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Journal

The American journal of tropical medicine and hygiene, 31(6)

ISSN

0002-9637

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Publication Date

1982-11-01

DOI

10.4269/ajtmh.1982.31.1080

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Peer reviewed

IN VITRO RELEASE OF LYMPHOTOXIN BY SPLEEN CELLS FROM C3H/HEJ AND C57BL/6 MICE INFECTED WITH *TRYPANOSOMA CRUZI*

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Abstract. Lectin (PHA-P) activated nonadherent spleen cells from uninfected inbred strains of mice known to exhibit high parasitemias when infected with *Trypanosoma cruzi* (A/J and C3H/HeJ), released in vitro significantly less lymphotoxin (LT) than did a mouse strain (C57BL/6) known to exhibit low parasitemia when infected with *T. cruzi*. The capacity of mice to release LT in vitro changed upon infection with *T. cruzi*. Cells from infected C57BL/6 mice released LT levels well above that from uninfected C57BL/6 animals within 4 days after initial infection, and their capacity to release LT remained high even after the parasites were not detectable in the blood. Cells from infected C3H/HeJ mice were not able to respond as rapidly as those from C57BL/6 animals; however, they were able to release LT at the same levels as the infected C57BL/6 by day 18. The parasitemia induced by *Trypanosoma cruzi* in C3H/HeJ and C57BL/6 mice was compared with the in vitro ability of their spleen cells to spontaneously release LT. Spontaneous release of LT was significantly higher by spleen cells from infected C57BL/6 mice than by cells from infected C3H/HeJ mice. The kinetics of natural LT release differed from that of mitogen-(PHA-P)-stimulated LT release; LT activity peaked at 3-6 hours of incubation in the former and then declined whereas LT activity in the latter reached a plateau after 3 hours of incubation and did not decline for at least 18 hours in the presence of the inducer. Supernatants from infected C57BL/6 splenocytes, when concentrated 5× by ultrafiltration, significantly inhibited both bloodstream trypomastigote motility in vitro and their infectivity (by 98%) for WI38 human embryonic lung cell cultures. Similar preparations from C3H/HeJ splenocytes only slightly inhibited bloodstream trypomastigote motility and delayed, but did not prevent, infection of WI38 cells. No agglutination of immobilized parasites was noted. Splenocyte supernatants did not affect culture epimastigote motility or growth. This is the first report of direct action by lymphokine-containing supernatants against *T. cruzi* bloodstream trypomastigotes.

Trypanosoma cruzi, the agent for Chagas' disease, is a major cause of cardiomyopathy in Central and South America.¹ It is therefore important to examine in experimental animal models the nature of the host-parasite relationship in this disease. It is clear that experimental *T. cruzi* infections differ in parasitemia, histopathology, and mortality rates between various inbred strains of mice during the acute phase of the disease.^{2,3} Therefore, we have initiated studies to define the basis of some of these differences, and to determine if they involve elements of the host-immune system.

One in vitro assay of host cellular immune reac-

tivity is to measure the capacity of stimulated cells to release lymphokines. The number of biologic activities attributed to lymphokines are diverse, and there are a number that could be measured. Recently Nogueira et al.⁴ demonstrated a correlation between the ability of spleen lymphocytes from infected inbred strains of mice to generate, in vitro a lymphokine(s) capable of activating macrophages to a trypanocidal state with susceptibility of the mice to the Y and CL strains of *T. cruzi*. The lymphocytes in their study released the macrophage activating factor(s) after incubation with heat-killed trypomastigotes. One of the few lymphokines that can be quantitated are the lymphotoxins (LT), an interrelated family of growth inhibitory and cell lytic glycoproteins.⁵⁻⁷ The molecules may have a role in tissue destructive (cell-mediated immune reactions) and could also be tested for direct effects on the parasites themselves.

Accepted 14 April 1982.

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We first examined the capacity of spleen cells from normal and infected C57BL/6 and A/J and C3H/HeJ mice to release LT in vitro after lectin-induced activation. We then investigated if lymphocytes from infected mice were naturally induced by the parasite to release LT.

