

# TRYPANOSOMA CRUZI: IN VITRO INDUCTION OF MACROPHAGE MICROBICIDAL ACTIVITY\*

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In previous papers we have shown that trypomastigotes of *Trypanosoma cruzi*, the etiological agent of Chagas' disease, survive quantitatively and replicate in the cytoplasm of both normal and inflammatory mouse peritoneal macrophages (1). Organisms enter the cell via phagocytosis, are initially enclosed within a phagocytic vacuole, and subsequently escape into the cytosol. In contrast, macrophages obtained from either *T. cruzi* or Bacille Calmette-Guérin (BCG)<sup>1</sup>-infected mice, which had been elicited by a secondary challenge with specific antigen, were capable of destroying a majority of the intracellular parasites (2). Exposure of normal macrophages to lymphokines failed to modify their microbicidal activity, but promptly stimulated their secretion of plasminogen activator. The generation of the active lymphocyte supernatant fluid(s) required the presence of thymus-derived lymphocytes (3).

In this study, we report the conditions under which we are now able to induce and maintain trypanocidal activity in both resident and inflammatory mouse peritoneal macrophages maintained in vitro.

## Materials and Methods

*Parasites.* The Y strain of *T. cruzi* was obtained from Dr. Ruth Nussenzweig (New York University School of Medicine, New York). Blood form trypomastigotes were obtained from infected NCS mice and grown in liver-infusion tryptose (LIT) medium (4). Parasites were harvested from 30-day-old cultures in LIT, washed five times in ice-cold phosphate-buffered saline (PBS) (Dulbecco's; Grand Island Biological Co., Grand Island, N. Y.) at 750 *g* for 15 min, resuspended in ice-cold PBS or Dulbecco's modified Eagle's medium (Grand Island Biological Co.) and counted in a hemocytometer with a  $\times 40$  objective. These cultures of Y strain in LIT contained >30% trypomastigotes. Trypomastigotes were purified by means of a modified procedure (5), utilizing a Metrizamide gradient (Nyegaard and Company A/S, Oslo, Norway). The gradients were prepared with 2 ml of 17.5% Metrizamide ( $d = 1.0924 \text{ g}\cdot\text{cm}^{-3}$ ) and 2 ml of 15% Metrizamide ( $d = 1.0787 \text{ g}\cdot\text{cm}^{-3}$ ) in Hepes buffer, pH 7.4, 260 mosmol (6).  $10^7$  parasites, after treatment with guinea-pig complement (Cordis Laboratories Inc., Miami, Fla.) were washed and resuspended in 1 ml of Hepes buffer, and overlaid on the Metrizamide gradient. The preparation was then centrifuged at 2,000 *g* for

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\* Supported by grants 5-29055 from the Rockefeller Foundation, and AI 07012 from the U. S. Public Health Service.

<sup>1</sup> *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin; Con A, concanavalin A; D<sub>2</sub>FBSME, Dulbecco's medium containing 2% fresh fetal bovine serum plus  $5 \times 10^{-5}$  M mercaptoethanol; D<sub>2</sub>HIFBS, Dulbecco's medium containing 2% heat-inactivated fetal bovine serum; FBS, fetal bovine serum; hkBCG, heat-killed BCG; HKT, heat-killed trypanosomes; LIT, liver-infusion tryptose; LPS, lipopolysaccharide; ME, mercaptoethanol; PBS, phosphate-buffered saline; PP, proteose peptone; PPD, purified protein derivative of tuberculin; SCF, spleen cell factor.

25 min; live trypomastigotes were collected at the 15/17.5% interface, washed once at 750 *g*/15 min in PBS, and resuspended at the desired concentration in Dulbecco's medium containing 2% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co.), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (D<sub>2</sub>HIFBS).

**Normal Macrophages.** Mouse peritoneal macrophages were obtained from female Swiss mice (NCS) maintained at The Rockefeller University. Cells were harvested according to the methods of Cohn and Benson (7), and cultivated on 13-mm round glass coverslips in 16-mm Linbro plates (Linbro Chemical Co., New Haven, Conn.) in D<sub>2</sub>HIFBS.  $1.5 \times 10^6$  total peritoneal cells were plated per well, 45–50% of which were macrophages.

