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Selective Suppressive Effects of *Trypanosoma cruzi* on Activated Human Lymphocytes

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The acute phase of Chagas' disease is accompanied by immunosuppression. To explore the underlying mechanism(s), we used an in vitro culture system in which the capacities of activated human peripheral blood mononuclear cells to express interleukin-2 receptors (IL-2R) and proliferate are markedly inhibited in the presence of *Trypanosoma cruzi*, the etiologic agent. The present work was designed to define the earliest time at which *T. cruzi*-induced suppression is manifested in terms of IL-2R expression on the cell surface and establish whether expression of other lymphocyte activation markers is also suppressed by the parasite. We found that expression of IL-2R by human peripheral blood mononuclear cells cocultured with *T. cruzi* and stimulated with either phytohemagglutinin or anti-CD3 (a monoclonal antibody specific for an epitope of the T cell receptor complex T3-Ti) was significantly suppressed as early as 12 h after culture initiation. Both the percentage of IL-2R$^+$ cells and the surface density of IL-2R, measured by flow cytometry, were affected. However, expression of EA1, a human lymphocyte activation antigen known to be expressed 4 to 6 h after stimulation, was not altered by *T. cruzi* whether phytohemagglutinin or anti-CD3 was used. On the other hand, expression of transferrin receptors (TfR), which first occurs between 20 and 24 h after lymphocyte activation, was markedly suppressed by *T. cruzi*. This effect was denoted by significant reductions in both the percentage of TfR$^+$ cells and the cell surface density of TfR whether phytohemagglutinin or anti-CD3 was used as the mitogen and was observed at all test times, i.e., at 24, 48, 72, and 96 h. Because expression of IL-2R and TfR is required for lymphoproliferation but that of the EA1 lymphocyte activation marker is apparently not, these results are consistent with the possibility that *T. cruzi*, at a relatively early stage during lymphocyte activation, selectively affects certain key events on which clonal expansion is dependent. Inhibition of IL-2R and TfR expression by the parasite might play a role in causing the suppressive effects associated with acute Chagas' disease.

Chagas' disease, caused by the unicellular parasite *Trypanosoma cruzi*, affects millions of people in South and Central America. Occasional cases have also been reported in the United States (20, 25, 31). Laboratory animals and patients in the acute stage of this disease exhibit immunosuppression, which is thought to diminish resistance to the establishment and dissemination of the organism in the host. In acutely infected mice, this suppression is manifested in many forms, including reduced lymphoproliferation in vitro in response to mitogens or trypanosomal antigens and impaired primary and secondary antibody responses and delayed hypersensitivity reactions (5–8, 13–15, 22, 24). In the mouse model system of Chagas' disease, these effects have been attributed to increased suppressive activities by splenic T cells (22) or macrophages (8, 15), reduced levels of T cells (13), and decreased interleukin-2 (IL-2) production (12, 26, 27). In patients with Chagas' disease, immunosuppression has been reported to occur in the acute and subacute phases, at least in the form of suppressed cellular immunity (28, 30). Whereas all of these studies have focused on the immunological alterations that occur in infected hosts, our understanding of the underlying mechanism(s) is minimal. While trying to develop an in vitro system to explore this mechanism, we were able to show that the presence of *T. cruzi* in cultures of lymphoid cells from either normal mice (17) or human volunteers (1, 2) markedly suppresses mitogen-induced lymphoproliferation. Whereas the parasite inhibits expression of IL-2 receptors (IL-2R) by human peripheral blood mononuclear cells (hPBMC) in this system, it does not alter production of interleukin-1 (IL-1), IL-2, or gamma interferon (2; L. A. Beltz, G. Sonnenfeld, and F. Kierszenbaum, unpublished data). Some of these findings represent glaring differences between mouse and human cells with respect to *T. cruzi*-induced immunosuppression. Thus, the capacities of mitogen-stimulated mouse cells to produce IL-2 and gamma interferon in the presence of *T. cruzi* (Beltz et al., unpublished data) are significantly diminished, but production of these cytokines by hPBMC under similar conditions is not affected (2; Beltz et al., unpublished data). Furthermore, exogenous IL-2 restores the responsiveness of lymphocytes from infected mice both in vitro and in vivo (5, 27) and corrects the in vitro suppressive effect of *T. cruzi* on normal mouse lymphocytes (2) but fails to do so in *T. cruzi*-induced suppression of hPBMC in vitro (2). Given these differences, it is important to characterize the suppressive effects of *T. cruzi* on human lymphocytes. The kinetic studies on the expression of several human lymphocyte activation markers that were conducted in this work had two main purposes. One was to define the earliest time at which manifestations of *T. cruzi*-induced suppression occur in hPBMC. The other one was to establish whether parasite-mediated suppression involved alteration of lymphocyte activation markers besides IL-2R. Selected for these purposes were the early activation antigen (EA1) and transferrin receptors (TfR), known to be first expressed by human lymphocytes at 4 to 6 (11) and 20 to 24 (10, 21) h after

