**Trypanosoma cruzi**–Infected Cardiomyocytes Produce Chemokines and Cytokines That Trigger Potent Nitric Oxide–Dependent Trypanocidal Activity

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**Background**—The pathogenesis of myocarditis that occurs in *Trypanosoma cruzi*–infected mice is still poorly understood. Therefore, it is important to know the mediators that trigger leukocyte migration to the heart as well as the cellular source of these possible mediators. In this study, we investigated (1) NO synthase (NOS) induction, (2) NO synthesis, (3) trypanocidal activity, and (4) chemokine and cytokine mRNA expression by isolated cardiomyocytes infected with *T. cruzi*.

**Methods and Results**—Mouse cardiomyocytes were isolated, infected with *T. cruzi*, and evaluated for induction of inducible NOS (iNOS), nitrite production, trypanocidal activity, and cytokine and chemokine mRNA expression. We found that *T. cruzi*–infected murine embryonic cardiomyocytes produced nitrite and expressed mRNAs for the chemokines chemokine growth-related oncogene, monokine induced by interferon-γ, macrophage inflammatory protein-2, interferon-γ-inducible protein, RANTES, and monocyte chemotactic protein, for iNOS, and for the cytokines tumor necrosis factor (TNF)-α and interferon (IFN)-γ. Separate addition of IL-1β, interferon-γ, TNF-α or monocyte chemotactic protein, macrophage inflammatory protein-2, and interferon-γ-inducible protein, to cultured cardiomyocytes resulted in NO production but low trypanocidal activity. However, simultaneous addition of IL-1β, interferon-γ, and TNF-α or the chemokines to cultures resulted in the induction of iNOS, high levels of nitrite, and a marked trypanocidal activity. The iNOS/L-arginine pathway mediated the latter activity, inasmuch as it was inhibited by treatment with N^G^-monomethyl-L-arginine.

**Conclusions**—These results indicate that iNOS activation and the proinflammatory cytokines and chemokines produced by cardiomyocytes are likely to control parasite growth and cell influx, thus contributing to the pathogenesis of chagasic cardiomyopathy seen in *T. cruzi*–infected mice. (*Circulation*. 2000;102:3003-3008.)

**Key Words:** heart diseases ■ myocarditis ■ myocytes ■ interleukins ■ nitric oxide

Chagasic cardiomyopathy is one of the most common forms of heart disease in the world and is a consequence of infection with the hemoflagellate parasite *Trypanosoma cruzi*, the causal agent of Chagas’ disease. In the acute phase of *T. cruzi* infection, an intense myocarditis is frequently found. Infected individuals may also experience a chronic phase, in which both cardiac fibrosis and dilatation are prominent. Alternatively, a progressive hypertrophy of the digestive organs may occur, characterizing the digestive form of chronic Chagas’ disease. In the heart or digestive tissue lesions, parasites are found only when more sensitive methods, such as polymerase chain reaction (PCR) or immunohistochemistry, are used.

Recently, we and others have shown that resistance to *T. cruzi* infection is associated with the capacity of lymphocytes to generate interferon (IFN)-γ, which in turn can activate macrophages to produce NO, the main effector molecule that controls intracellular *T. cruzi* multiplication. NO is synthesized in macrophages by inducible NO synthase (iNOS), which is induced by IFN-γ acting synergistically with tumor necrosis factor (TNF)-α. Both of these cytokines are produced during the acute phase of *T. cruzi* infection with concomitantly an increase in the plasma concentration of nitrite, the end product of the activation of the L-arginine/NO pathway. In addition to macrophages, many other cells are able to produce NO by use of the inducible or constitutive isoforms of the NO synthase (NOS) enzyme. For example, murine myocardial cells produce NO in vitro via the induction of NOS by cytokines such as IFN-γ and interleukin (IL)-1β or by IL-6 and TNF-α in the presence of lipopolysaccharide. Furthermore, iNOS has been shown to be inducible in the...
myocardium of animals infected with coxsackievirus \(^{12}\) or with *T. cruzi* \(^{13}\) and in mice with experimental autoimmune myocarditis.\(^{14}\)