**Inflammatory Macrophages.** Female Swiss mice were injected intraperitoneally 4 days earlier with 1 ml of 1% solution of proteose-peptone (PP) (Difco Laboratories, Detroit, Mich.). Macrophages were harvested and cultivated as described above.

**Immune Macrophages.** Immune macrophages were obtained from animals previously infected intraperitoneally with  $5 \times 10^6$  live culture forms of *T. cruzi*, Y strain, in PBS, collected as described above, or from animals infected intravenously with  $2-6 \times 10^7$  viable BCG (strain 1011; Trudeau Institute, Inc., Saranac Lake, N. Y.). Peritoneal cells were harvested from 3-wk-infected mice, 2 days after a secondary challenge with the respective antigen; heat-killed trypanosomes (HKT) ( $5 \times 10^6$  culture forms in 1 ml PBS), or purified protein derivative of tuberculin (PPD) (50  $\mu$ g in 1 ml PBS; Connaught Medical Research Laboratory, Willowdale, Ontario, Canada). Macrophages were cultivated as described above.

***T. cruzi* and BCG-Primed Spleen Cells.** Swiss mice were infected with *T. cruzi* or BCG as described above. 2–5 wk after infection, spleens were removed, placed in ice-cold Dulbecco's medium, tested with forceps, and passed through a sterile stainless steel wire mesh. The screen was washed with cold medium, and the cells were dispersed by pipetting the suspension up and down several times. The cells were then washed once in cold Dulbecco's, and resuspended in Dulbecco's medium containing 2% fresh FBS,  $5 \times 10^{-5}$  M mercaptoethanol (ME), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin and glutamine (D<sub>2</sub>FBSME), in the presence of the specific antigens.

**Concanavalin A- and Lipopolysaccharide-Stimulated Spleen Cells.** Spleens from normal Swiss mice were removed, and a cell suspension was prepared as described above in D<sub>2</sub>FBSME in the presence of either 3  $\mu$ g/ml concanavalin A (Con A) (Miles Laboratories Inc., Elkhart, Ind.), or 100  $\mu$ g/ml lipopolysaccharide (LPS) (*Escherichia coli* W 055:B5; Difco Laboratories).

**Antigens.** HKT were obtained by mildly heating (80°C/10 min) a suspension of  $5 \times 10^6$ /ml Y strain culture forms in PBS. PPD was obtained from Connaught Medical Research Laboratory. Heat-killed BCG (hkBCG) was obtained by autoclaving a suspension of  $2-6 \times 10^6$ /ml viable BCG, Pasteur strain, at 15 lb/in<sup>2</sup> for 15 min.

**Preparation of Spleen Cell Factor(s) (SCF).**  $10^8$  spleen cells from normal, *T. cruzi*- or BCG-infected Swiss mice were incubated with the respective antigens (PPD, 50  $\mu$ g/ml; HKT, 10<sup>6</sup>/ml; hkBCG, 10<sup>6</sup>/ml) or mitogens (Con A, 3  $\mu$ g/ml; LPS, 100  $\mu$ g/ml) in 6.5 ml of D<sub>2</sub>FBSME, at 37°C in a CO<sub>2</sub> atmosphere for 48 h. The supernates were then collected, centrifuged at 750 *g* for 15 min to remove cells and debris, and filtered through a 0.45- $\mu$ m Millex filter (Millipore Corp., Bedford, Mass.). *T. cruzi* and BCG control supernates consisted of normal spleen cells incubated with HKT or PPD (or hkBCG) for 48 h. Con A control supernates received Con A at the end of the incubation period.