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activation, respectively. These times compare with 6 to 8 h for the appearance on the activated lymphocyte surface of Tac (4, 9, 16, 23), an epitope of the p55 chain of IL-2R.

MATERIALS AND METHODS

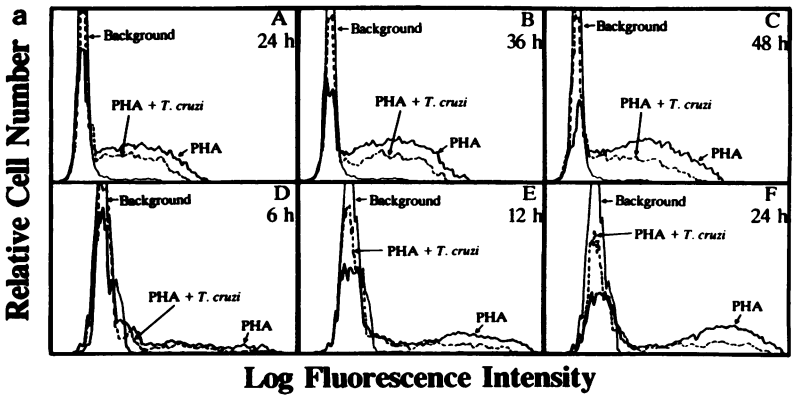
Parasites. *T. cruzi* trypomastigotes (Tulahuen isolate) were isolated from the blood of Crl-CD1 (ICR) Swiss mice (Charles River Breeding Laboratories, Inc., Portage, Mich.) infected intraperitoneally 2 weeks previously with 2×10^5 organisms. The parasites were purified by centrifugation ($350 \times g$, 20°C, 45 min) over a mixture of Ficoll-Hypaque with a density of 1.077 (3), followed by chromatography through DEAE-cellulose (18). After two washings with RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 100 U of penicillin and 100 μg of streptomycin per ml, the parasites were suspended at the desired concentrations in the same medium supplemented with 5% heat-inactivated (56°C, 20 min) fetal bovine serum. The suspension consisted of 100% trypomastigotes (>99% viable).

hPBMC. Normal hPBMC from healthy volunteers were purified by density gradient centrifugation ($350 \times g$, 20°C, 45 min) over a mixture of Ficoll-Hypaque with a density of 1.077. After three washings with RPMI 1640 medium, the cells were suspended at the desired concentration in RPMI-fetal bovine serum. Cell viability, determined by trypan blue exclusion, was >99%.

Reagents. Phytohemagglutinin (PHA) was purchased from Sigma Chemical Co., St. Louis, Mo. Anti-Tac, a monoclonal antibody (MAb) specific for an epitope of the p55 chain of human IL-2R (29), and anti-EA1, a MAb which recognizes an early human lymphocyte activation marker (11), were generous gifts from T. A. Waldmann (National Institutes of Health, Bethesda, Md.) and S. M. Fu (Oklahoma Medical Research Foundation, Oklahoma City, Okla.), respectively. MAbs that recognize epitopes of human TfR (OKT9) and an epitope of the T cell antigen receptor complex T3-Ti (OKT3, anti-CD3) (19) were purchased from Ortho Diagnostics, Inc., Raritan, N.J.