MATERIALS AND METHODS

Infection of mice

Trypanosoma cruzi, CL strain (obtained from Dr. W. Leon, Univ. Rio de Janeiro, Brazil), was maintained by bimonthly subcutaneous injection of 10^5 bloodstream trypomastigotes in C3H/HeJ mice. The blood was collected in a heparinized syringe and diluted in Hanks' balanced salts solution (DIFCO) supplemented with 0.5% albumin. The diluted blood was centrifuged at low speed ($120 \times g$ for 15 min) to remove most of the host cells. The supernatant containing the trypomastigotes was diluted to a final concentration of 5×10^5 parasites/ml. The animals were lightly anesthetized with ether and injected subcutaneously with 0.1 ml of the final trypomastigote suspension. Parasitemia was determined by collecting tail blood in 1- μ l heparinized Microcaps (Drummond Scientific Co.), diluting the blood with 9.0 μ l 0.89% NH_4Cl and counting the number of trypomastigotes with a hemocytometer.

Large numbers of parasites were required for the experiments on the correlation between parasitemia and in vitro natural lymphokine release and for our measurements of lymphokine inhibitory activity against *T. cruzi*. In order to obtain sufficient numbers of bloodstream trypomastigotes we decided to use the Peru strain of *T. cruzi* because it gave rise to a much higher parasitemia than did the CL strain.

The cloned Peru strain of *T. cruzi* was derived from the stabilate LUMP-722 obtained from Dr. Lumsden.⁸ Characteristics of this strain have previously been described.⁹ Parasitemia in and survival of inbred strains of mice infected with the Peru strain have been studied by Wrightsman et al.¹⁰ The Peru strain has been maintained in our laboratory by bimonthly subcutaneous injection of 10^5 bloodstream trypomastigotes in BALB/c mice since 1974. Unlike mice infected with the CL strain of *T. cruzi*, Peru strain-infected C57BL/6 mice did not survive the infection. Therefore it was not possible to obtain LT activity values much beyond 18 days of infection. Bloodstream trypomastigotes

used for injections were obtained by cardiac puncture of BALB/c mice (8–12 weeks old) 11 days after infection with the parasite. Collection of blood and preparation of the final concentration of trypomastigotes were as above, except that the final concentration of parasites was 1×10^4 /ml. Mice were injected with 0.1 ml and their parasitemia was determined as above. Culture epimastigotes were maintained in tubes containing 1.0 ml BHI Medium (GIBCO, Grand Island, New York) supplemented with 10% newborn calf serum (NCS) (GIBCO) and 0.025 mg hemin/ml and grown at 28°C. The epimastigotes in these studies had been established in culture for 6 months.

Culture media and cell lines

The culture medium employed in these studies consisted of RPMI 1640 medium supplemented with 3% heat-inactivated (56°C, 30 min) fetal (FCS) or newborn (NCS) calf serum (GIBCO), 20 μ g/ml streptomycin and 100 units/ml penicillin (RPMI + 3% FCS or NCS). NCS was used in the last half of our studies because it was less expensive than FCS and was as effective as FCS in maintaining all of the cell cultures used in these studies. The D98 subline of human HeLa cells maintained as monolayer cultures in RPMI + 10% FCS were passed biweekly. An LT-sensitive substrain of L-929 cells developed by Kramer and Granger¹¹ was obtained from stock monolayer cultures and passed biweekly in RPMI + 3% FCS or NCS.

In the cell infectivity studies, the human embryonic lung cell line, WI38 (ATCC No. CCL75) was cultured in Eagle's diploid basal medium (BME) (GIBCO) supplemented with 10% heat inactivated FCS, 10 mM HEPES buffer, 20 mM sodium bicarbonate, 20 μ g/ml streptomycin and 100 units/ml penicillin. WI38 cells were maintained as monolayer cultures and passed biweekly.

Preparation of murine spleen cells

Spleens from C3H/HeJ, A/J and C57BL/6 8- to 10-week-old mice (obtained from the Jackson Laboratory, Bar Harbor, Maine) were aseptically removed, and a single cell suspension prepared in RPMI + 10% FCS or NCS as described by Granger and Kolb.¹² Cell viability was determined by exclusion of 0.1% Eosin Y and was routinely 90–100%. The lymphoid cell suspensions

were placed in 32-oz prescription bottles at a density of approximately 5×10^6 cells/ml for 1–2 hours at 37°C to remove adherent cells. The nonadherent cells were then collected, washed, and resuspended to a final density of 9×10^6 cells/ml for CL strain studies and 2×10^7 cells/ml for Peru strain studies and incubated at 37°C RPMI and either FCS or NCS. Differential and viable cell counts revealed these cells were 90% lymphocytes and 90–100% viable.