The ability of NO to mediate microbicidal activity against a wide variety of parasites,\(^{15}\) including *T. cruzi*,\(^{4,5,9}\) the observation that myocytes produce NO in response to TNF-\(\alpha\) and IL-1\(\beta\)\(^{10,11}\) and the fact that these cytokines are detected in the heart of *T. cruzi*-infected rats\(^{13}\) prompted us to investigate whether cardiac myocytes could be involved in NO-dependent trypanocidal activity. Also, because we have shown that *T. cruzi*-infected macrophages produce chemokines that enhance NO production and microbicidal activity\(^{16}\) and because rat myocardial cells stimulated with TNF-\(\alpha\) and IL-1\(\beta\) produce chemokines,\(^{17}\) we investigated whether *T. cruzi*-infected myocytes could express chemokines and whether these mediators could stimulate cultured cardiac myocytes to produce NO.

We showed that parasite-infected cardiomyocytes express message for TNF-\(\alpha\), IL-1\(\beta\), and iNOS and for the chemokines chemokine growth-related oncogene (KC/GRO), macrophage inflammatory protein (MIP)-2, monokine induced by interferon-\(\gamma\) (Mig), interferon-\(\gamma\)-inducible protein (Crg-2/IP-10), RANTES, and monocyte chemotactic protein (JE/MCP-1). IFN-\(\gamma\), IL-1\(\beta\), and TNF-\(\alpha\)-stimulated or chemokine-stimulated cardiomyocytes produce critical levels of NO that control *T. cruzi* replication. The release of cytokines and chemokines by infected cardiomyocytes may be essential not only for NO production locally but also for the modulation of leukocyte recruitment at the site of the infected tissue.

**Methods**

**Parasites and Experimental Infection**

The Y strain of *T. cruzi* was used in all experiments. Trypomastigote forms were grown and purified from a monkey kidney fibroblast cell line (LLC-MK\(\_\_\)). BALB/c mice were infected with 1000 blood-derived trypomastigotes and euthanized 9 days later. The hearts were rapidly removed and minced, and the cardiac fragments were exhaustively washed, added to Trizol LS reagent (Life Technologies), and stored at \(-70^\circ\)C until mRNA isolation.

**Embryonic Cardiomyocyte Cultures**

Heart cell cultures were prepared as previously described.\(^{18}\) Briefly, hearts from 19- to 20-day-old BALB/c mouse embryos were dissected, minced, and incubated for 5 minutes at 37°C in 0.05% trypsin (GIBCO-BRL) and 0.01% collagenase type II (Worthington Biochemical Corp) in a Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS solution. Trypsin activity was interrupted by adding 10% FBS (HyClone). Tissue clumps were incubated 8 or 9 times in a trypsin-collagenase solution. After trypsinization, the suspension was centrifuged, and the cell pellet was suspended in DMEM supplemented with 15% horse serum, 5% FBS, 2% chick embryo extract, 1 mmol/L glutamine, 1000 U/mL penicillin, and 50 mg/mL streptomycin (all from Sigma Chemical Co). For cardiomyocyte enrichment, the cell suspension was preplated in tissue culture flasks and incubated at 37°C for 45 minutes in a 5% CO\(_2\) atmosphere, after which time the cultures flask were gently shaken, and the unattached myocytes were withdrawn with a pipette. The cells were then plated on a gelatin-treated 24-well tissue plate (Corning), and the medium was replaced daily. The percentage of CD14\(^+\) and CD11b\(^+\) cells decreased significantly after cell preadhesion, reaching values close to zero (0.57% and 0.04%, respectively) when the cells were preincubated in uncoated plates for 45 minutes.