Lymphoproliferation assay. In all experiments, parallel cultures were set up to monitor the effects of *T. cruzi* on stimulated hPBMC. Cultures of hPBMC in RPMI-fetal bovine serum were incubated (5% CO_2; 96-well plates) at 37°C for 96 h (final concentration, 1.25×10^6 cells per ml) with or without *T. cruzi* (5×10^6 organisms per ml) in the presence or absence of optimal concentrations of PHA (5 μg/ml) or anti-CD3 (25 ng/ml). In all cases, the final culture volume was adjusted to 100 μl. All conditions were tested in triplicate. Each culture received 1 μCi of [^{3}H]thymidine (specific activity, 2 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) at 24 h before termination by automated harvesting. Radioactivity was determined in a liquid scintillation counter. The results were expressed as mean counts per minute ± one standard deviation. However, results for the lymphoproliferation assays are not shown because, had they failed to demonstrate significant suppression, the parallel flow cytometric analyses (see below) would not have been performed. The effects of *T. cruzi* on the proliferation of mitogen-stimulated hPBMC have been described in detail elsewhere (2).

Flow cytometric determinations. Cultures of hPBMC (1.25×10^6 cells per ml) were incubated in RPMI-fetal bovine serum (5% CO_2; 24-well plates) at 37°C for various periods (see Results) with or without *T. cruzi* in the presence or absence of PHA (5 μg/ml) or anti-CD3 (25 ng/ml). The cells were washed three times with phosphate-buffered saline solution containing 1% bovine serum albumin. Cells were incubated with the appropriate MAb (i.e., anti-Tac, anti-EA1, or anti-TfR) for 30 min at 4°C, followed by one wash with phosphate-buffered saline–1% bovine serum albumin and incubation with 25 μl of a 1:6 dilution of fluorescein-labeled $F(ab')_2$ goat anti-mouse immunoglobulin G antibody (Tago Immunodiagnostics, Burlingame, Calif.) for 30 min at 4°C. Cells stained with normal mouse immunoglobulin G, followed by fluorescein-labeled $F(ab')_2$ goat anti-mouse immunoglobulin G antibody, were used as controls for background fluorescence. The stained cells were fixed in 1% formaldehyde and stored at 4°C in the dark until analyzed with a FACS IV flow cytometer (Becton Dickinson and Co., Mountain View, Calif.). At least 10,000 cells, gated to exclude erythrocytes, platelets, nonviable cells, and *T. cruzi*, were accumulated for each histogram. The percentage of positive cells was estimated against a background of cells stained with normal mouse immunoglobulin G. Mean channel numbers of the logarithm of fluorescence intensities of the positive cell populations were used to compare the relative density of the relevant lymphocyte marker in the



b

Panel	hPBMC+PHA	hPBMC+PHA+T. cruzi
A	53.5 (93)	41.5 (80)
B	60.6 (95)	44.9 (87)
C	69.0 (101)	42.7 (86)
D	14.4 (146)	7.9 (145)
E	34.3 (171)	14.9 (156)
F	48.8 (191)	23.6 (160)

