Immunology 1994 **81** 462–467

Inhibition of *Trypanosoma cruzi*-specific immune responses by a protein produced by *T. cruzi* in the course of Chagas' disease

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SUMMARY

Immunosuppression is readily demonstrable in the acute phase of *Trypanosoma cruzi* infection but subsides during the chronic phase. *In vitro*, living *T. cruzi* induces important alterations in mitogen-activated human T and B lymphocytes and inhibits their capacity to proliferate. These effects are reproduced by a protein spontaneously released by this parasite, termed trypanosomal immunosuppressive factor (TIF). In this study we asked whether TIF would also inhibit a *T. cruzi*-specific immune response and if it is produced in a mammalian host during infection. A significant reduction in the level of [^{3}H]thymidine incorporation by spleen cells from chronically infected mice stimulated with a *T. cruzi* antigen preparation ensued when TIF was added to the cultures. Production of TIF in *T. cruzi*-infected individuals was denoted by the ability of serum IgG from either chronically infected patients or mice to abolish, in a concentration-dependent manner, the capacity of TIF to suppress interleukin-2 receptor expression by phytohaemagglutinin-stimulated human lymphocytes. This neutralizing activity was absent in the IgG fractions prepared from sera of healthy volunteers, non-infected mice or mice killed at different times during acute *T. cruzi* infection. Circulating anti-TIF antibodies represent indirect evidence of TIF production *in vivo* which, together with TIF-mediated inhibition of *T. cruzi*-specific lymphoproliferation, raise the possibility that TIF controls anti-parasite immune responses *in vivo*. The presence of TIF-neutralizing antibodies during chronic but not acute *T. cruzi* infection may be one of the reasons why immunosuppression is confined to the acute stage.

INTRODUCTION

Acute infection with *Trypanosoma cruzi* (the aetiological agent of Chagas' disease) in both laboratory animals and patients is accompanied by multiple manifestations of immunosuppression.[1,2] However, our understanding of the role of *T. cruzi* in the production of these immunological alterations—believed to facilitate the establishment and dissemination of this parasite in mammalian hosts—is very limited. To disclose the molecular alterations induced by *T. cruzi* in activated lymphocytes, identify the parasite product(s) that trigger these alterations and define the underlying mechanisms we have used an *in vitro* system in which either the parasite or its products are added to cultures of normal lymphocytes activated with different stimuli.[2] In this manner, we were able to establish that, in the presence of *T. cruzi*, a massive arrest of mitogen-activated lymphocytes occurs at the G_0/G_{1a} phase of the cell cycle.[3] This blockade is likely to result, in part, from the inhibitory effects that *T. cruzi* exerts on the expression of high-affinity interleukin-2 receptors (IL-2R),[4,5] IL-2 production,[2,6] and the modulation of constitutive T-lymphocyte molecules playing critical roles in the activation process [e.g. T-cell receptor (TcR), CD3, CD4 and CD8].[7] All of these effects are readily reproduced *in vitro* by trypanosomal immunosuppressive factor (TIF), a protease-sensitive molecule spontaneously released by *T. cruzi* whose molecular weight has been estimated to fall between 30,000 and 100,000.[8] In this study, we sought evidence of TIF production during *T. cruzi* infection and the potential of this molecule to inhibit the host's immune response to *T. cruzi* antigens.

Received 4 October 1993; revised 28 October 1993; accepted 17 November 1993.

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Abbreviations: DPBS, Dulbecco's modified phosphate-buffered saline; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; I-IgG, IgG from chronic chagasic sera; IL, interleukin; IL-2R, interleukin-2 receptor(s); N-IgG, normal human IgG; RPMI+2%FBS, RPMI-1640 medium containing 2% FBS and antibiotics; RPMI+5% FBS, RPMI-1640 medium containing 5% FBS and antibiotics; TcAg, *Trypanosoma cruzi* antigen; TIF, trypanosomal immunosuppressive factor.

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MATERIALS AND METHODS

Parasites

We used *T. cruzi* trypomastigotes (Tulahuen isolate) purified from either blood of infected mice or infected cell cultures.