**Evaluation of NO\(_2^-\) Production by Cardiomyocytes**

Cardiomyocyte suspensions (1x10\(^6\) cells/mL) were plated onto 24-well tissue culture plates and incubated overnight until spontaneous contractility was observed. The cardiomyocytes were then infected at a parasite-to-cell ratio of 5:1 for 3 hours, after which the extracellular parasites were removed, and the cells were incubated at 37°C in 5% CO\(_2\) in the presence or absence of 100 U/mL of recombinant murine IFN-\(\gamma\), IL-1\(\beta\), and TNF-\(\alpha\) (GIBCO), 100 ng/mL of monocyte chemotactic protein (MCP)-1, MIP-2, Crg-2, and RANTES (all from R&D Systems), and 400 \(\mu\)mol/L of \(N^4\)-monomethyl-L-arginine (L-NMMA, an L-arginine analogue NOS inhibitor). The supernatants were harvested at different times after infection and assayed for nitrite concentration by mixing 0.1 mL of culture supernatant with 0.1 mL of Griess reagent.\(^{19}\) Absorbance at 540 nm was read 10 minutes later, and NO\(_2^-\) concentration was determined by reference to a standard curve of 1 to 200 \(\mu\)mol/L NaNO\(_2\).

**Determination of NOS Activity: Effect of Different NOS Inhibitors and EGTA**

NOS activity of infected cardiomyocytes was evaluated 72 hours after incubation with DMEM or stimulation with IFN-\(\gamma\), TNF-\(\alpha\), and IL-1\(\beta\) (100 U/mL) in the presence or absence of 0.5 mmol/L \(L\)-arginine in 5% CO\(_2\) (Life Technologies), and stored at \(-70^\circ\)C until mRNA isolation.

**Cardiomyocyte and Macrophage Cultures for RNA Extraction**

BALB/c mouse inflammatory macrophages were harvested from peritoneal cavities 3 days after injection of 1 mL of 3% sodium thioglycollate. The cells were washed and suspended to 1x10\(^6\) cells/mL in RPMI-1640 (Sigma), supplemented with 5% FBS, 5x10\(^{-4}\) mol/L 2-mercaptoethanol, 2 mmol/L \(L\)-glutamine, and antibiotics. The adherent cells were obtained after 2 to 4 hours of incubation of single-cell suspensions in 24-well tissue culture plates at 37°C. Nonadherent cells were removed, and trypomastigote forms were added at a 5:1 parasite-to-cell ratio to macrophages or cardiomyocytes and incubated for 12 hours at 37°C in a humidified chamber containing 5% CO\(_2\). The cells were then washed 3 times, and 0.5 mL of Trizol LS reagent was added to each well, followed by incubation at room temperature for 5 minutes and storage at \(-70^\circ\)C until RNA extraction.

**Chemokine and Cytokine mRNA Detection**

Total RNA was extracted by use of the Trizol LS reagent (GIBCO). Expression of mRNA was analyzed by reverse transcription-PCR for the following: chemokine growth-related oncogene (KC/GRO), MIP-2, MIP-1\(\alpha\), MIP-1\(\beta\), monokine induced by IFN-\(\gamma\) (Mig), interferon-induced protein (Crg-2/IP10), RANTES, and JE/MCP-1; cytokines TNF-\(\alpha\), IL-1\(\beta\), and IFN-\(\gamma\); iNOS; and \(\beta\)-actin. PCRs were performed by use of Taq polymerase (GIBCO) in a PTC-100 thermal cycler (MJ Research). Reaction conditions were 35 cycles of 1 minute at 94°C, 1 minute at 54°C, and 2 minutes at 72°C, with a final extension step of 7 minutes at 72°C. For each set of primers, a negative sample (water) was run in parallel. PCR products were separated by acrylamide gel electrophoresis and stained with silver nitrate. The PCR method for the chemokines tested has been...
validated in the laboratory by using plasmids containing the gene for each chemokine (kindly provided by J. Farber, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Md). The CXC and CC chemokines, cytokines, and β-actin sequences used have been previously described.22,23

Cardiomyocyte Microbicidal Activity

To evaluate parasite growth, cardiac cell suspensions presenting spontaneous contractility were infected, the extracellular parasites were removed, and the cells were incubated with or without cytokines, chemokines, or L-NMMA as described above. Parasite growth was evaluated by daily counting of the trypomastigote forms released on days 3 to 6 after infection. The intracellular amastigote growth rate was evaluated in cardiomyocytes plated (5 × 10^5 cell/mL) onto chamber slides (Nunc Inc), as described.9

Statistical Analysis

The results are expressed as mean ± SEM of the triplicate cultures or experiments. Statistical analysis was performed by ANOVA followed by the Student-Newman-Keuls test (INSTAT software, GraphPad). A value of *P*<0.05 was considered to indicate significance.