Stimulation of LT release in vitro

Previous studies by Hiserodt et al. revealed that murine cells release more LT activity when stimulated with lectin in the presence of allogenic or xenogenic cells.¹³ One million five hundred thousand mitomycin-C treated human D98 cells were established as a monolayer in 30 ml plastic T flasks in RPMI + 10% FCS or NCS 10–20 hours before use. After this time period, the medium was poured off, washed three times with RPMI without FCS or NCS, and these monolayers were treated with phytohemagglutinin-P (PHA-P, Difco, Detroit, Mich.) at 100 µg/ml per 10^5 cells in RPMI without FCS or NCS for 1 hour at 37°C. In all experiments, the PHA-P solution was freshly prepared before use. The medium was again poured off, the monolayers were washed three times with RPMI without FCS or NCS and 5 ml of a cell suspension containing 45×10^6 lymphoid cells in RPMI without FCS or NCS were added (ratio of lymphoid cells to D98 cells 30:1). After 7 hours at 37°C, the supernatants were collected, cleared of cells by centrifugation ($500 \times g$, 10 min) and immediately tested for LT activity.

Quantitation of LT levels

The amount of LT in a given supernatant, expressed as units of biologic activity, was determined according to the method of Spofford, Daynes and Granger.¹⁴ Briefly, serial dilutions of each supernatant were placed in duplicate 1-ml tube cultures of L-929 cells containing 100,000 mitomycin-C-treated L cells in RPMI + 3% FCS or NCS. Sixteen to 24 hours later the medium was discarded and the remaining viable adherent L cells were enumerated in a Coulter counter. The highest dilution that killed 50% of the cells was then determined. One unit of LT activity is defined as that amount of LT that will destroy 50% of the cells (50,000) in these tubes. The reciprocal of the

dilution giving 50% destruction provided the number of units present per milliliter in the original undiluted supernatant.

Binding of 125 I-PHA to lymphoid cells

Phytohemagglutinin was labeled with 125 I by the iodogen method.^{15,16} The lectin, at a concentration of 2.0 mg/ml in phosphate buffered saline (PBS) (pH 7.2, 10 mM phosphate) was exposed to 250 µCi 125 I (NEN, Boston, Mass) in 10 µl PBS in a 10×75 mm tube coated with 10 µg 1,3,4,6-tetrachloro-3 α , 6 α -diphenylglycouril (Pierce Chemical Co., Rockford, Ill.) After 5 min, the reaction was quenched by decanting and the labeled protein was freed of unincorporated iodide by overnight dialysis against PBS containing 1.0 mM KI. The net efficiency was about 15%. One-milliliter samples containing 5×10^6 A/J or C57BL/6 lymphoid cells were incubated with 1.0–50 µg labeled PHA in microcentrifuge tubes at 4°C for 30–40 min. Duplicate samples were tested for each PHA concentration. Control tubes (without cells) contained 1 ml RPMI without FCS. The cell suspension was pelleted, the fluid aspirated off, cells were washed twice with PBS, and the pellet was directly subjected to counting (Beckman Biogamma, Beckman Industries, Fullerton, Calif.).

Natural release of LT in vitro

Supernatants from non-adherent spleen cell cultures were collected at various times (1–18 hours), cleared of cells by centrifugation ($500 \times g$) for 10 min and immediately tested for LT activity against L-929 cells. The cell pellet was resuspended to 2×10^7 cells/ml in RPMI + 0.5% NCS and incubated at 37°C until the next time for collection of supernatants when the above process was repeated. LT levels were quantitated as described above.

Concentration of naturally released supernatants containing LT

Although supernatants containing LT showed cytolytic activity against target L-929 cells, they did not affect the motility of bloodstream trypomastigotes nor the infectivity of these parasites for WI38 cells. The supernatants were therefore concentrated as follows. Supernatants from peak release times (these depended primarily on the time elapsed from spleen removal rather than strictly

on non-adherent cell incubation time) were concentrated through an Amicon PM10 ultrafiltration membrane (Amicon Corp., Lexington, Mass.). The 10 kilodalton retentate ($5\times$ concentrated) retained 90% or more of the total LT activity; the filtrate showed no activity. Retentates were sterilized by filtration through a Millipore 0.22 μ m filter before use.