Blood was drawn from ether-anaesthetized Crl-CD1(ICR) Swiss mice (CD1 mice; Charles River Laboratories, Portage, MI) infected subcutaneously with 1×10^6 organisms 10–11 days previously. The flagellates were separated from blood cells by centrifugation over Ficoll–Hypaque (specific gravity 1·077), followed by chromatography through diethylaminoethyl-cellulose.[9,10] Trypomastigotes grown in rat heart myoblast (American Type Culture Collection CRL 1446; ATCC, Rockville, MD) cultures[11] were separated from other forms of the parasite and host cell debris by centrifugation (400 *g*, 20°, 45 min) through two layers of metrizamide solutions (specific gravities 1·086 and 1·064), recovering the upper metrizamide layer. In all cases, the recovered trypanosomes were washed twice with, and resuspended in RPMI-1640 medium (Gibco, Grand Island, NY) alone or containing 5% heat-inactivated (56°, 1 hr) fetal bovine serum (FBS; Hyclone, Logan, UT) (RPMI + 5%FBS). Blood and cultured parasite suspensions consisted of 100% and > 93% trypomastigotes, respectively; the remainder, if any, being amastigotes. Trypomastigote viability was established by the criterion of motility and was determined to be > 99% in all cases. All media contained 100 IU penicillin and 100 μg streptomycin/ml.

Mouse spleen cells

Spleens were aseptically removed from ether-anaesthetized CD1 (Charles River Laboratory) or inbred CBA/J (Jackson Laboratory, Bar Harbor, ME) mice 150–250 days after intraperitoneal infection with 25 blood *T. cruzi* trypomastigotes. Spleens from age-matched non-infected mice were used for control purposes. Single-cell suspensions were made in ice-cold RPMI-1640 medium supplemented with 2% FBS and antibiotics at the concentrations described above (RPMI + 2%FBS) using a Ten-Broeck tissue grinder. Tissue debris were removed by filtration through sterile nylon gauze and the cells were centrifuged over a cushion of Ficoll–Hypaque (specific gravity 1·077; 400 *g*, 45°, 30 min). The mononuclear cells recovered at the interface were washed twice with RPMI + 2%FBS, counted microscopically using a Neubauer haemocytometer and adjusted to the appropriate concentration of viable (i.e. trypan blue-excluding) cells. Cell viability was consistently > 98%.

Human cells

Heparinized blood from healthy volunteers was centrifuged through Ficoll–Hypaque (see above). The peripheral blood mononuclear cells (PBMC) accumulating at the interface were washed twice with RPMI + 5%FBS, counted microscopically and adjusted to the desired concentration in the same medium. The viability of the PBMC, determined by the criterion of trypan blue exclusion, was routinely > 98%.

Preparation of TIF

The preparation of TIF has been described in detail.[8] Briefly, suspensions of *T. cruzi* at 2×10^7 blood- or cell-culture-derived trypomastigotes/ml in either serum-free RPMI-1640 medium or RPMI + 5%FBS were incubated at 37° and 5% CO_2 for 15–18 hr. The parasites were then removed by filtration (0·45 μm pore size filter). The filtrates were divided into aliquots and stored at −20° until used.

Trypanosoma cruzi *antigen (TcAg)*

The *T. cruzi* antigen used in lymphoproliferation assays was a lysate of purified blood-derived trypomastigotes (TcAg) prepared by three cycles of freezing and thawing.[12] The stock suspension contained 4×10^7 parasite equivalents/ml of RPMI + 5%FBS, was divided into aliquots and was stored at −20° until used.

Human and mouse IgG preparations

Pooled sera from chronic chagasic patients (kindly provided by Dr D. Allain, Centers for Disease Control, Atlanta, GA) and healthy volunteers were used as the sources of anti-*T. cruzi* human IgG (I-IgG) and normal human IgG (N-IgG), respectively. Pooled sera from groups ($n = 5$) of chronically infected mice (killed on day 230 after intraperitoneal infection with 25 trypomastigotes) and age-matched non-infected mice were the sources of mouse anti-*T. cruzi* and normal mouse IgG, respectively. Preparations of IgG were also made from pools ($n = 5$) of sera from acutely infected CD1 mice killed on days 7, 14 and 19 days after intraperitoneal infection with 1×10^4 blood trypomastigotes. Regardless of the serum source, IgG was prepared by two steps of precipitation with ammonium sulphate at 33% saturation followed by extensive dialysis at 4° against phosphate buffer (0·0016 M NaH_2PO_4, 0·0084 M Na_2HPO_4, 0·015 M NaCl) pH 7·55 and subsequent chromatography through diethylaminoethyl-cellulose equilibrated with the same buffer. All of the preparations produced single immunoprecipitation bands with typical IgG mobility when tested by immunoelectrophoresis against goat and sheep antisera specific for human and mouse serum, as appropriate.