**Induction and Evaluation of Macrophage Trypanocidal Activity.** Monolayers of normal and PP-induced macrophages were prepared, and nonadherent cells were removed after a 2-h incubation at 37°C (7). SCF was then added at a concentration of 25% in D<sub>2</sub>HIFBS. 18 h later, purified trypomastigotes were added in a 100- $\mu$ l volume to yield an organism/cell ratio of 2:1, and then incubated for 120–180 min at 37°C. At the end of the exposure period, coverslips were washed extensively to remove all extracellular parasites, and they were either fixed for microscopic observation or replenished with complete medium (D<sub>2</sub>HIFBS) in the presence or absence of SCF, and incubation was continued at 37°C for the desired time. The percentage of macrophages infected, number of parasites/100 macrophages, and total cell number were counted in Giemsa-stained samples as previously described (1).

## Results

The behavior of trypomastigotes of *T. cruzi* in mouse peritoneal macrophages

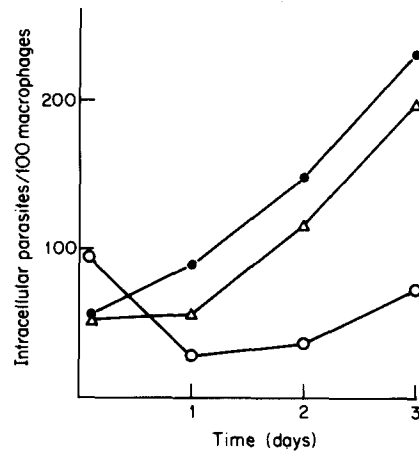


FIG. 1. The behavior of *T. cruzi* in three macrophage populations: normal resident peritoneal macrophages (●); macrophages harvested 4 days after an intraperitoneal injection of 1% PP (Δ); macrophages obtained from *T. cruzi*-infected mice, harvested 3 wk after infection and 2 days after an i.p. challenge with  $5 \times 10^6$  HKT (○). 24-h explanted macrophages were exposed to purified trypomastigotes at 2:1 multiplicity for (●) and (Δ), and a 3:1 multiplicity in (○). Thioglycollate-stimulated macrophages follow the same pattern as PP-induced macrophages, except that they take up twice as many trypomastigotes during the exposure period of 120 min.

obtained from normal, PP-stimulated, and *T. cruzi*-infected animals is illustrated in Fig. 1. In both the resident and inflammatory macrophage populations, intracellular parasites begin to replicate after a short lag period, and by 72 h, the resulting amastigotes fill the cytoplasm (1). These forms may differentiate into trypomastigotes, and then enter the extracellular space after cell disruption and infect other macrophages. In contrast, macrophages obtained from *T. cruzi*-infected animals kill  $\approx 75\%$  of the initial inoculum in the first 24 h. Thereafter, the remaining organisms begin to divide and will eventually destroy the monolayer. This escape could be explained by heterogeneity in either the macrophage or trypomastigote populations, or else by a loss of microbicidal activity of the immune macrophages under these particular *in vitro* conditions.

*Induction of Trypanocidal Activity on Inflammatory Macrophages by Factor(s) From Antigen-Stimulated Sensitized Spleen Cells.* Initial attempts to induce trypanocidal activity in normal, resident macrophages with lymphocyte products were unsuccessful (3). For this reason, we decided to investigate the conditions necessary for such induction by using macrophages obtained from a mildly inflamed peritoneal cavity. Exposure of PP-induced peritoneal macrophages to SCF from spleen cells of BCG- or *T. cruzi*-infected mice, incubated with the specific antigen resulted in the induction of trypanocidal activity (Fig. 2). Preincubation of macrophage cultures for 24 h before infection with active SCF (Fig. 2 A) resulted in intracellular killing of a portion of the phagocytized inoculum. In the absence of additional active SCF (days 0-3), organismal growth promptly resumed. Neither medium alone (nor control SCF) influenced the growth of the intracellular organisms. Fig. 2 B shows the added influence of having the active SCF present not only in the preinfection period, but added