Tests with concentrated LT supernatants

Duplicate tubes containing 1×10^6 Ficoll-Hypaque cleaned¹⁷ bloodstream trypomastigotes obtained from BALB/c mice infected 14–15 days previously with trypomastigotes were incubated overnight (18–20 hours at 37°C) in 0.5 ml of $5\times$ concentrated supernatants or $5\times$ concentrated media controls. The trypomastigotes were centrifuged at $500 \times g$ for 10 min and the pellet was added to 1×10^6 WI38 cells in 25-cm² flasks containing 5 ml BME and/or observed for viability by phase microscopy. Twenty-four hours after adding the incubated trypomastigotes to the WI38 cells, the medium containing free parasites was poured off, the host cells were washed one time with BME, 5 ml BME plus 10% FCS was added, the cultures were gassed with 5% CO₂ and the flasks were incubated at 37°C for another 24 hours. Cell cultures were then kept at 32°C for the remainder of the observation period. This procedure enhances diploid cell survival time by slowing monolayer growth.¹⁸ Invasion of WI38 cells was measured by counting the number of host cells containing amastigotes in 10–50 fields at 100 $\times$ with an inverted phase microscope, every 24 hours. It is difficult to see a single parasite in a cell; at least five amastigotes must be present in order to be sure that the WI38 cell is parasitized. We estimated that ≈ 50 WI38 cells were visible per field. Observations were made only for 3–4 days because trypomastigote release from infected cells followed by the invasion of new cells resulted in increasing numbers of parasitized cells when anti-trypomastigote activity was not present.

RESULTS

PHA-induced LT release from uninfected mouse splenocytes

A significant difference in the capacity to release LT was found between spleen cells from uninfected C57BL/6, A/J and C3H/HeJ strains of mice.

FIGURE 1. Comparison of lymphotoxin (LT) secretion between C3H/HeJ, A/J and C57BL/6 spleen non-adherent lymphocytes. Serial dilutions of LT were placed in 1-ml tube cultures of target (L-929) cells containing 100,000 mitomycin-C L cells in RPMI + 3% FCS. The remaining viable adherent L cells were enumerated 16–24 hours later in a Coulter counter and the highest dilution killing 50% of the cells was determined. One unit of LT activity is that amount of LT that destroyed 50% of the cells (50,000). The reciprocal of the dilution giving 50% destruction provided the number of units present/ml in the original undiluted supernatant. Preparation of LT, methods of cell culture and determination of cell viability are described in the text.

C57BL/6 cells released 6.7 ± 4.6 (mean $\pm$ SD) (range 2.0–17.0) ($n = 40$) units of LT activity whereas A/J and C3H/HeJ cells released $< 1.00 \pm 0.0$ ($n = 40$ for C3H/HeJ, $n = 14$ for A/J) units of LT activity. An example of a typical assay is shown in Figure 1; this is characteristic of approximately 40 different experiments. To determine if the difference in LT production between C57BL/6 and A/J cells was due to a differential stimulation effect or to the number of lectin (PHA) binding sites on lymphoid cells we measured binding of ¹²⁵I-PHA to the nonadherent lymphoid cells of A/J and C57BL/6 mice. No significant difference in the capacity of A/J and C57BL/6 spleen cells to bind the radio-labeled lectin was noted: A/J cells showed saturation counts of $124,794 \pm 2,477$ CPM and C57BL/6 cells showed counts of $119,454 \pm 33,037$ ($n = 4$). An example of the binding of ¹²⁵I-PHA to the two cell lines is depicted in Figure 2.

