule (ICAM)-1. Most of these primers and probes were designed by Villinger et al. (1993, 1995) based on conserved human cytokine gene sequences to detect both human and nonhuman primate (Old World monkeys) cytokine mRNA transcripts. The IFN-\( \gamma \) sequences were as reported by Yamamura et al. (1991). PCR was performed and products detected and confirmed by dot-blot hybridization using an internal probe labelled with digoxigenin (Samudio et al. 1998). The analysis of specific mRNA for cytokine expression by semiquantitative RT-PCR involved the comparison of signals for each cytokine under study with respect to \( \beta \)-actin (housekeeping gene). Equal amounts of cDNA obtained following reverse transcription of cellular RNA were normalized to the amount of \( \beta \)-actin and amplified. Following dot-blot hybridization, films were scanned by densitometry. Density values for cytokines were provided as a ratio of each cytokine to the \( \beta \)-actin signal (expressed as arbitrary densitometric unit estimates 1.0, 0.8, 0.6, 0.4, 0.2, 0; Diaz-Mitroma et al. 1995).

**Gross and histopathologic examination of cardiac tissues**

Hearts from necropsied, \textit{T. cruzi}-infected monkeys were weighed and examined for gross pathology. Cardiac tissue specimens were also prepared for histopathologic examination. Samples were collected from four different sites: posterior left ventricle, anterior interventricular septum, posterior interventricular septum, and longitudinal section of the apex. Tissues samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with haematoxylin-eosin (Mallory trichrome stain was used for the specific assessment of fibrosis). Each specimen was assessed for the presence of: inflammatory infiltrates; endocardial, interstitial, and perivascular fibrosis; cellular hypertrophy; myocytolysis; \textit{T. cruzi} amastigote foci.
**T. cruzi-specific PCR**

To confirm the presence of *T. cruzi* in blood and/or cardiac tissue of infected monkeys, a nested PCR with outer and inner primers was used to amplify a genomic repetitive DNA sequence of the parasite (Moser et al. 1989, Ochs et al. 1996). DNA was isolated from whole blood and cardiac tissue stored at – 80°C with a Puregene™ DNA Isolation kit according to the manufacturer’s instructions (Gentra Systems, Inc., Cambridge, MA, USA). The nested strategy served to optimize reaction sensitivity and specificity for *T. cruzi* DNA over that originally observed with primers (TCZ1 and TCZ2) which amplified a 188-nucleotide segment of a 195-nucleotide repetitive DNA sequence (Moser et al. 1989). By using the inner primers TCZ3 and TCZ4, the sensitivity of the PCR assay for detection of *T. cruzi* was determined to be equivalent to one parasite in 10 ml infected blood.

**RESULTS**

Two monkeys (M116 and M120) failed to control the *T. cruzi* parasitemias (peaking at 165,000 and 70,000 parasites per ml) and succumbed to a hyperacute, fulminating infection on days 21 and 40 p.i., respectively. The remaining eight monkeys showed varying levels and duration of parasitemias. Five animals exhibited high parasitemias that ranged from 15,531 to 34,300 parasites per ml. Parasitemias dropped to subpatent levels as early as day 87 p.i. in some monkeys, while persisting in others. Conversely, parasitemias were relatively lower in three monkeys, with peak parasitemias ranging from 859 to 4,695 parasites per ml. These monkeys remained patent for periods ranging from 116 to 270 days p.i.. All monkeys showed anti-*T. cruzi* IgM and IgG responses as mean IgM levels peaked on day 40 and remained constant for 1 year p.i.; mean IgG responses increased gradually, reaching a plateau by day 87 that continued throughout the 19-month experimental period (data not shown). EKG determinations were carried out at approximately 1-month intervals. All monkeys presented with normal EKG at baseline. Abnormal EKG were observed in seven out of 10 monkeys with alterations as follows: first degree atrioventricular block, left ventricular hypertrophy, left anterior hemifascicular block, and left ventricular dilatation (data not shown). EKG alterations began to appear during the acute stage of infection. Two monkeys with low parasitemias showed normal EKG tracings throughout the follow-up period. The course of *T. cruzi* parasitemias in the five animals in which sequential cytokine analyses were carried out is depicted in Figure 1. Systemic cytokine analysis of M120