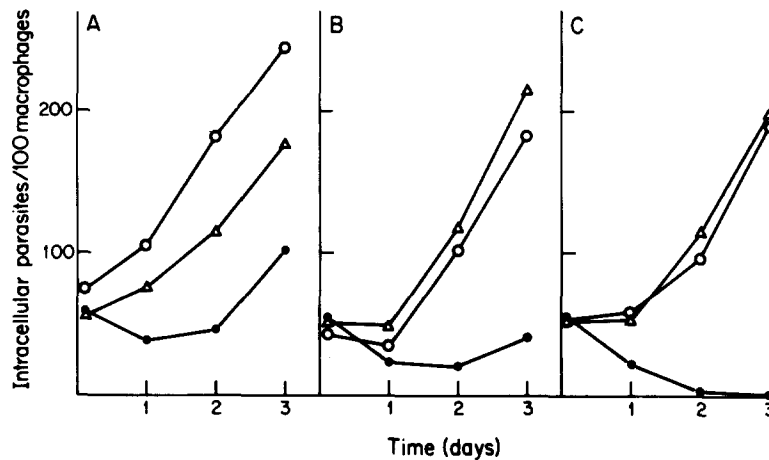


FIG. 2. The induction of trypanocidal activity in PP-induced macrophages. Freshly explanted cells were exposed to the following media for 18 h before infection with purified trypomastigotes (day -1 to 0): ( $\Delta$ ), D<sub>2</sub>HIFBS alone; ( $\circ$ ), D<sub>2</sub>HIFBS plus 25% control SCF (normal spleen cells + 10<sup>6</sup>/ml HKT); ( $\bullet$ ), D<sub>2</sub>HIFBS plus 25% active SCF (sensitized spleen cells + 10<sup>6</sup>/ml HKT). After infection with a 2:1 multiplicity (day 0), all subsets of (A) received D<sub>2</sub>HIFBS. (B) received the same media as in the above preinfection period at day 0, and this was left unchanged for the next 3 days. In contrast, (C) received the same media as (B), but fresh media was replaced every 24 h.

once after infection. However, preincubation plus the daily addition of active SCF led to the complete destruction of the intracellular parasites (Fig. 2C) within 48 h after infection. Fresh medium alone or control SCF were without significant effect. Although Fig. 2 only shows the number of parasites/100 macrophages, the percentage of cells infected and the total cell number were always evaluated.

*Induction of Trypanocidal Activity in Cultures of Normal, Resident Macrophages.* Similar trypanocidal properties could also be induced in unstimulated, resident macrophages by using the optimal conditions derived from studies with inflammatory cells; namely, the continuous presence and daily renewal of the active SCF (Fig. 3). Fig. 3A shows that preincubation of unstimulated cells with the active SCF, followed by its constant replenishment, resulted in dramatic trypanocidal activity 48 h after infection and the absence of parasites at 72 h. A parallel experiment with PP-induced macrophages is shown in Fig. 3B, and this illustrates trypanocidal activity at day 1. Therefore, normal unstimulated macrophages are capable of displaying trypanocidal activity, but they require an additional 24 h of exposure to exhibit the activated state.

Phase-contrast micrographs of macrophage cultures infected 72 h before with trypomastigotes of *T. cruzi* are seen in Fig. 4. Figure 4A shows the appearance of cells cultured in medium alone, whereas the cells in Fig. 4B have been exposed to active SCF for 24 h before infection, and to its daily renewal. Parasites are abundant (arrows) in cells cultivated in medium alone, and they are absent in the activated cells. Cells cultivated in the presence of control SCF (not shown) display the same pattern as those cultivated in medium alone.