Results

Trypmastigote Forms Trigger Expression of TNF-α, IL-1β, and iNOS mRNA in Cardiomyocytes

Expression of TNF-α, IL-1β, IFN-γ, and iNOS mRNA was detected in hearts obtained from mice on day 9 after infection with *T cruzi* but not in hearts from uninfected control mice (Figure 1A). We next evaluated whether infected cardiomyocytes could be the source of these messages. We found messages for TNF-α, IL-1β, and iNOS but not for IFN-γ (Figure 1B). Time course studies showed that maximal expression of mRNA was observed 12 hours after infection with *T cruzi*.

NO_2^- Production by Infected Cardiomyocytes

Significant levels of nitrite were detected in supernatants of cardiomyocytes incubated for 120 hours with IFN-γ, IL-1β, and/or TNF-α and infected with *T cruzi*. Nitrite was not detected in the supernatants of control cultures (Figure 2). Simultaneous addition of these 3 cytokines to the cells resulted in an even higher amount of NO_2^- production. Addition of L-NMMA resulted in a drastic decrease in the production of NO_2^-.

Figure 1. IL-1β, TNF-α, IFN-γ, and iNOS mRNA expression in myocardium and cardiomyocytes infected with *T cruzi*. RNA was extracted from mice heart cells (A) of uninfected (U) or *T cruzi*-infected (I) mice or from cardiomyocytes (B) cultured in medium alone (M) or with *T cruzi* trypomastigotes (Tc). Equal amounts of cDNA were loaded in each lane. Results shown are representative of 3 different experiments.

Figure 2. IL-1β, TNF-α, and IFN-γ induce NO synthesis by infected cardiomyocytes. Nitrite levels were assayed in supernatants of *T cruzi* (Tc)-infected cardiomyocytes stimulated with IFN-γ (▲), TNF-α (■), and IL-1β (○) alone or simultaneously (ILs), with ▲ or without ● L-NMMA. Uninfected (○) or infected (100 U/mL ILs; ●) cells cultured with medium alone were used as controls. Supernatants of cultured cells were harvested, and nitrite concentration was assayed. Data represent mean ± SD of triplicate samples and are representative of 3 separate experiments. *P*<0.05 compared with values in absence of parasite and cytokines.

Figure 3. NOS activity in *T cruzi*-infected cardiomyocytes stimulated with IL-1β, TNF-α, and IFN-γ. Cardiomyocytes were infected with *T cruzi*, stimulated with the cytokines (ILs,100 U/mL) with or without aminoguanidine (●), L-NIO (●), and L-NMMA (■), and NOS activity (A) was evaluated. As controls, cells were incubated with medium only (○). The Ca^{2+}-dependent (cNOS) and Ca^{2+}-independent (iNOS) enzyme activities (B) were determined in normal or infected cardiac myocytes cultured with cytokines for 24 and 48 hours. Each point represents mean ± SD of triplicate samples and is representative of 3 separate experiments. *P*<0.05 compared with value obtained in absence of NOS inhibitor.
levels of nitrite detected in the supernatants (Figure 2). We next observed that the cytokine mixture enhanced NOS activity, which was inhibited in a dose-dependent manner by L-NMMA, L-NIO, and aminoguanidine. Uninfected cardiomyocytes had no detectable NOS activity (Figure 3A).

