BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **223,** 365–371 (1996)
ARTICLE NO. 0900

Induction of Proinflammatory Cytokine Expression in Experimental Acute Chagasic Cardiomyopathy

Bysani Chandrasekar, Peter C. Melby, Dean A. Troyer,* and Gregory L. Freeman[1]

*Department of Medicine and *Department of Pathology, University of Texas Health Science Center and Audie L. Murphy Memorial Veterans Hospital, San Antonio, Texas*

Received April 8, 1996

One of the hallmarks of Chagas' disease (caused by *Trypanosoma cruzi*) is progressive cardiomyopathy. The disease is associated with increased serum TNF-α levels, and TNF-α is known to depress cardiac function. It is, however, not known whether the cytokines are produced within the infected myocardium. One-month-old male Lewis rats were injected with cell culture-derived *T. cruzi* trypomastigotes and killed 15 days post-infection. As compared to normal animals, histologic analysis of infected animals revealed dense infection with amastigotes within myocytes and a minimal inflammatory infiltrate in the myocardium. Northern blot analysis of total RNA revealed no signal for IL-1β or TNF-α, and a weak signal for IL-6 in the control rat hearts, and high levels of expression for the three genes in the infected rats. Western blots revealed results similar to that of mRNA levels, suggesting that, in addition to mechanical damage, infection by *T. cruzi* induces proinflammatory cytokine production in the myocardium itself, which may further exacerbate the pathology, and affect adversely myocardial function.

Chagas' disease follows systemic infection by the flagellate *T. cruzi.* Seroepidemiological studies indicate that nearly 15–20 million people are infected in the American continents, and more than 65 million are at risk of being infected with this pathogen (1). Although the disease confers a low rate of mortality, it is associated with high morbidity. The heart is a target organ for this pathogen, which causes both an acute inflammatory cardiomyopathy, and a chronic condition characterized by cardiac dilatation, fibrosis, and contractile depression (2). The acute form of the disease occurs shortly after infection, and is felt to represent an inflammatory condition disproportionate to the cardiac burden of organisms.

In animal models, the mortality of acute cardiomyopathy varies by strain of infecting parasite. Lewis rats have been shown to be a good model of acute disease when infected by the Sylvio X10/7 strain (3). The peak level of parasitemia occurs 14–21 days after the infection, and histopathology of the acute stage demonstrates focal inflammatory infiltrates throughout the myocardium (2). Further, during the acute stage, increased serum levels of tumor necrosis factor (TNF)-α has been demonstrated (4). In other inflammatory models, such as endotoxemia, TNF-α plays a role in cardiac depression (5). In addition, systemic administration of TNF-α to normal animals causes acute reversible myocardial depression (6, 7). Thus, it appears that proinflammatory cytokines have negative inotropic impact; whether locally produced cytokines may participate in this pathological process is not known. The purpose of the present study was to determine if acute infection with *T. cruzi* would induce production of proinflammatory cytokines within the myocardium.

MATERIALS AND METHODS

Animals. One month-old male Lewis rats ($\sim$100g) obtained from Charles River Laboratories, were inoculated intraperitoneally with 1×10^6 cell culture (LLC MK2 rat kidney fibroblasts)-derived *T. cruzi* trypomastigotes (Sylvio X10/7 strain; n = 6). Control rats (n = 4) received 0.9% saline placebo. Animals were housed in a climate- and light-controlled

[1] Address correspondence to Gregory L. Freeman. FAX: (210) 567-6960. E-mail: freeman@uthscsa.edu.

0006-291X/96 $18.00

environment at the Laboratory Animal Care facility at the University of Texas Health Science Center, San Antonio. The animals were euthanized 15 days after inoculation; hearts were collected for histology, and for mRNA and protein analyses. Tissue intended for mRNA and protein analyses were frozen immediately in liquid nitrogen and stored for not more than 3 days at −82° C before extraction.

Histology and histomorphometric analyses. At necropsy, part of the hearts were harvested and immediately fixed in 10% buffered formalin. The tissues were then processed, embedded in paraffin, sectioned at 5 mm, and stained with hematoxylin-eosin (H&E) using standard techniques.