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**Figure 4** *T. cruzi* parasitemias and systemic Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10)-type cytokine responses in Monkeys M136 and M140 (low parasitemias) after experimental infection. Parasitemia, parasites/ml blood; cytokine expression, cytokine densitometric unit (DU) ratio.

(hyperacute, high parasitemia) revealed an early type 1 response by day 7 p.i., characterized by elevated IFN-γ expression (Figure 2). IL-10 mRNA transcripts were elevated during patentcy in a pattern similar to that of IFN-γ. Histopathological examination of cardiac tissue from both monkeys M116 and M120 revealed severe myocarditis (Figure 3, M120). Numerous, disseminated mononuclear inflammatory foci were observed. Acute cell damage (myocytolysis) was also evident. The lesions were observed in both the atrial and ventricular walls. A severe periendocarditis was also seen in M120. In addition, several inflammatory foci were found to be associated with the presence of pseudocysts containing *T. cruzi* amastigotes (Figure 3). *T. cruzi* genomic DNA was amplified in cardiac tissue collected from the left ventricle and apex of monkey M116. This was correlated with the detection of parasites in histological sections. Gross pathologic examination in this monkey revealed concentric left ventricular hypertrophy.

Two animals with low parasitemias (M136: 3528 parasites per ml; M140: 4695 parasites per ml) showed differences in the length of the patent period (116 and 270 days, respectively). In monkey M136, elevated expression of IFN-γ coincided with the decline in parasitemia and remained high throughout the experimental period (Figure 4). The level of IL-2 mRNA transcripts was low during patentcy, but once parasites were cleared, a gradual increase in IL-2 expression was noted. IL-4 expression was moderate, however, IL-10 levels were persistently elevated throughout the postacute period. In contrast, the cytokine pattern seen in response to the persistent parasitemia in M140 was characterized by type 2 (IL-4, IL-10) responses at the time of patent parasitemia. Type 1 (IFN-γ, IL-2) cytokine expression was manifested only after parasites were cleared (Figure 4).

Monkeys M125 and M143 had high parasitemias (M125: 34 300 parasites per ml; M143: 31 416 parasites per ml) soon after infection. The parasitemia in M125 declined rapidly and was no longer detectable by day 87 p.i. (Figure 1). The expression of IFN-γ and IL-2 mRNA transcripts increased on day 7 p.i. and remained moderately elevated after parasitemia declined on day 28 p.i. (Figure 5). Thereafter, IL-4 and IL-10 expression paralleled that of IFN-γ, resulting in a Th0-type cytokine pattern. Monkey M143 sustained a high parasitemia that persisted until day 228 p.i. (Figure 1). A relatively stronger type 2 response was noted in this monkey as the level of antigen-specific IL-4 transcripts began to rise by

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**Figure 5** *T. cruzi* parasitemias and systemic Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10)-type cytokine responses in Monkeys M125 (high parasitemia, rapid clearance) and M143 (high parasitemia, persistent infection). Parasitemia, parasites/ml blood; cytokine expression, cytokine densitometric unit (DU) ratio.
day 14 p.i. and remained elevated throughout the postacute phase (Figure 5). IFN-γ expression was approximately one-half the level of IL-4 during the same time period.

Monkeys M136 and M143 were sacrificed after the 19-month follow-up period for further pathologic analyses. Histopathological examination showed areas of focal inflammatory infiltrates in the myocardium associated with myocytolysis (Figure 6, M143). Focal areas of interstitial fibrosis were also observed. Lesions were more severe in the anterior portion of the left ventricle. These findings correlated with the left ventricular dilatation observed upon gross examination. Finally, the posterior portion of the left ventricle and the anterior interventricular septum were found to be positive for T. cruzi genomic DNA.