Next, we evaluated Ca²⁺-independent (iNOS) and Ca²⁺-dependent (constitutive NOS [cNOS]) activity in cardiomyocyte lysates previously infected and cultured in the presence of cytokines. We found that most [¹⁴C] L-arginine that converted to [¹⁴C] L-citrulline after a 24- or 48-hour culture was due to iNOS (>87%), inasmuch as it was not inhibited by EGTA (Figure 3B), which blocks cNOS activity. We concluded that iNOS was the major isof orm of NOS present in infected murine cardiomyocytes stimulated with IFN-γ, TNF-α, and IL-1β. A significant expression of iNOS enzyme was detected by immunohistochemistry in cells infected with T cruzi but not in control myocytes (data not shown).

**Microbicidal Activity of Cytokine-Stimulated Cardiomyocytes**

To address the question of whether NO produced by cytokine-stimulated cardiomyocytes could control parasite replication, established cultures were infected and cultured with IFN-γ, TNF-α, and/or IL-1β, and the growth of parasites was determined. As shown in Figure 4, all cytokines significantly inhibited parasite growth. The addition of IFN-γ or of all cytokines simultaneously resulted in a further reduction of the numbers of parasites released (Figure 4A). The inhibition of parasite growth was significantly blocked by the addition of L-NMMA to the cultures, suggesting that NO production was critical for the microbicidal activity of cytokine-stimulated myocytes. These results were confirmed by determining the number of intracellular amastigotes in infected cardiomyocytes (Figure 4B).

**Trypomastigote-Induced Chemokine mRNA Expression in Infected Cardiac Myocytes**

We next investigated whether T cruzi–infected cardiomyocytes and macrophages expressed chemokine mRNA. A message for RANTES but not for other chemokines was detected in cardiomyocytes incubated with medium only. Control uninfected macrophages expressed mRNA for RANTES and Mig. However, after T cruzi infection, the cardiomyocytes expressed messages for JE, RANTES, KC, MIP-2, Mig, and Crg-2 chemokines. For comparison, positive messages for JE, RANTES, MIP-1α, MIP-1β, KC, MIP-2, Mig, and Crg-2 were found in T cruzi–infected macrophages (Figure 5). The message for lymphotactin, T-cell activation gene, lipopolysaccharide-induced CXC chemokine, stromal cell-derived factor 1α, stromal cell-derived factor 1β, and osteopontin was not detected in control or infected cardiomyocytes and macrophages (data not shown).

**NO₂⁻ Production and Microbicidal Activity of Chemokine-Stimulated Cardiomyocytes**

We also examined whether the chemokines could be involved in the regulation of NO production and microbicidal activity by infected cardiomyocytes. The addition of parasites with or without RANTES (Figure 6A) or MIG, MIP-1β, and KC (data not shown) resulted in small amounts of nitrite production. The addition of JE, MIP-2, or Crg-2 together with parasites led to significant nitrite production (Figure 6A) that

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Effects of cytokine-induced trypanocidal activity in cardiomyocytes. A, Infected cardiomyocytes were cultured with IFN-γ ( ), TNF-α ( ), and IL-1β ( ) alone or in combination (ILs), with ( ) or without ( ) L-NMMA, and released trypomastigote forms were counted daily. Infected cells with ( ) or without ( ) L-NMMA were used as controls. B, Cells were infected and treated as above, cultured for 48 hours, washed, fixed, and stained, and intracellular amastigotes were counted in 500 cells. C indicates control. Each point represents mean ± SD of triplicate samples and is representative of 3 separate experiments. *P<0.05 compared with values obtained in absence of parasites and cytokines.

![Figure 5](http://circ.ahajournals.org/)

**Figure 5.** T cruzi trypomastigotes induce chemokine mRNA expression by cardiomyocytes. Total RNA was extracted from cardiomyocytes (MY) or macrophages (MØ) and cultured in presence of medium alone (M) or medium containing T cruzi (Tc). CDNA was synthesized, and PCR was performed by using specific primers. Equal amounts of cDNA were loaded in each lane. Results shown are representative of 3 experiments.
the inflammatory phenomena, secreting cytokines, chemokines, and NO, which might attract leukocytes to the inflammatory site and control intracellular parasite replication.