RNA extraction and Northern blot analysis. Total RNA was extracted from frozen heart tissue using acid-guanidium isothiocyanate-phenol-chloroform, and equal amounts (30 μg) of RNA were denatured in 2.2M formaldehyde and analyzed on 0.8% agarose gels containing 0.5 μg/ml ethidium bromide to check RNA integrity and loading equivalency, and electroblotted at 4°C onto a nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) membrane in 0.025M phosphate buffer, pH 6.5 (8, 9). RNA was UV cross linked (Stratalinker 2400, Stratagene, La Jolla, CA) to the membrane. The blot was prehybridized for 1 h at 42°C in a prehybridization buffer that contained 50% formamide, 0.1% SDS, 5X SSC, 2.5X Denhardt's, 250 μg/ml salmon sperm DNA, 50 mM Na_2PO_4, at pH 6.5. The blots were then be hybridized at 42°C for 16 h with the labeled cDNA probe (6×10^4 cpm/ml), washed twice at 23°C in 6X SSPE/0.5% SDS, twice at 37°C in 1X SSPE/0.5% SDS, and once at 57°C in 0.1X SSPE/0.5% SDS. All blots will be exposed at −80°C to Kodak XAR-5 film with Kodak intensifying screens, and the intensity of the autoradiographic bands was semi-quantified by videoimage analysis (8, 9).

The cDNA probes (American Type Culture Collection, Rockville, MD; cross-react with rat) used were mIL-1α (2 kb, *Bam* HI-*Hind* III), hIL-1β (0.6 kb, *Bam* HI-*Sma* I), mIL-6, (1.0 kb, *Eco* RI), hTNF-α (1.3 kb, *Bam* HI-*Hind* III) and hGAPDH ($\sim$1.0 kb, *Bgl* 1-*Pst* I), and were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) to a specific activity of 0.5-1 × 109 cpm/μg, using random hexanucleotide primers (Boehringer Mannheim, Indianapolis, IN). mRNA size was determined in relation to the relative mobility of 28S and 18S rRNA, and an mRNA ladder (GibcoBRL, Grand Island, NY).

Protein extraction and Western blot analysis. Equal amounts of protein (60 μg) per well were separated by 16.5% SDS-PAGE, electrotransferred onto nitrocellulose membranes using 20% methanol, 25 mM Tris, pH 8.3 and 192 mM glycine (8, 9). The membranes were saturated with 10% normal goat serum (pre-immune; Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) to block for non-specificity followed by incubation at 23°C for 1 h and 18 h at 4°C with respective primary antibody (after determining the optimal concentrations of antibodies). Antibodies that cross-react with rat IL-1β, IL-6 and TNF-α were obtained commercially from R & D Systems, Minneapolis, MN. Specificity was verified by incubating these antibodies with their respective antigens for 1 h at 37°C followed by 14 h at 4°C. The membranes were washed with a buffer containing 20 mM Tris (pH 7.5), 500 mM NaCl, and 0.05% (v/v) Tween-20, incubated with respective secondary antibody for 2 h at 23°C, washed, and incubated further at 23°C for 2 h with ^{125}I-protein A (0.33 μCi/ml). Autoradiography was performed by exposing the blots to Kodak XAR-5 film at −80°C with intensifying screens. The intensity of autoradiographic bands was semi-quantified by videoimage analysis (8, 9).

Statistical analysis. The data (mean±SEM) for levels of expression of individual mRNA (Northern blot), individual protein level (Western blot) were analyzed using Student's t test, and $p < 0.05$ was considered significant.

RESULTS

Histomorphometric analysis. Histopathologic study of the myocardium from the infected animals revealed dense infection with amastigotes within myocytes (Figure 1); these aggregates incited a relatively minimal inflammatory cell response. There appeared to be some wavy fibers and edema as compared to the uninfected normal animals. In addition, there were some mononuclear cells scattered throughout the myocardium. The myocyte nuclei appeared to be somewhat enlarged compared to the uninfected normal animals. At this stage of infection there appeared to be no increase in myocardial fibrosis.

mRNA analysis. Myocardium from animals killed 15 days post-infection and from controls were analyzed for proinflammatory cytokine mRNA expression. Northern blot analysis of total RNA revealed that, in control animals at steady state there was no detectable mRNA for IL-1β or TNF-α, and either weak or undetectable signal for IL-6 mRNA (Fig. 2). Even after 8 days of prolonged autoradiographic exposure specific signal could not be detected for either IL-1β and TNF-α in tissue from control animals. In *T. cruzi* infected rats, however, the signals were readily detectable after 2 days of autoradiographic exposure. As a separate control, we tested for the presence of IL-1α*;* no mRNA for this molecule was detected in either control or in infected animals. Semi-quantification by means of densitometric analysis of the autoradiographic bands seen in Figure 2 showed that the levels were at least 3.3-fold higher than the controls (Table 1).