PHA-induced LT release from CL strain infected mouse splenocytes

The next series of experiments was conducted with spleen cells from infected animals taken at different intervals during the infection. The re-

FIGURE 2. Binding of ^{125}I -PHA to A/J and C57BL/6 spleen nonadherent lymphocytes. Two mg/ml lectin in PBS (pH 7.2., 10 mM phosphate) was exposed to 250 $\mu\text{Ci } ^{125}\text{I}$ in 10 μl PBS in a 10×75 mm tube coated with 10 μM 1,3,4,6-tetrachloro-3 α , 6 α -diphenylglycouril. The reaction was quenched after 5 min by decanting and the labeled protein was freed of unincorporated iodide by overnight dialysis against PBS containing 1.0 mM KI. Samples contained 5×10^6 A/J or C57BL/6 lymphoid cells incubated with 1.0–50 μg labeled PHA in microcentrifuge tubes at $4^\circ\text{C}/30$ –40 min. Control tubes (without cells) contained 1 ml RPMI without FCS. Preparation of lymphoid cells, harvesting of incubated cells and counting procedures are described in the text.

sults of those experiments are shown in Figure 3. Spleen cells from infected C57BL/6 animals could be stimulated to release high levels of LT activity at day 11 which remained high for at least 25 days, whereas cells from uninfected animals released a lower and constant level. Although the capacity to release LT from cells of infected C57BL/6 mice appeared to decline by day 25, subsequent experiments showed high LT values in these hosts even after 2 months of infection. The C57BL/6 animals routinely survived the infection. In contrast spleen cells from infected C3H/HeJ animals released lower levels of LT at 11 days but could be stimulated to release high levels at 18 days which remained high until death between 22 and 26 days. High levels of parasites were evident in the blood of C3H/HeJ mice until 18 days where

FIGURE 3. Comparison of lymphotoxin (LT) production by lymphocytes from C3H/HeJ and C57BL/6 mice infected with 5×10^4 bloodstream trypomastigotes of *Trypanosoma cruzi*, CL strain. Data presented as percent target cell destruction above that induced by lymphocytes from C3H/HeJ uninfected control mice. Each point represents the average cell destruction by LT from four or five mice. Four hundred microliters of LT from infected and uninfected control mice were added to 1-ml tube cultures of target (L-929) cells containing 15,000 mitomycin-C L cells in RPMI + 3.0% FCS. The remaining viable adherent L cells were enumerated 16–24 hours later in a Coulter Counter and the percentage of cells destroyed was determined. Preparation of LT, methods of cell culture and determination of cell viability are described in the text.

two patterns were seen: (a) In most cases they decreased until death 3–6 days later, and (b) levels increased and animals died early. In contrast, parasite blood levels were low in C57BL/6 mice and at 18 days rapidly disappeared.

PHA-induced LT release from Peru strain-infected mouse splenocytes

Since the Peru strain of *T. cruzi* used for the latter portions of our study was different from the one employed above (CL strain), we repeated the assays for LT release by splenocytes from hosts infected with Peru strain *T. cruzi* using mitogenic (PHA-P) stimulation. A typical assay is shown in Figure 4; these results were characteristic of five experiments. The lymphocyte response pattern in Peru strain-infected C57BL/6 mice appeared to be similar to that found in lymphocytes from CL strain-infected mice. C57BL/6 mice released significantly more LT than did C3H/HeJ mice: 1,000 μl of LT supernatant from infected C57BL/6 mouse cells destroyed $41.5 \pm 15.8\%$ (mean $\pm$ SD) ($n = 6$) more target L-929 cells than did C3H/HeJ

FIGURE 4. Comparison of lymphotoxin (LT) release between PHA-P stimulated spleen non-adherent lymphocytes from C3H/HeJ and C57BL/6 mice infected 14 days before subcutaneously with 10^5 bloodstream trypomastigotes of *Trypanosoma cruzi*, Peru strain. The data are presented as percent target cell destruction above that induced by lymphocytes from C3H/HeJ uninfected control mice. Each point represents the average cell destruction by LT from 2-5 mice. One thousand microliters of supernatant LT from infected and uninfected control mice were added to 1.0 ml tube cultures of target (L-929) cells containing 15,000 mitomycin-C L cells in RPMI + 3.0% FCS. The remaining viable adherent L cells were enumerated 16-24 hours later in a Coulter Counter and the percentage of cells destroyed was determined. Preparation of LT, methods of cell culture, and determination of cell viability are described in the text.

uninfected controls whereas the equivalent volume of LT supernatant from infected C3H/HeJ cells destroyed $13 \pm 5.7\%$ ($n = 5$) more of the target cells than did C3H/HeJ uninfected controls.