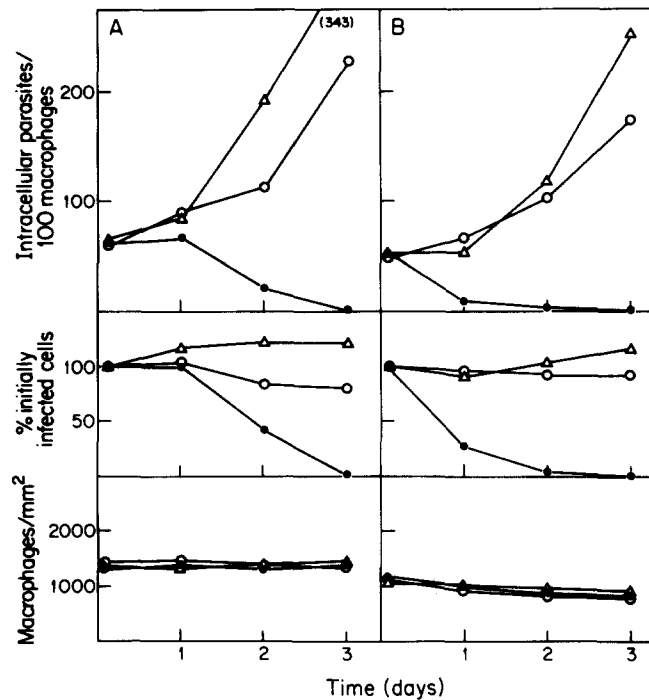


FIG. 3. Induction of trypanocidal activity in normal, resident macrophages. Unstimulated (A), or PP-induced (B) mouse peritoneal macrophages were explanted, washed at 2 h to remove nonadherent cells, and fed either D<sub>2</sub>HIFBS alone ( $\Delta$ ), D<sub>2</sub>HIFBS plus 25% control supernate ( $\circ$ ), or D<sub>2</sub>HIFBS plus 25% active BCG-induced SCF ( $\bullet$ ). 18 h later, cells were infected with a 2:1 multiplicity of purified trypomastigotes for 2½ h, washed to remove extracellular parasites, and new medium  $\pm$  supernates was added each day.

*The Morphological Response of Macrophages Exposed to Active SCF.* The exposure of either unstimulated or PP-stimulated macrophages to active SCF leads to characteristic changes in macrophage morphology. As shown in Fig. 4, these include symmetrical spreading, intensely ruffled plasma membrane, and the presence of numerous pinocytotic vesicles and lysosomes in the perinuclear region. Such changes are present as early as 24 h after the addition of active SCF, they are similar to the appearance of cells activated *in vivo* (2), and they are often an accurate indication of microbicidal activity.

*The Specificity of the SCF and its Ability to Enhance the Trypanocidal Activity of In Vivo Activated Macrophages.* Macrophages activated *in vivo* initially kill a portion of the inoculum, but upon further *in vitro* cultivation, they lose this ability with the subsequent growth of the organisms. This is illustrated in Fig. 5 in which cells obtained from animals infected with *T. cruzi* (Fig. 5 A) or BCG (Fig. 5 B), and cultivated with medium alone allowed growth to occur. The presence of an SCF in the *in vitro* system, however, led to rapid killing and disappearance of the intracellular parasites. Although antigenic

FIG. 4. Phase-contrast micrographs of PP-induced macrophages 72 h after infection with *T. cruzi*. (A), Cells cultivated in medium alone for the whole time. (B), Cells cultivated in medium containing 25% of BCG-induced SCF.  $\times 600$ .