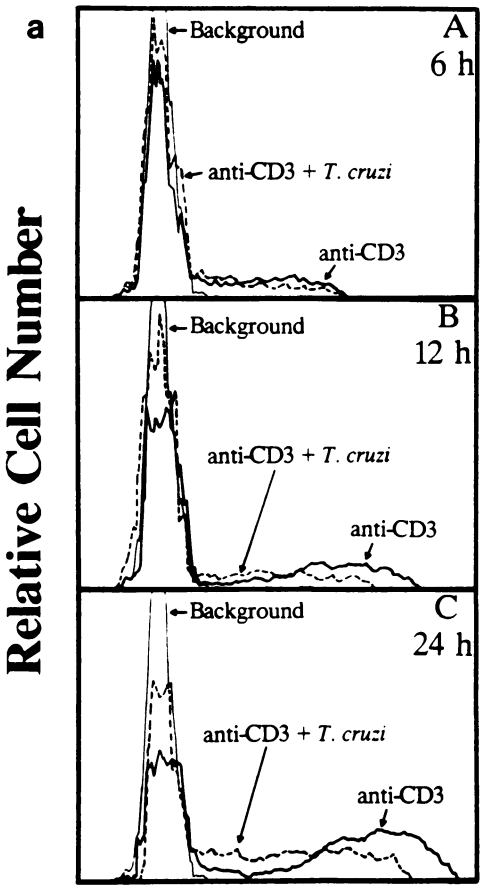
FIG. 1. Kinetics of *T. cruzi*-induced suppression of expression of IL-2R by PHA-stimulated hPBMC. (a) PHA-stimulated cultures were collected at the indicated times, stained with anti-Tac, and analyzed by flow cytometry. hPBMC and *T. cruzi* were used at 1.25×10^6 and 5×10^6 cells per ml, respectively. Panels A to C and D and E represent experiments performed at different times with cells from different donors. Each set of results is representative of two repeat experiments performed with cells from different donors. (b) Percentages of Tac^+ cells and mean fluorescence channel (in parentheses) values for panels in part a.

presence or absence of *T. cruzi*. The logarithm of fluorescence intensities was distributed over 256 channels.

RESULTS

Kinetics of IL-2R expression by hPBMC cocultured with *T. cruzi*. Previous flow cytometry studies with anti-Tac established that IL-2R expression by PHA- or anti-CD3-stimulated hPBMC is suppressed by *T. cruzi* at 48 h after activation (2). The results of an initial kinetic study (Fig. 1a, panels A, B, and C) confirmed this observation and showed that the effect was demonstrable on PHA-stimulated hPBMC at 24 and 36 h as well. At all of these times, the hPBMC population cocultured with *T. cruzi* was found to contain significantly lower proportions of IL-2R$^+$. In addition, the surface density of Tac antigen on IL-2R$^+$ cells was markedly reduced, as denoted by significant decreases in fluorescence intensity with respect to the values obtained with PHA-stimulated cells cultured in the absence of parasites. To establish whether these inhibitory effects occurred at times earlier than 24 h, we performed additional experiments, harvesting the hPBMC at 6, 12, and 24 h after stimulation. A typical set of results is shown in Fig. 1a (panels D, E, and F), demonstrating significant inhibition of IL-2R expression as early as 12 h, with borderline effects occurring at 6 h. To examine whether this effect was also produced under conditions known to mimic antigen-induced lymphocyte activation (16), we performed similar experiments with anti-CD3 as the mitogenic stimulus. The kinetic pattern of suppression of IL-2R expression under these conditions was essentially the same as that seen when PHA was used (Fig. 2).

Effects of *T. cruzi* on expression of other markers of human lymphocyte activation. To establish whether the suppressive effects of *T. cruzi* on activated human lymphocytes were confined to IL-2R expression or were a part of a wider phenomenon involving alterations in the expression of other markers, we monitored the kinetics of expression of EA1 and TfR. Expression of EA1 was readily demonstrable on either PHA- or anti-CD3-activated hPBMC at all tested times after 6 h (Fig. 3). The presence of the parasite appeared to have had no effect on EA1 expression, since there was no significant difference in either the percentage of EA1$^+$ cells (Fig. 3) or the surface density of the marker (data not shown). In contrast, expression of TfR after triggering with PHA or anti-CD3 was markedly inhibited by the presence of *T. cruzi* (Fig. 4). The effect was evidenced by reductions in both the percentage of TfR$^+$ cells and surface TfR density, which were consistently demonstrable at 24, 48, 72, and 96 h after initiation of the cultures.



b

Panel	hPBMC+anti-CD3	hPBMC+anti-CD3+T. cruzi
A	19.5 (118)	14.6 (117)
B	26.9 (142)	15.0 (131)
C	48.0 (181)	38.7 (154)

FIG. 2. Kinetics of *T. cruzi*-induced suppression of expression of IL-2R by anti-CD3-stimulated hPBMC. (a) Anti-CD3-stimulated cultures were collected at the indicated times, stained with anti-Tac, and analyzed by flow cytometry. Cell concentrations were as described in the legend to Fig. 1. This set of results is representative of two repeat experiments performed with cells from different donors. (b) Percentages of Tac$^+$ cells and mean fluorescence channel (in parentheses) values for panels in part a.