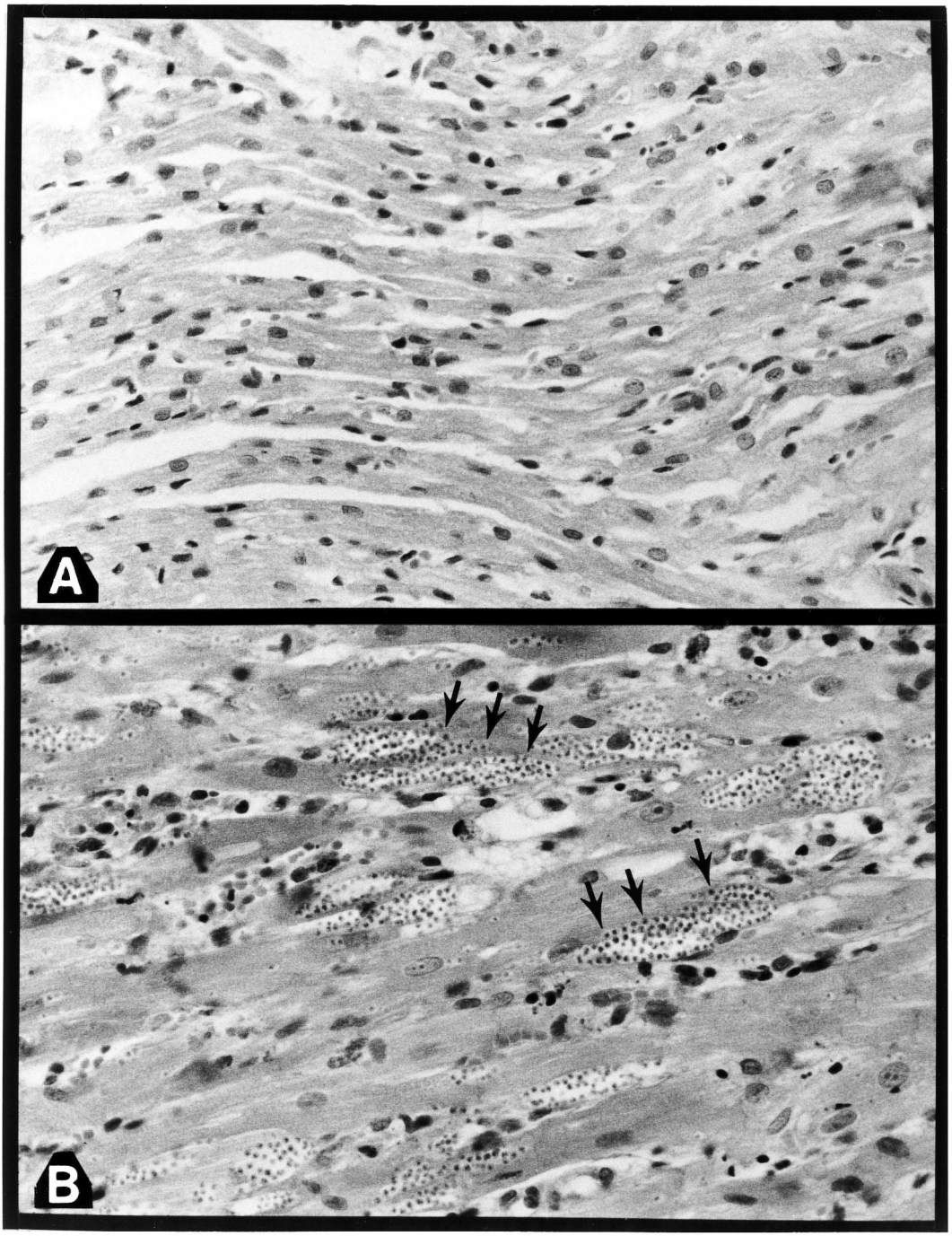

FIG. 1. A representative photomicrograph of myocardium from a control (A) and a *T. cruzi* infected (B) Lewis rat sacrificed 15 days post-inoculation. As compared to controls, infected animals revealed dense infection with amastigotes within myocytes (arrows), and a relatively minimal inflammatory infiltrate in the myocardium. Also in infected animals, some wavy fibers, edema, and enlarged myocyte nuclei were noted.