Natural LT release from Peru strain infected mouse splenocytes

During these studies we observed large numbers of blast cells in the spleens of infected C57BL/6 mice. It was possible that some of these cells could be spontaneously releasing LT because of stimulation by the parasite. So we then measured the release of LT from non-mitogen stimulated spleen cells obtained from Peru strain infected mice. The results from a number of time course experiments with C57BL/6 mice are summarized

FIGURE 5. Kinetics of lymphotoxin (LT) release by non-mitogen stimulated spleen non-adherent lymphocytes from C57BL/6 mice infected 15 days previously with 10^5 bloodstream trypomastigotes of *Trypanosoma cruzi*, Peru strain. Data are presented as percent target cell destruction above C3H/HeJ uninfected control mice. Each bar represents the mean $\pm$ SD percent destruction by LT from two to eight mice. Collection, preparation, and testing of LT activity from the spleen lymphocytes as well as methods of culture and determination of cell viability are described in the text.

in Figure 5. Lymphocytes from these infected mice secreted readily detectable LT activity after only 1 hour of incubation; LT activity peaked between 3 and 6 hours and then gradually declined. Spleen cells from uninfected control C57BL/6 mice also spontaneously released measurable amounts of LT but these were significantly less ($24.5 \pm 10.5\%$ target cell destruction above C3H/HeJ controls by 6-hour supernatants) than those observed in infected cell supernatants. Splenocytes from infected high parasitemia mice secreted little or no detectable LT; thus there appears to be a major qualitative difference between the capacity of C57BL/6 and C3H/H3J mice to release LT. Also noteworthy was the difference in LT release kinetics between PHA-P stimulated and non-stimulated (spontaneous) lymphocytes of infected C57BL/6 mice. Natural LT release began early, peaked and then declined after 6 hours of incubation (Fig. 5) whereas mitogen stimulated LT release began early, peaked but did not decline for at least 18 hours (Fig. 4).

Anti-trypanosome activity by lymphocyte supernatants

We then asked whether the supernatants had any direct in vitro effect upon bloodstream trypomastigotes. This was determined by testing supernatants for inhibition of motility and infectivity of parasites for WI38 cells after incubation with lymphokine-containing supernatants. There was no consistent inhibitory activity by non-concentrated (1×) supernatants against the parasites. The supernatants were concentrated five times with a PM10 Amicon ultrafilter and the 5× concentrate was tested for anti-trypanosome activity. The results from three experiments showing the effect of 5× concentrate from C57BL/6 and C3H/H3J lymphocytes against bloodstream trypomastigotes are summarized in Table 1.

Concentrated supernatants obtained from infected low parasitemia (C57BL/6) mouse lymphocytes immobilized the trypomastigotes. Concentrated supernatants from infected C3H/HeJ cells slightly reduced the number of motile trypomastigotes, whereas similar preparations from normal, uninfected C3H/HeJ cells showed no effect upon trypomastigote motility. Anti-trypanosomal activity of the supernatants therefore parallels their ability to destroy L-929 target cells.

We then measured the effect of concentrated supernatants from infected C57BL/6 mouse lymphocytes upon the infectivity of bloodstream trypomastigotes for WI38 cells. The results of these experiments are summarized in Table 2. Prein-

TABLE 1

Direct effect of 5× concentrated supernatants from 2.0×10^7 lymphocyte-enriched spleen cells on motility of *Trypanosoma cruzi* bloodstream trypomastigotes*

Test supernatants	% Motile trypomastigotes		
	Exp 1	Exp 2	Exp 3
Medium control	100	100	100
C3H/HeJ			
Uninfected control	100	100	100
C3H/HeJ			
<i>T. cruzi</i> infected	±	N.D.	±
C57BL/6			
<i>T. cruzi</i> infected	<33%	0	0

* 1×10^6 trypomastigotes incubated in 0.5 ml of concentrated supernatants 18–20 hours/37°C, washed, then transferred to 0.1 ml medium L-15 (GIBCO) in microtiter wells and observed for 4–5 days. 100 = no effect, ± = slight effect, 0 = no motile trypomastigotes, N.D. = not done.

cubation of the parasite with concentrated supernatants for 20 hours from infected low parasitemia mice inhibited their ability to subsequently infect WI38 cells. By the 4th day of culture this inhibition was ≈98%. Similar preparations from infected C3H/HeJ cells initially delayed parasite invasion of the WI38 cells (93% inhibition on Day 1, 78% inhibition on Day 2) but the *T. cruzi* eventually overcame the inhibition. By the 3rd and 4th days more host cells were parasitized than in the concentrated medium control cultures.