Lymphoproliferation assay

Mouse spleen cell proliferation assays were set up in quadruplicate in sterile, 96-well, flat-bottomed plates. Each culture consisted of 2×10^5 mononuclear cells in a final volume of 100 μl. The antigen was TcAg (1×10^6 parasite equivalents/well). When present, TIF substituted for 85% (v/v) of the culture medium. After incubation at 37° and 5% CO_2 for 48 hr, each well received 1 μCi [^{3}H]thymidine (Amersham, Arlington Heights, IL; specific activity 2 Ci/mmol). The cultures were terminated 24 hr later by automated harvesting and were then processed for measurement of incorporated radioactivity in a liquid scintillation counter.

Flow cytometric analysis

Cultures of PBMC containing $1{\cdot}25 \times 10^6$ cells/ml in RPMI + 5%FBS were set up in 24-well plates. Phytohaemagglutinin (PHA; Burroughs Wellcome, Research Triangle Park, NC) was used as the mitogen, at 1 μg/ml. When present, TIF substituted for 85% (v/v) of the culture medium. After 16–18 hr of incubation at 37° and 5% CO_2, the cells were harvested, washed with Dulbecco's modified phosphate-buffered saline (DPBS) and suspended in DPBS containing 1% bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO) and 0·1% sodium azide. To stain for IL-2R, the cells were incubated at 0° for 30 min with a fluorescein isothiocyanate (FITC)-labelled anti-human CD25 monoclonal antibody (Becton Dickinson, San Jose, CA). A FITC-labelled monoclonal antibody of the same isotype and irrelevant specificity was used to determine background fluorescence. The cells were then fixed with 1% formaldehyde and stored at 4° until analysed in an EPICS

ELITE flow cytometer/cell sorter (Coulter Cytometry, Hialeah, FL). Erythrocytes and platelets were excluded from analysis by setting an appropriate gate on the forward versus 90° light scatter parameters. Twenty thousand cells were collected for each sample. The mean channel number of the logarithm of the mean fluorescence intensity of the IL-2R$^+$ cell populations was used to compare the relative density of IL-2R on lymphocytes subjected to the various test conditions.

Assay of TIF neutralization

A volume of sterile IgG solution (not exceeding 33 μl) sufficient to provide the desired final IgG concentration was added to 1100 μl of TIF (made in RPMI + 5% FBS) and the mixture was incubated at 4° for 15 min. Control mixtures were prepared using RPMI + 5% FBS instead of TIF. In addition, an aliquot of TIF was mock treated with medium and subjected to the same physical manipulations. After centrifugation at 16,000 *g* and 4° for 30 min, the supernatants, or fresh RPMI + 5% FBS, were used to set up cell cultures, representing 90% of the final volume. Each culture contained $1{\cdot}25 \times 10^6$ PBMC/ml and, where appropriate, PHA at 1 μg/ml. After 16 hr of incubation at 37° and 5% CO_2, the cultures were harvested and processed for flow cytometric analysis of IL-2R expression as described above.

RESULTS

Inhibition by TIF of *T. cruzi*-specific proliferation by lymphocytes from chronically infected mice

Spleens from chronically infected outbred (CD1) or inbred (CBA/J) mice were used as sources of *T. cruzi*-primed lymphocytes in experiments designed to establish whether TIF would affect a parasite-specific immune response. The level of [^{3}H]thymidine incorporation elicited by stimulation with TcAg was found to be significantly decreased when TIF was present in the cultures (Fig. 1). As can be seen, the extent of the proliferative responses induced by TcAg varied among individual mice but the immunosuppressive effect of TIF was always demonstrable and statistically significant ($P \leqslant 0{\cdot}05$, Student's *t*-test). The levels of [^{3}H]thymidine incorporation in cultures of chronically infected or age-matched non-infected mouse spleen cells containing TIF and lacking TcAg were comparable and statistically indistinguishable from the baseline levels obtained with normal spleen cells incubated in medium alone (data not shown). Spleen cells from non-infected mice did not respond to TcAg to any significant extent (data not shown).