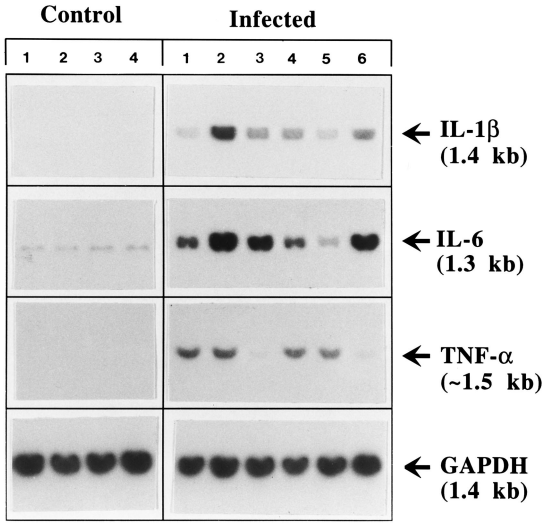

FIG. 2. Proinflammatory cytokine mRNA expression in the myocardium of control and *Trypanosoma cruzi* inoculated male Lewis rats. Thirty micrograms of total RNA per lane was electrophoresed, electroblotted onto nitrocellulose, and fixed by UV irradiation. The blot was reprobed after stripping off previous probe. mRNA size was determined in comparison to the relative mobility of 28S and 18S and to that of the mRNA ladder (0.2–9.5 kb). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control and indicates equal levels of RNA loading in all lanes of the gel. The autoradiographic time was 2 days for interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, and 12 h for GAPDH. Even after 8 days of prolonged exposure, no signal was detected in lanes that did not have detectable levels of mRNA.

Immunoblotting. We also studied the expression of IL-1β, IL-6 and TNF-α by Western blotting. Similar to the results for mRNA, control animals showed only a weak signal for IL-6 and no detectable IL-1β or TNF-α proteins (Figure 3). The signals were, however, readily detectable in

TABLE 1
Densitometric Analysis of Autoradiographic Bands Obtained in Northern and Western Blot Analyses

	Control (n = 4)	Infected (n = 6)
	A. Northern blot analysis	
IL-1α	ND	ND
IL-1β	ND	0.23 ± 0.04
IL-6	0.14 ± 0.01	0.46 ± 0.08*
TNF-α	ND	0.23 ± 0.05
	B. Western blot analysis	
IL-1α	ND	ND
IL-1β	ND	125 ± 29.4
IL-6	38 ± 2.4	119 ± 11.7**
TNF-α	ND	184 ± 36.9

ND: not detected even after 8 days of autoradiographic exposure.

A. The autoradiographic bands shown in Fig. 2 are semi-quantitated by videoimage analysis using NIH Image 1.4 program. The values are mean ± SEM of arbitrary numbers obtained, and are represented as ratio of specific gene expression to that of GAPDH to compensate for any loading differences. *$p < 0.005$ (Vs. control; Student's t test).

B. Values show the result in arbitrary units as determined by image analysis of band densities seen in Fig. 3, and are the mean ± SEM. **$p < 0.001$ (Vs. control; Student's t test).