The effect of these supernatants on culture epimastigotes of Peru strain *T. cruzi* was also tested. Culture epimastigotes were incubated with LT su-

TABLE 2

Direct effect of naturally released 5× concentrated supernatants from spleen lymphocytes on infectivity of bloodstream trypomastigotes of *T. cruzi* for the human diploid cell line WI38 (ATCC No. CCL75)*

Lymphocytes	Time of observation							
	Day 1		Day 2		Day 3		Day 4	
	No. INF cells/ 50 fields	% inhib.	No. INF cells/ 50 fields	% inhib.	No. INF cells/ 50 fields	% inhib.	No. INF cells/ 50 fields	% inhib.
Medium control	76.5 ± 6.4	—	74.0 ± 1.4	—	120.0 ± 7.0	—	112.0 ± 24.7	—
Uninfected C3H/HeJ lymphocytes	Not done	—	50.5 ± 0.7	32	136.4 ± 60.0	(190)*	202.0 ± 44.8	(180)†
Infected C3H/HeJ lymphocytes	6.0 ± 0.0	93	16.8 ± 1.7	78	300.0 ± 21.2	(250)*	240.0 ± 28.3	(214)†
Uninfected C57BL/6 lymphocytes	Not done	—	39.0 ± 4.2	48	Not done	—	Not done	Not done
Infected C57BL/6 lymphocytes	17.0 ± 1.4	78	7.7 ± 6.6	90	8.25 ± 8.46	94	2.5 ± 3.5	98

* Supernatants collected and concentrated as in Table 1.

† Percent infective (INF) cells above media control.

pernatants from infected C57BL/6 and C3H/HeJ cells for 3 days at 28°C. Epimastigote motility and cell division appeared to be unaffected by the lymphokine preparations.

Restimulation of lymphocytes

In a series of four experiments, lymphocytes tested for natural LT release were "restimulated," 18–20 hours after initiation of culture, by incubation with equal (1:1) numbers of 0.25% glutaraldehyde-fixed trypomastigotes or with a 1:10 ratio (splenocytes : trypomastigotes) of parasites. In neither case was further LT activity found in the supernatants from "restimulated" cells.

DISCUSSION

The present results reveal that infected C3H/HeJ and A/J mice differ markedly from C57BL/6 mice in their capacity to release LT in vitro in response to a polyclonal PHA signal. C57BL/6 mice release from 2–17 times more LT than the C3H/HeJ animals. This difference may be due to the inherent capacity of cells from the different inbred mice to be stimulated by the lectin, for they each bind the same amount of I^{125} -labeled PHA. It should be stressed that we measured I^{125} binding by the total cell population; it is possible that there are a number of subpopulations which differentially bind the mitogen or bind but do not respond, results that would not be detected using our methods. Although both B (G. A. Granger, unpublished data) and T-effector¹⁹ cells can release LT in humans, there are no data on the subpopulation(s) of cells releasing LT in mice.

The capacity of both C3H/HeJ and C57BL/6 mice to release LT in vitro underwent changes upon infection with CL strain *T. cruzi*. Cells from C57BL/6 mice were able to release high levels of LT, well above that for cells from uninfected C57BL/6 animals within 4 days after initial infection (data not shown), and the capacity to release remained high even after the parasites were cleared from the blood vascular system. In contrast, C3H/HeJ mice were not able to respond as fast; however, they were able to release at the same levels as the C57BL/6 by day 18. These animals usually died by 22–26 days. The numbers of parasites in the blood reached high levels in the C3H/HeJ but began to decline about the same time as the LT-releasing capacity peaked, 18 days. In contrast, parasitemia was initially much lower in the C57BL/

6, remained constant, then dropped to below countable levels at 18 days.