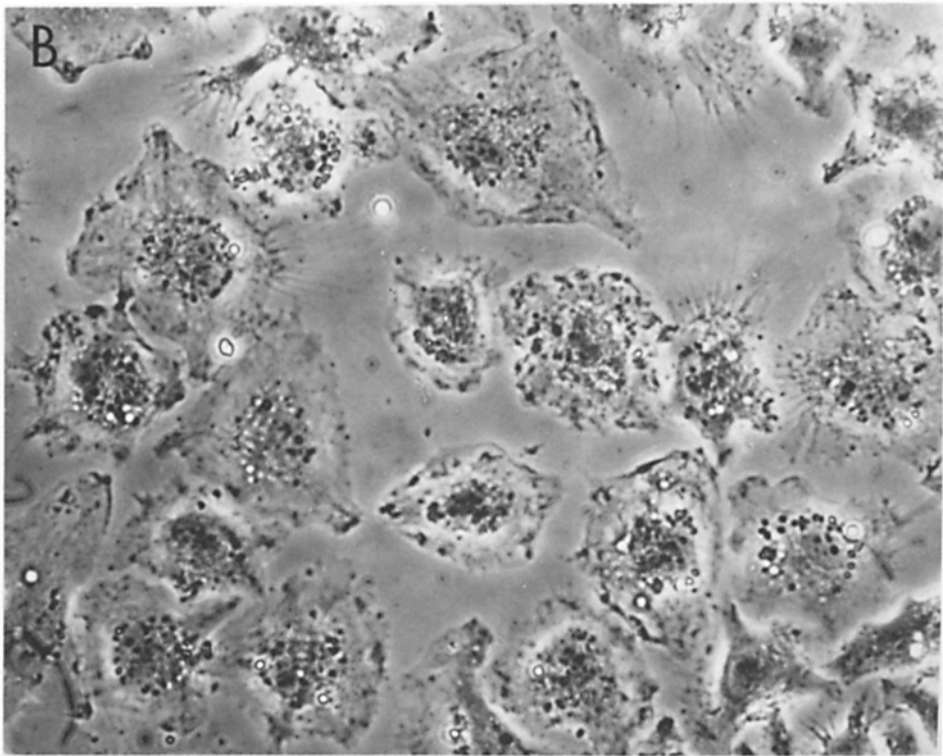
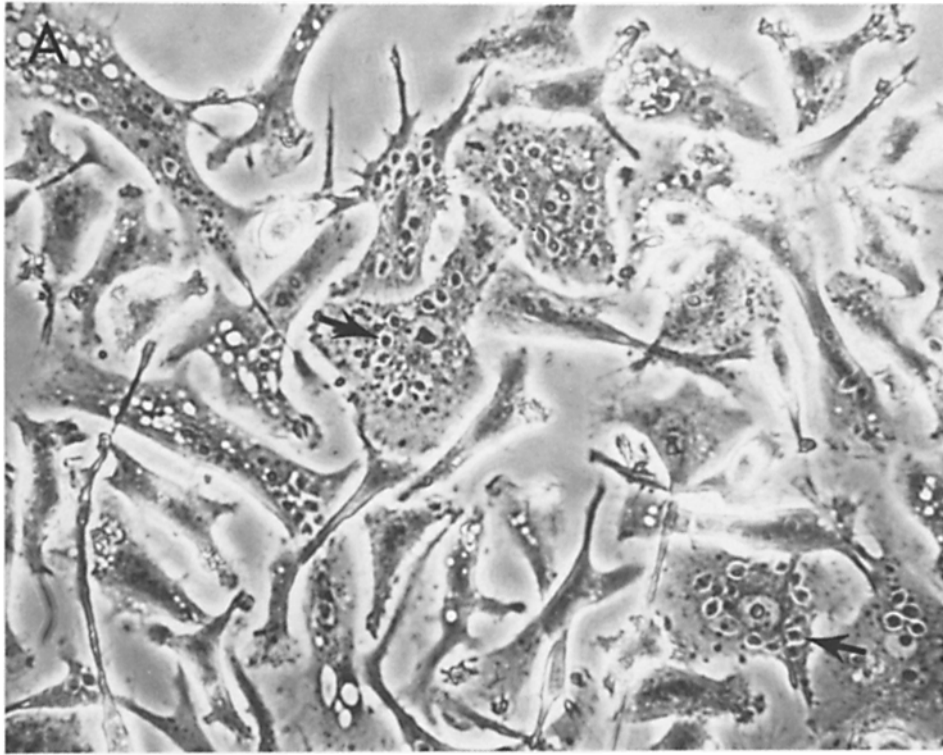