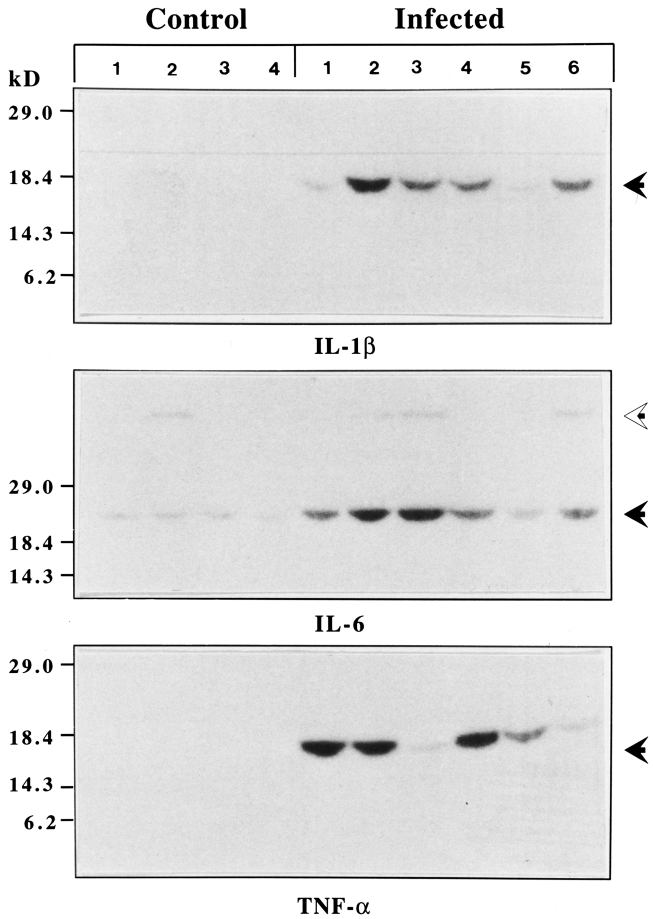

FIG. 3. Western blot analysis of proteins isolated from myocardium of control and *Trypanosoma cruzi* inoculated male Lewis rats. Equal amounts of protein per lane were electrophoresed using glycine-16.5% SDS–PAGE. The separated proteins were electroblotted onto nitrocellulose. After blocking with 10% normal goat serum, the membrane was sequentially incubated with the primary, secondary and ^{125}I-Protein A. Autoradiographic exposure time was 4 days for all three cytokines. Protein molecular weights were determined in comparison to respective recombinant cytokines, and pre-stained low molecular weight standards (shown on the left). Arrow indicates the relative position of protein detected by the respective antibody. Open arrow indicates what may be the glycosylated form of IL-6. No signal was detected even after 8 days of autoradiographic exposure in case of controls.

infected animals (Figure 3). Densitometric analysi s revealed, compared to controls, at least 3-fold higher proinflammatory cytokine proteins levels in the myocardium of infected animals.

DISCUSSION

Our results show for the first time that hearts subjected to acute chagasic cardiomyopathy have increased expression of mRNA for IL-1β, IL-6, and TNF-α, and that these proteins are present in the myocardium. Thus, response to this pathogen includes alteration in local transcription and translation of proinflammatory cytokines.

The impact of cytokines on myocardial contractile function has recently received substantial attention. The original thrust of this work arose from studies of septic shock, a condition in which bacterial infection leads to cardiac depression due to circulating factors (6). A number of cytokines, including IL-1, IL-6 and TNF-α, have been shown to depress myocardial performance. Increased circulating TNF-α secondary to endotoxemia or following systemic administration of TNF-α is

known to cause reversible cardiac depression in humans and in experimental animals (7, 10, 11). Whether these proteins participate in the contractile depression occurring in acute chagasic cardiomyopathy is not known.

Although IL-1β, IL-6, and TNF-α are the products of different genes which bind to distinct receptors, they exert a number of very similar biological activities (12). These cytokines can act by both autocrine and paracrine pathways, in several instances synergistically. While it appears that their purpose is predominantly related to combating infectious pathology, in excess they may have adverse impact on the host organism, which may even be fatal. Of note, correlation of in vitro and in vivo studies is not simple. In an in vitro study it was shown that macrophages treated with TNF-α inhibited intracellular replication of *T. cruzi* (13). On the other hand, in an in vivo study higher mortality was noted when TNF-α was systemically administered to mice subsequently infected with *T. cruzi* (14). Assessment of the impact of local cytokines on contractile performance in this model is critical to a better understanding of the pathogenesis of this disease.

Prior work has shown that the proinflammatory cytokines are highly chemotactic, inducing migration of inflammatory mononuclear cells to their site of production. It is of interest that our results showed that after 15 days of infection, *T. cruzi* infected rats had a minimal inflammatory myocardial infiltrate, despite large numbers of intracellular amastigotes. While the site of origin of these cytokines in the chagasic myocardium cannot be determined from our studies, the lack of white cell infiltrate suggests they arose from cells native to the heart; further work will be needed to establish whether the cytokines were made by the myocardium, vascular cells, or fibroblasts. We and others have observed earlier cytokine gene expression in post-ischemic myocardium in which no inflammatory infiltrate was present (15, 16). Further, the absence of IL-1α expression supports the notion that the observed cytokine expression originated from myocardial rather than inflammatory cells. Since these mediators are also known to stimulate production of toxic free radicals (reactive oxygen intermediates and nitric oxide) (17–19), which are known to adversely affect the myocardial function, their role in contractile depression may be generally underestimated.

In summary, the present study demonstrates that both mRNA and protein levels for IL-1β, IL-6 and TNF-α are elevated in myocardium of rats infected with *T. cruzi*. Thus, in addition to parasitism of the cardiac myocyte, with subsequent myocytolysis, local synthesis of proinflammatory cytokines may promote myocardial dysfunction. Further studies will be required to define the magnitude of the effect of each of these individual factors, a process which may aid in development of treatment strategies for this important problem.

ACKNOWLEDGMENTS

This research was supported by the Research Service of the Department of Veterans Affairs. The authors thank Emilio R. Garcia for excellent technical assistance, and Dr. James Dvorak, Laboratory of Parasitic Diseases, NIH for kindly providing *T. cruzi.*

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