Type 1/proinflammatory cytokines IL-1β and TNF-α were consistently expressed during the acute and postacute periods in all infected monkeys (Figure 7, M136 only). An increase in anti-inflammatory TGF-β coincided with peak parasitemia, followed by a decline during the descending phase of parasitemia, with a subsequent wave of expression in the postacute period (Figure 7).

Ten chronically infected monkeys were sampled cross-sectionally for PBMC cytokine expression after infection periods of 5, 10 and 12 years. Although none of the monkeys demonstrated patent parasitemias by microscopy at the time of sampling, 60% were PCR-positive (blood) for T. cruzi DNA. Systemic cytokine expression patterns were similar in all monkeys. A nonpolarized, Th0-type response was found to predominate in the chronic stage of infection. Typical pathology in chronically infected monkeys revealed concentric left ventricular hypertrophy and apical aneurysm, with myocardial lesions consisting of progressive and chronic inflammatory processes with fibrotic areas and variable mononuclear infiltrates. No T. cruzi amastigotes were found in any of the

**Figure 6** Photomicrographs of histological cardiac sections from Monkey M143. (a) Focus of mononuclear cell infiltration (arrow) (haematoxylin and eosin stain, 100x magnification); (b) Inflammatory mononuclear infiltrate with myocytolysis (haematoxylin and eosin stain, 400x magnification); (c) Focal fibrosis (haematoxylin and eosin stain, 200x magnification); (d) Dense fibrosis with destruction of muscle fibers (Mallory-Trichrome stain, 400x magnification).
The role for an immunological component of pathogenesis during the acute phase of *T. cruzi* infections is still not well documented. However, there is mounting evidence to implicate the presence of parasites as the determining factor in the development of tissue lesions (Jones *et al.*, 1993, Brandariz *et al.* 1995, Belloti *et al.* 1996, Vago *et al.* 1996). Acute *T. cruzi* infections in humans generally results in a mild illness, but fatal cases with severe myocarditis have been reported (Laranja *et al.*, 1956, Dias 1984, Ochs *et al.* 1996). In such cases, the degree of inflammation may not be proportional to the number of parasitized cells. Similarly in the present study, monkeys that succumbed to acute infection revealed disseminated inflammatory foci with few amastigote clusters. In severe cases of acute human myocarditis and in the *Cebus apella* monkey model, the cells associated with myocytolysis are monocellular. Sun & Tarleton (1993) showed that the majority of infiltrating cells in acute inflammatory lesions are CD8⁺ T cells. The authors suggested a direct role for cytotoxic CD8⁺ T cells in the lysis of *T. cruzi*-infected target cells.

In the present study, systemic cytokine expression was analysed in PBMC collected from one (M120) of the two monkeys that succumbed to acute infection. Expression of IFN-γ (type 1 response) was dominant on day 7 p.i. as compared with IL-4 levels (type 2 response). Local cytokine expression in cardiac tissue revealed high levels of growth factor (TGF-β, PDGF-α) and proinflammatory cytokines (IL-1β, TNF-α, IL-6). Increased production of IFN-γ and TNF-α, particularly when mediated by IL-12, has been associated with resistance to *T. cruzi* (Hunter *et al.* 1996). However, when the host is confronted with a high number of virulent parasites, together with a detrimental milieu of cytokines, normally protective type 1 responses when overproduced may become inflammatory. Cytokines such as TNF-α and IL-1β may also alter myocyte function, possibly through the activation of endothelial cells and the production of adhesion molecules, e.g. ICAM-1. Moreover, direct myocyte damage may be caused by CD8⁺ cytotoxic lymphocyte activity (Gorelik *et al.* 1992). It is possible that during acute *T. cruzi* infections, the presence of parasites or parasite antigen on parasitized and nonparasitized myocardial cells is responsible for the induction of CD8⁺ cytotoxicity that results in severe myocarditis.