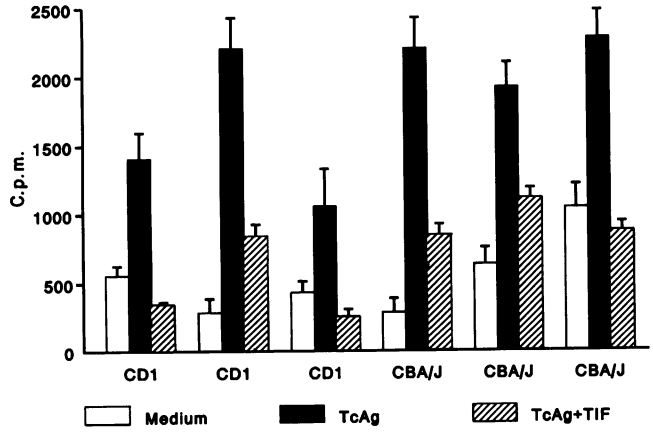

Figure 1. Suppressive effect of TIF on TcAg-induced lymphoproliferation by spleen mononuclear cells from chronically infected outbred (CD1) or inbred (CBA/J) mice. Each set of histograms represents results obtained with cells from a single mouse. Spleens were removed from mice 150–250 days after intraperitoneal infection with 25 blood *T. cruzi* trypomastigotes. All reductions in c.p.m. caused by TIF, relative to the positive control value obtained with TcAg alone, were statistically significant ($P \leqslant 0{\cdot}05$, Student's *t*-test). Bars and vertical lines represent the mean of quadruplicate determinations and the corresponding standard deviation, respectively.

Evidence of TIF production during *T. cruzi* infection

An indirect approach was used to establish whether TIF is produced during *T. cruzi* infection. This approach was based on testing whether anti-TIF antibodies are present in the sera of *T. cruzi*-infected patients or mice. To this end, we monitored the level of inhibition of IL-2R expression by PHA-stimulated PBMC as evidence of TIF activity.[4] Whereas pretreatment of TIF with N-IgG failed to affect its suppressive activity at any of the N-IgG concentrations tested (Fig. 2, upper panels), incubation with I-IgG displayed an inhibitory effect in a concentration-dependent manner (Fig. 2, lower panels). Thus, the capacity of TIF to reduce the proportion of activated PBMC expressing IL-2R was gradually curtailed as the I-IgG concentration increased and was completely abrogated at 30 μg I-IgG/ml (Fig. 3). Similar experiments performed with IgG from chronically infected or non-infected mice also showed that only the chronic mouse IgG could neutralize TIF-induced suppression of IL-2R expression (Fig. 4). In the absence of TIF, none of the IgG preparations, whether from humans or mice, healthy or chronically infected, affected significantly the level of IL-2R expression by PHA-stimulated PBMC at concentrations of up to 30 μg IgG/ml (Fig. 4 for mouse IgG and data not shown for human IgG).

Is anti-TIF activity detectable in IgG from acutely infected mice?

During acute infection, CD1 mice develop parasite levels in their body fluids and tissues which should be more than adequate for antigenic stimulation. However, chronic but not acute chagasic mouse spleen cells mount significant proliferative responses upon stimulation with TcAg.[12] Because of this observation and the fact that other manifestations of immunosuppression are readily demonstrable in mice during acute but not chronic *T. cruzi* infection,[13–16] we were interested in finding out if circulating, TIF-neutralizing antibodies were present in the sera of acutely infected animals. To this end, mice were infected intraperitoneally with 10,000 trypomastigotes, a dose that rapidly leads to relatively high parasitaemias and acute disease.[17] The IgG preparations used in these experiments were purified from the sera of infected mice killed at three different times during the acute phase, 7 (when parasitaemias became patent in all of the infected animals), 14 (after parasitaemias had risen considerably and signs of the disease had become apparent) and 19 (when all of the mice remaining in the lot died) days post-infection (dpi). None of these IgG preparations displayed any significant TIF-neutralizing activity, whereas an aliquot of the TIF preparation incubated with chronic mouse IgG lost its immunosuppressive activity almost completely (Fig. 5).