DISCUSSION

These results showed that the suppressive effects of *T. cruzi* on activated human lymphocytes are selective. Thus, the trypanosome altered the expression of IL-2R and TfR but failed to affect EA1 expression to a detectable extent. Similar findings were made whether the hPBMC were stimulated with PHA- or anti-CD3 and therefore appeared to represent parasite effects on activated hPBMC unrelated to the nature of the mitogenic stimulus.

We previously reported markedly suppressed expression of IL-2R expression by *T. cruzi* at 48 h after lymphocyte activation (2). The present kinetic studies place the earliest manifestation of suppression by the parasite as early as 12 h after activation, with borderline effects at 6 h. The suppressive effect at 12 h was documented by decreases in both the percentage of IL-2R$^+$ cells and Tac antigen density on the surface of positive cells. These findings and the time of their occurrence demonstrated the ability of the parasite to affect an early event(s) during lymphocyte activation. Interestingly, these early alterations induced by *T. cruzi* are followed by both impaired expression of TfR (Fig. 4) and marked inhibition of lymphocyte proliferation (1, 2). Expression of both IL-2R and TfR is required for lymphocytes to proceed in their division cycle (4, 10, 11, 16, 19, 21, 23) but apparently not that of EA1 since a monoclonal antibody to the latter marker does not block lymphocyte proliferation (11). Therefore, it is tempting to speculate that the immunosuppression caused by *T. cruzi* in vitro, and possibly that

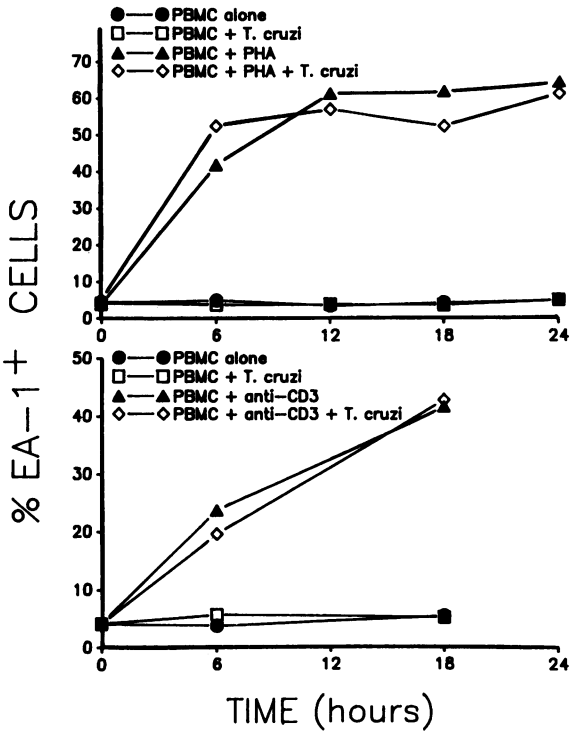
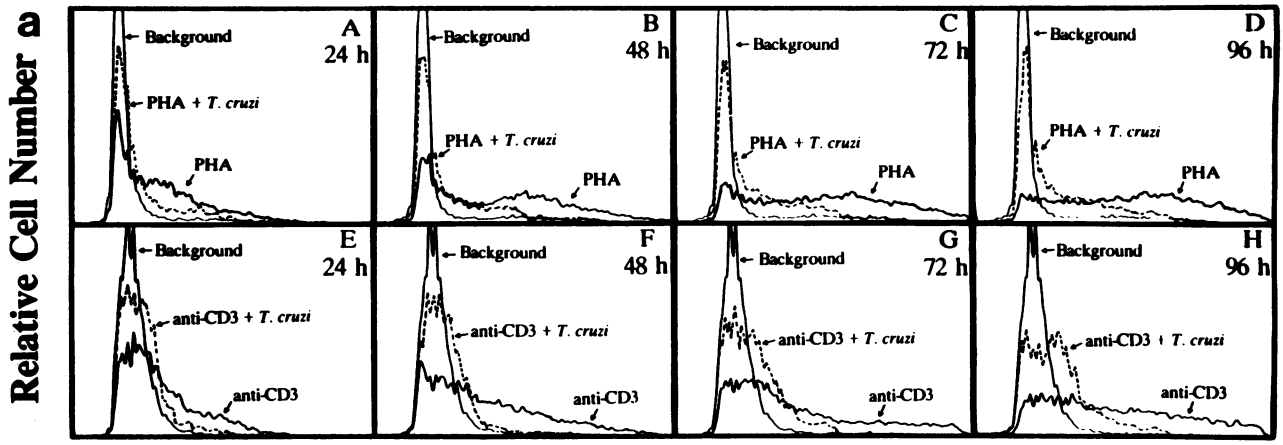