Eight out of 10 monkeys in our study were able to control the acute stage of infection and progressed to the chronic phase of disease. During this period, five animals presented high parasitemias and three exhibited low parasitemia levels (approximately eightfold differences in peak parasitemias). The presence of IFN-γ transcripts correlated with early parasite clearance from the circulation. In contrast, a
predominant expression of IL-4 correlated with persistence of parasitemia. In addition, IL-10 and TGF-β were prominently expressed in infected monkeys. It has been proposed that parasite-induced production of cytokines IL-10 and TGF-β may influence pathogenicity (Silva et al. 1991, 1992). These cytokines may inhibit protective immune responses, resulting in parasite persistence. However, endogenous IL-10 synthesis may play an important role in down-regulating monokine and IFN-γ responses during infection, thereby preventing host immunopathology caused by the detrimental effects of excessive cell-mediated immune responses (Hunter et al. 1997).

No polarized expression of type 1/type 2 cytokines was evident during the chronic stage of infection in the T. cruzi-infected Cebus model. These findings are in agreement with those of Zhang & Tarleton (1996) and Powell et al. (1998) who found an overlapping cytokine pattern (Th0-type) in two murine models that differed in the outcome of infection. Recently, Samudio et al. (1998) observed a similar pattern in T. cruzi-infected children as they progressed from the acute to indeterminate stage of infection. These authors proposed that multiple Th0 responses suppress parasite burdens to subpatent levels as the cumulative effects of persistent, localized inflammatory responses to sequestered parasites result in chronic disease.

Analysis of local cytokine expression in all chronically infected monkeys revealed a high level of growth factors (TGF-β, PDGF-α). It is possible that the early, intense fibrosis associated with chagasic cardiomyopathy is caused by excessive TGF-β production. Excess TGF-β within a lesion has been associated with unresolved inflammation and fibrotic events (Wahl 1994). TGF-β is also known to induce the expression of PDGF-α in myocytes and vascular structures (Zhao et al. 1995).

Cytokines IFN-γ, IL-1, IL-4 and TNF-α have been shown to enhance expression of MHC class I antigens in human
cardiac myocytes in vitro (Wang et al. 1991). Furthermore, an increased expression of MHC class I molecules has been observed in myocardial cells from chronic chagasic patients (Reis et al. 1993a). Cells expressing TNF-α have been identified in heart tissues from the same patients (Reis et al. 1993b). In the present study, moderate expression of the adhesion molecule ICAM-1 was demonstrated in cardiac tissues of all monkeys examined. Lymphocytes may recognize adhesion molecules on the surface of vascular endothelial cells at sites of inflammation (Tanowitz et al. 1992). It has been suggested that these endothelial cells have an important role in the pathogenesis of Chagas’ disease. Tanowitz et al. (1992) postulated that local production of IL-1β, IL-6, PGE2-α and other cytokines by endothelial cells may lead to a variety of alterations in endothelial cell functions. Discrete foci of necrosis and cellular infiltrates may represent areas where microvasculature alterations have occurred over a period of time. The monkeys under study manifested cardiac damage as determined by gross pathology and histopathological findings regardless of parasitemia level and systemic cytokine pattern. In addition, elevated cardiac cytokine expression correlated with the presence of T. cruzi DNA as determined by PCR. The present study is significant in that it is the first to report on cytokine expression in an appropriate nonhuman primate T. cruzi model that correlates potential immunopathogenic responses to chronic infection with clinical outcome.

ACKNOWLEDGEMENTS

This work was supported by the WHO/TDR Special Program for Research and Training in Tropical Diseases (doctoral fellowship to Margarita Samudio for study in the Department of Parasitology, Tulane University).

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