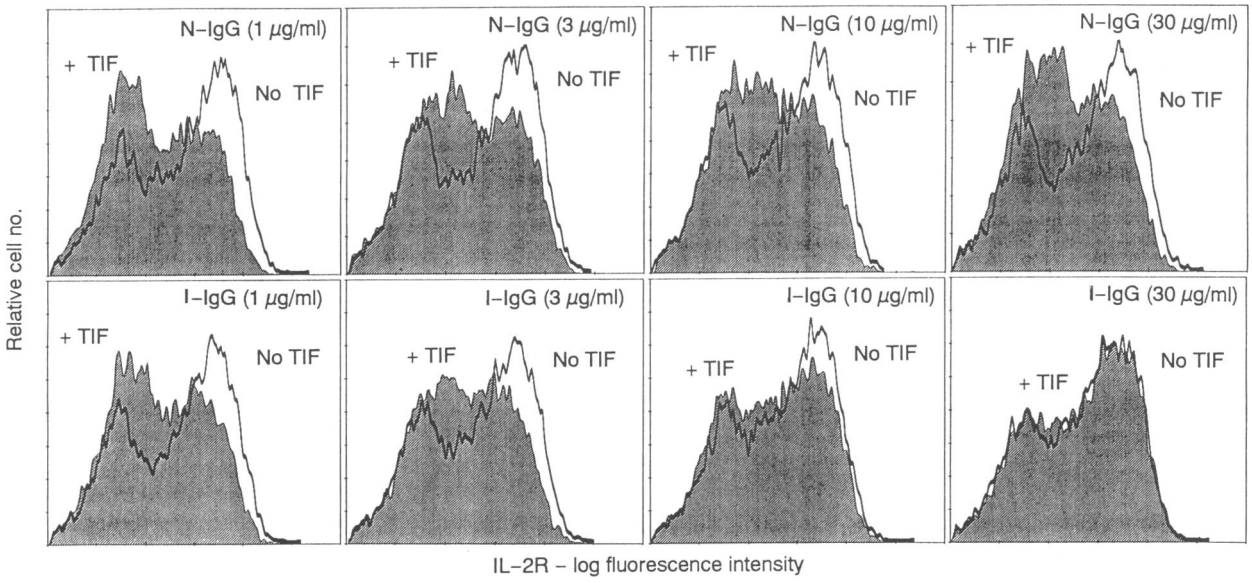

Figure 2. Inhibition by IgG from chronic chagasic patients (I-IgG) of TIF-induced suppression of IL-2R expression by PHA-stimulated PBMC. The concentration-dependent inhibitory effect of I-IgG on TIF activity (lower panels) contrasted with the lack of neutralization afforded by normal human IgG (N-IgG) (upper panels). This set of data is representative of two repeat experiments performed with different batches of TIF and PBMC from different donors.

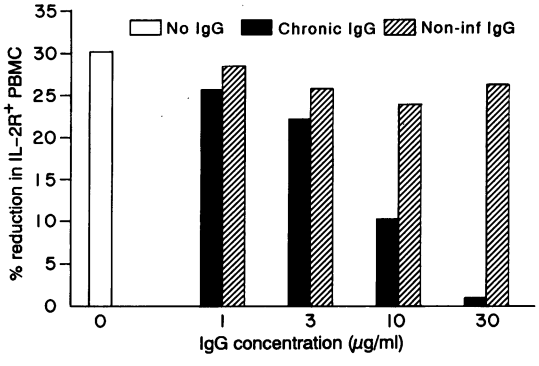

Figure 3. Proportions of PHA-stimulated PBMC losing their capacity to express a detectable level of surface IL-2R in the absence or presence of IgG from chronic chagasic or healthy donors. In this experiment, the proportions of IL-2R$^+$ PBMC in parallel cultures containing medium alone and PHA were 5·7% and 56·0%, respectively. The percentage reduction in the proportion of IL-2R$^+$ PBMC was calculated by the equation:

$$\% \text{ reduction} = \frac{(\% \text{ IL-2R}^+ \text{ cells}_{PHA} - \% \text{ IL-2R}^+ \text{ cells}_{PHA+TIF})}{(\% \text{ IL-2R}^+ \text{cells}_{PHA})} \times 100;$$

where cells_{PHA} and $\text{cells}_{PHA+TIF}$ refer to PBMC cultured with PHA in medium containing the tested IgG in the absence and presence of TIF, respectively. This set of results is representative of two repeat experiments performed with different batches of TIF and cells from different donors.