FIG. 4  
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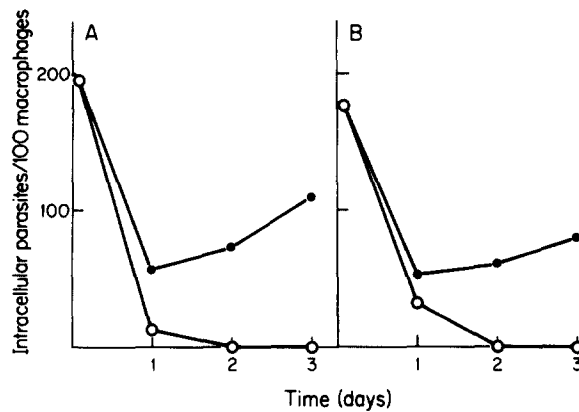


FIG. 5. The ability and specificity of SCF to enhance the trypanocidal activity of in vivo activated macrophages. (A) Macrophages obtained 3 wk after infection with  $5 \times 10^6$  *T. cruzi* and 2 days after an i.p. challenge with HKT. (B) Macrophages obtained after infection with  $2-6 \times 10^7$  BCG intravenously, and 2 days after i.p. challenge with PPD. After 24 h cultivation in D<sub>2</sub>HIFBS, the cells were infected with a 10:1 multiplicity of purified trypomastigotes for 2½ h and the extracellular parasites were removed. The cultures then received either D<sub>2</sub>HIFBS alone (●); or in series (A, ○) medium containing a 25% supernate from BCG-sensitized spleen cells exposed to PPD, or in (B, ○) a 25% supernate prepared from *T. cruzi*-sensitized spleen cells exposed to HKT. The details of preparing the spleen cell supernatant factor are found in Materials and Methods.

specificity in the generation of the SCF is observed, the factor itself and its resulting enhancement of macrophage microbicidal activity is quite nonspecific. In Fig. 5 A, the addition of a factor generated by the addition of PPD to BCG-sensitized spleen cells led to the trypanocidal activity of *T. cruzi*-activated macrophages. Similarly, in Fig. 5 B, a factor generated from the interaction of HKT with *T. cruzi*-sensitized spleen cells led to enhanced trypanocidal activity of BCG-activated cells. This degree of enhanced microbicidal action is identical to the previous effects noted in the homologous system (Fig. 3). It should also be noted that these experiments were performed with a very high parasite/cell ratio of 10:1, and still led to complete elimination of the organisms.

*Induction of Macrophage Trypanocidal Activity by Supernates of Con A- and LPS-Stimulated Lymphocytes.* The use of T-cell and B-cell mitogens such as Con A and LPS on the production of SCF was next investigated. Incubation of macrophages with supernatant fluids from Con A- or LPS-stimulated normal spleen cells resulted in a similar induction of trypanocidal activity (Fig. 6). The magnitude of the microbicidal activity induced by these supernates was, however, lower than that produced by antigen-pulsed sensitized lymphocytes. Con A ( $3 \mu\text{g/ml}$ ) alone had no effect on the trypanocidal activity of macrophages. These results suggest the involvement of T cells in the generation of the supernatant factor(s). Preliminary experiments suggest that LPS alone is not capable of inducing macrophage microbicidal activity, but more detailed experiments are in progress to elucidate this point.

*Effect of SCF Concentration on the Trypanocidal Activity of Macrophages.* A dose-response of SCF concentration versus the percent of intracellular parasites destroyed at 24 and 72 h after infection is shown in Fig. 7. SCF concentrations of 6.25% result in a significant trypanocidal activity. The curve plateaus at