FIG. 3. Kinetics of EA1 expression by PHA- or anti-CD3-stimulated hPBMC in the absence or presence of *T. cruzi*. The experimental conditions are described in Materials and Methods.

accompanying the acute phase of Chagas' disease, could result from selective inhibition of certain crucial events on which effective lymphoproliferation is dependent.

Since *T. cruzi* does not absorb, consume, or inactivate IL-2 (1), suppressed mitogenic responses are unlikely to result from removal of this essential cytokine by the parasite. Furthermore, because under optimal stimulatory conditions IL-2 production by hPBMC is not reduced by the presence of *T. cruzi* in the cultures, suppression could be due, to a large extent, to impaired IL-2 utilization resulting from reduced IL-2R expression. We have also shown previously that the parasite itself stimulates IL-1 production by adherent hPBMC and does not affect bacterial lipopolysaccharide-induced IL-1 production by these cells (2). Therefore, the suppressed expression of IL-2R and TfR seems unlikely to be the consequence of insufficient IL-1 levels. Additionally, our earlier work has shown that mitogen absorption or consumption of essential medium nutrients by *T. cruzi* is not responsible for its suppressive effects (1). Increased hPBMC death due to the presence of the parasite has also been ruled out (2). Finally, culture crowding because of the presence of the parasite is unlikely to be responsible for the noted suppression; cultures containing up to twice as many hPBMC alone as hPBMC plus parasites used in the present study effectively mounted lymphoproliferative responses after mitogenic stimulation (data not shown).

Although the present data improve our understanding of the kinetics of *T. cruzi*-induced suppression of hPBMC and indicate that important and very early events during lympho-



b

Time	hPBMC+PHA	hPBMC+PHA+T. cruzi	hPBMC+α-CD3	hPBMC+α-CD3+T. cruzi
24 h	34.5 (97)	18.6 (84)	30.0 (99)	13.6 (81)
48 h	48.8 (134)	21.1 (79)	42.4 (137)	14.4 (82)
72 h	63.2 (150)	23.0 (71)	50.5 (144)	20.0 (79)
96 h	71.2 (151)	23.0 (78)	61.8 (155)	32.8 (89)

FIG. 4. Kinetics of *T. cruzi*-induced suppression of expression of TfR by PHA- or anti-CD3-stimulated hPBMC. (a) Cultures stimulated with PHA (panels A to D) or anti-CD3 (panels E to H) were harvested at the indicated times, stained by using MAb OKT9, and analyzed by flow cytometry. Cell concentrations were as described in the legend to Fig. 1. This set of results is representative of four repeat experiments performed with cells from different donors. (b) Percentages of TfR^+ cells and mean fluorescence channel (in parentheses) values for panels in part a.

cyte activation are altered by the parasite, they do not define the precise mechanisms which affect the suppression of IL-2R and TfR. These mechanisms could involve alterations in transcription of the genes that code for receptor proteins, mRNA stability or translation, receptor protein transport to the lymphocyte membrane, and/or increased receptor shedding. These possibilities, as well as identification of the parasite factor(s) that mediates the suppressive effect, are currently receiving attention in our laboratories.

Finally, it is worth mentioning that exploration of the mechanism by which *T. cruzi* induces suppression of lymphocyte functions could also help advance our understanding of the early regulatory events that govern lymphocyte activation.

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