DISCUSSION

The presence of TIF-blocking antibodies in the sera of chronic chagasic patients or mice represents indirect evidence of TIF production during *T. cruzi* infection. Viewed together with the capacity of TIF to inhibit *T. cruzi*-specific lymphocyte proliferation, this observation suggests a potential for TIF to down-regulate immune responses against this parasite in the host.

Antibodies capable of neutralizing TIF immunosuppressive activity were demonstrable in IgG from chronically infected hosts but not in IgG from animals traversing different periods of the acute phase. This contrast suggests possible explanations for the virtual confinement of immunosuppression to the acute period of *T. cruzi* infection and the re-establishment of immune responsiveness seen during the chronic stage.[17] The absence of TIF-neutralizing activity in IgG preparations from acutely infected mice might be due to suppressed production of blocking antibodies as one more component of the massive immunosuppression characteristic of the acute phase. Alternatively, any anti-TIF antibodies conceivably produced during acute infection could be complexed by an excess of TIF molecules. The largest amounts of TIF produced in the host are anticipated to occur during the acute phase, i.e. when the numbers of *T. cruzi* in the tissues and body fluids are at their highest levels. In the chronic phase, when the parasitosis subsides to such low levels that it becomes difficult to demonstrate, TIF production should be minimal, i.e. insufficient to display immunosuppressive activity. These low levels could, however, be sufficient and persistent to elicit and maintain a humoral response.

Measurements of p55 IL-2R mRNA by Northern blot analyses and of cytoplasmic levels of p55 IL-2R antigen by either flow cytometric analyses of permeabilized lymphocytes or ELISA assays performed on cytosol extracts have shown that TIF reduces significantly the levels of IL-2R message and intracellular IL-2R protein in PHA-stimulated PBMC (F. Kierszenbaum, E. Moretti and M. B. Sztein, submitted for publication; and F. Kierszenbaum, S. Majumder, H. Mejia Lopez and M. B. Sztein, unpublished results). The cytoplasmic localization of these effects rules out the possibility that the reduced expression of IL-2R on the cell surface by activated lymphocytes exposed to TIF results from mere IL-2R cleavage by a parasite-released enzyme. Instead, these and other lymphocyte alterations induced by TIF appear to reflect a disturbance in the regulatory mechanisms governing lymphocyte activation.