We first showed that heart tissue collected from *T. cruzi*–infected mice expresses mRNA to IFN-γ, TNF-α, IL-1β, and iNOS. Hearts from rats infected with *T. cruzi* also express iNOS and proinflammatory cytokines.13 Because the expression of cytokines and iNOS has been evaluated only in whole cardiac tissue, we investigated whether cardiomyocytes could be one of the cellular sources of cytokine mRNA expression. We detected mRNA expression for the cytokines TNF-α and IL-1β and for iNOS in cardiomyocytes cultured with trypanostigote forms. These results show that cardiomyocytes are potential cellular sources of cytokines and iNOS in vivo.

iNOS protein induction in cardiomyocytes was confirmed by its ability to convert [14C]L-arginine to [14C]L-citrulline in an EGTA-independent manner and is supported by the fact that 2 known selective iNOS inhibitors (L-NIO and amino-guanidine) significantly blocked this conversion. Not only were the iNOS message and activity upregulated, but there was also significant NO synthesis after infection of cultured myocytes. These results demonstrate that parasites induce NO production in cardiomyocytes via upregulation of the expression of the inducible isofrom of NOS.

The mechanisms by which iNOS expression is induced by *T. cruzi* in cardiac myocytes remain to be determined. One possibility is that parasite-secreted products, such as trypanostigones glycosylphosphatidylinositol–anchored mucin-like glycoproteins24 and lipopolysaccharide-like molecules,25 may induce the enzyme directly. Alternatively, iNOS expression may result from autocrine stimulation by cytokines and chemokines released by cardiomyocytes after *T. cruzi* infection. In this regard, *T. cruzi* induces the production of β-chemokines by macrophages16 and the expression of mRNA for JE, RANTES, KC, MIP-2, Mig, and Crg-2 by cardiomyocytes (Figure 5). The parasites also induce TNF-α and IL-12 synthesis by macrophages, which results in IFN-γ production by T and NK cells.9,26 The presence of IFN-γ and chemokines in heart tissue of infected mice, in association with IL-1 and TNF-α, could lead to the induction of iNOS. The TNF-α produced may also trigger chemokine production, as previously described.27

Inasmuch as there was considerable induction of iNOS on stimulation of infected cardiomyocytes with cytokines, we investigated whether iNOS-derived NO could mediate trypanocidal activity. Incubation of cardiomyocytes with cytokines or chemokines resulted not only in NO synthesis but also in significant trypanocidal activity. We also found that the addition of selective iNOS inhibitors significantly inhibited NO production and parasite killing, convincingly demonstrating that cardiomyocyte-derived NO possesses signficant trypanocidal activity. The partial inhibition of parasite killing observed in infected cells cultured with cytokines or chemokines in the presence of L-NMMA could occur because the dose of NOS inhibitor used (400 μmol/L) was unable to completely abrogate the NO production.

The importance of the production of these chemotactic cytokines by cardiomyocytes in disease outcome and host immunopathology during *T. cruzi* infection is not known, but
these cells may play an important role in driving or maintaining the local inflammatory response. It is possible that the early chemokine-and cytokine-mediated cardiomyocyte activation may play an essential role in the containment of parasite dissemination during the acute phase of infection. On the other hand, the release of parasites from the amastigotes within cardiac tissue may act locally to enhance the control of parasite replication and spread in the host tissues. Nevertheless, the induction of these molecules in cardiomyocytes could also play a detrimental role in chronic Chagas’ heart disease.1,28 Nitric oxide may directly regulate the contractile properties of muscle cells10 and lead to depressed cardiac function29 and myocardial damage.20 This could further potentiate chemokine-driven inflammation and tissue damage. Moreover, TNF-α may be an autocrine contributor to the myocardial dysfunction seen in patients with chagasic cardiomyopathy.28 We believe that elucidation of the role of chemokines and cytokines in the mediation of inflammatory responses in patients with chagasic cardiomyopathy may have important implications for the development of a therapy designed to protect the host against the infection and the pathology induced by Trypanosoma cruzi infections.

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