We observed a parallel correlation between natural in vitro release of lymphotoxin by lymphocyte-enriched spleen cells and parasitemia with Peru strain *T. cruzi* infection in the intact host. At present, however, all we can say about lymphotoxin release is that it is a measure of infected host cell activation. In addition we found a direct immobilizing action by splenocyte supernatants on bloodstream trypomastigotes of Peru strain *T. cruzi*. Previous studies in our laboratory on the in vivo pattern of parasitemia and survival of infection with Peru strain-infected inbred strains of mice showed that the C57BL/6 mouse experienced a low parasitemia ($<5 \times 10^6$ trypomastigotes/ml blood on the 18th day of infection) and that the C3H/HeJ mouse exhibited a high parasitemia ($>1 \times 10^7$ trypomastigotes/ml blood on the 18th day of infection).¹⁰ These responses in C57BL/6 and C3H/HeJ mice were similar to those observed in other investigations with inbred mice.^{3,4}

There was a difference in the kinetics of LT release between PHA-stimulated and non-stimulated (spontaneous) lymphocytes of infected C57BL/6 mice. The difference between the two systems may be that an inducer is continuously present in the PHA cultures and absent in the spontaneous release culture; thus, the latter cells stop releasing LT after a short period. Further studies are underway to test this hypothesis.

It should be emphasized that definitive proof for a direct role against *T. cruzi* by LT in splenocyte supernatants is lacking. Anti-trypanosome activity in the supernatants may be due to another lymphokine whose secretion pattern is similar to that of LT or perhaps by antibody released by immune cells. Experiments are currently underway to identify the active material in these supernatants.

Of relevance to our study is the recent paper by Nogueira et al.⁴ suggesting a direct correlation between in vitro cell-mediated effector mechanisms and protection against *T. cruzi* infection in the intact host. They found that activated T lymphocytes from resistant mice were capable of generating lymphokine(s) which induced macrophage trypanocidal activity under in vitro conditions. Nogueira and her coworkers concluded that cell-mediated mechanisms were responsible for the acquired immunity observed in the acute phase of a *T. cruzi* infection in mice. Since both resistant and susceptible inbred strains of mice were able to

mount protective immunity with non-lethal infections they suggested that the difference between the two types of mice might be the result of a slower and less vigorous immune response insufficient to handle a larger and/or more virulent challenge dose of organisms. These authors are unclear about the cellular basis for the variation except that it was not dependent upon the macrophages.

Although Nogueira and her coworkers have been able to protect mice from bloodstream trypomastigote attack by passive transfer of T cells from immune mice (see discussion in ref. 4) (see also, e.g. Burgess and Hansen²⁰), recent work by Scott^{21,22} indicates that the nature of immunity against *T. cruzi* in mice recovered from an acute infection may be predominantly B cell-mediated with T cell involvement being restricted to a helper role. Scott was unable to find a specific delayed type hypersensitivity (DTH) response (as measured by footpad swelling after antigen injection) to *T. cruzi* in chronic *T. cruzi* infected mice; these mice were still able to develop an unimpaired DTH response to an unrelated antigen (keyhole limpet haemocyanin). It should be pointed out that other workers have also established a major role for specific antibodies in protective immunity against *T. cruzi* infections in mice (e.g. references 2, 23, 24). Scott relegated the role of cell-mediated immunity as implicated in the pathology of chronic *T. cruzi* infection (see also ref. 25).

At this time it is not possible to reconcile the two contrasting views concerning the effector cell type(s) primarily responsible for development of immunity against *T. cruzi* in acutely infected mice. Of interest in this regard are the studies by Kuhn and coworkers suggesting that susceptible and resistant inbred strains of mice vary in their induction of parasite specific helper T lymphocytes during *T. cruzi* infections.²⁶

Based on our results (see Table 2) we suggest that lymphocytes may be able to control *T. cruzi* directly via lymphokines. It will be necessary to test more strains of mice to accurately correlate the release of lymphokines with parasitemia, and it will also be necessary to do F₁ backcross studies to show specific linkage vis-a-vis the release of lymphokine and parasite level. The high release of LT in infected animals may represent a natural response to infection or it may simply be an indication of host lymphocyte activation.

ACKNOWLEDGMENTS

This research was supported by Grants AI 14828 (SMK) and AI 09460 (GAG) from the Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland and a grant from UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (SMK). We are indebted to Dr. J. Klostergaard for advice and discussion, and to R. Yamamoto and Sue Tripp for technical assistance.

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