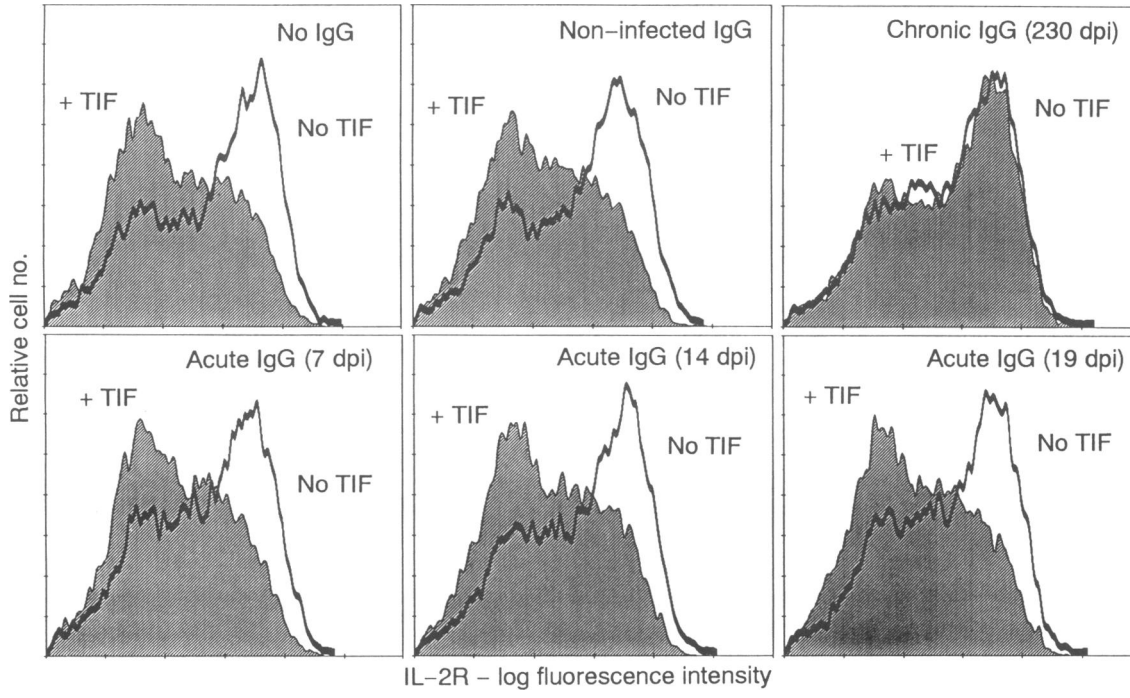

Figure 4. Levels of suppression of IL-2R expression by PHA-stimulated PBMC cultured in the absence or presence of TIF treated with IgG from non-infected, acutely infected or chronically infected mice. Aliquots of TIF alone or pretreated with the indicated IgG preparation (at 30 μg/ml) constituted 85% (v/v) of the culture volume. The chronic mouse IgG was purified from serum collected on day 230 post-infection with 25 *T. cruzi*. The IgG from acutely infected mice were derived from groups of mice killed at 7, 14 and 19 days post-infection with 1×10^4 trypomastigotes. In this experiment, normal IgG was obtained from pooled sera of 47-day-old CD1 mice (i.e. age matched to the mice killed on day 19 post-infection). This set of results is representative of two repeat experiments performed with different batches of TIF and PBMC from different donors.

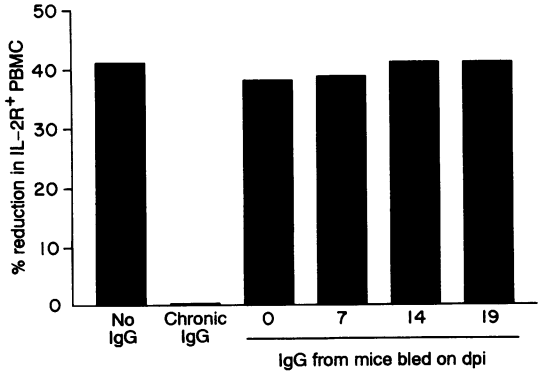

Figure 5. Proportions of PHA-stimulated PBMC losing their capacity to express a detectable level of surface IL-2R in the absence or presence of IgG from non-infected, chronically infected or acutely infected mice. The IgG from acute mice were obtained 7, 14 and 19 days post-infection (dpi). In this experiment, the proportions of IL-2R$^+$ PBMC in parallel cultures containing medium alone and medium with PHA were 5·6% and 57·8%, respectively. The percentage reduction in IL-2R$^+$ PBMC was calculated as described in the legend to Fig. 3. This set of results is representative of two repeat experiments performed with different batches of TIF and cells from different donors.

Such disturbance causes a massive arrest of mitogen-activated lymphocytes at the G_0/G_{1a} phase of the cell cycle[3] which might also underlie the impaired capacity of splenic lymphocytes from mice acutely infected with *T. cruzi* to express IL-2R upon mitogenic stimulation.[5]

Our results indicate that *T. cruzi* releases a protein capable of suppressing a lymphocyte response against its own antigens. Being sensitive to proteases[8] but not removable by immobilized concanavalin A, soybean agglutinin and wheat germ agglutinin (F. Kierszenbaum, H. Mejia Lopez and M. B. Sztein, unpublished results), TIF activity can be inferred to reside in a protein likely devoid of a carbohydrate moiety. Efforts to purify and clone this protein are underway; working amounts of recombinant TIF would facilitate further characterization of this T- and B-lymphocyte immunosuppressant and unravel its role in immunosuppression associated with acute *T. cruzi* infection.

Why and when does the immune system ultimately prevail and conquer immunosuppression, allowing the host to survive the acute infection and enter the chronic phase, are intriguing questions. In pondering possible answers, it must be borne in mind that the host succeeds when the infection is sublethal[17] and is conceivably accompanied by production of low levels of TIF. In more severe infections, often initiated by relatively large doses of *T. cruzi* (as was the case of the acutely infected mice used in this study), the host may not be afforded an opportunity to compromise with the parasite for mutual survival due to unrelenting immunosuppression.

ACKNOWLEDGMENTS

We thank M. K. Tanner for his technical assistance in the flow cytometric determinations. This work was supported by USPHS Grant AI-26542.

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