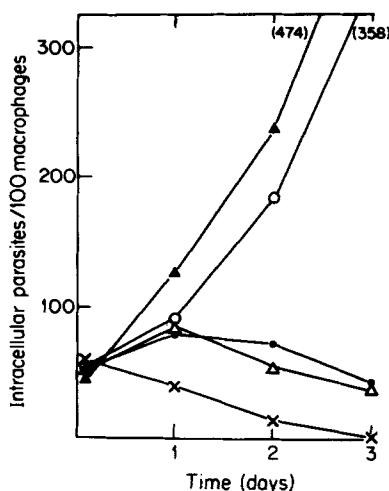


FIG. 6. Effect of Con A- and LPS-stimulated spleen cell supernates on the trypanocidal activity of macrophages. Macrophages were harvested from Swiss mice 4 days after an intraperitoneal injection of 1% solution of PP. 2 h after plating, cells were washed and fed either medium alone (▲), medium + 25% supernate from normal spleen cells incubated for 48 h, at the end of which 3  $\mu\text{g}/\text{ml}$  Con A was added (control supernate) (○), medium + 25% supernate from normal spleen cells incubated for 48 h with 3  $\mu\text{g}/\text{ml}$  Con A (●), medium + 25% supernate from normal spleen cells incubated for 48 h with 100  $\mu\text{g}/\text{ml}$  LPS (△), or medium + 25% supernate from spleen cells from 3-wk BCG-infected mice incubated for 48 h with PPD (×). Cells were infected with purified trypomastigotes 18 h later, and medium  $\pm$  supernates was added fresh daily.

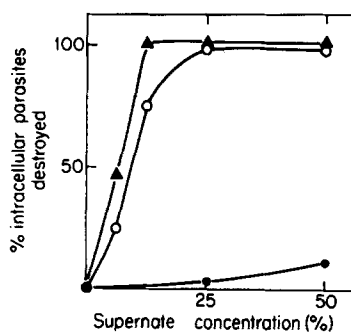


FIG. 7. Dose response titration of supernatant factor(s) activity.  $10^8$  spleen cells from control (●) or 3-wk BCG-infected mice (○, ▲) were exposed to hkBCG for 48 h. Supernatant fluids were added at different concentrations to PP-induced peritoneal macrophages 2 h after explanting. Cells were infected 18 h later with purified trypomastigotes at a 2:1 multiplicity for 2½ h, extracellular parasites were removed by washing, and SCF was read daily. Values represent percent parasites destroyed at 24 h (○, ●) and 72 h (▲).

a concentration of 25% and a concentration of 12.5% resulted in destruction of all intracellular parasites at 72 h. Control supernates did not result in significant trypanocidal activity at 24 h, and parasite growth was similar to control cells cultivated in medium alone at 72 h, as seen in Table I.

*Effect of the Time of Addition of SCF on the Trypanocidal Activity of Macrophages.* Phagocytized trypomastigotes within normal or inflammatory macrophages leave the confines of the vacuolar system and multiply free in



TABLE I  
*Effect of SCF Concentration on the Induction of Trypanocidal Activity of Macrophages\**

Macrophage treatment	Intracellular parasites/ 100 macrophages			Infected macrophages %		
	3 h	24 h	72 h	3 h	24 h	72 h
None	50	50	216	34	32	34
+ BCG SCF 50%	44	4	0	33	3	0
+ BCG SCF 25%	47	2	0	33	2	0
+ BCG SCF 12.5%	45	11	0	34	10	0
+ BCG SCF 6.25%	48	36	26	33	23	13
+ Control SCF 25%	46	45	205	33	31	31

\* SCF was obtained from control or 3-wk BCG-infected mice, incubated with hkBCG for 48 h. Macrophages were harvested from Swiss mice 4 days after an i.p. injection of 1% solution of PP. 2 h after explanting, SCF were added at different concentrations. Cells were infected 18 h later with purified trypomastigotes at a 2:1 multiplicity for 150 min, extracellular parasites were removed by washing, and SCF was re-added daily.

the cytoplasmic matrix. In macrophages activated *in vitro* by preincubation and constant exposure to lymphocyte products, electron micrographs indicate that partially digested parasites are found within phagocytic vacuoles. This suggests that the lymphokines prevent the parasites from escaping the phagocytic vacuole and allow its digestion within the phagolysosomal system. The question asked in this series of experiments was whether the lymphokines would modify the trypanocidal activity of the cell after the escape of the organism from the phagosome. To answer this question, SCF was added to macrophage cultures at 24 and 48 h after infection. Fig. 8 shows the results of such experiments. In both cases, a marked trypanocidal activity was observed, with a time course similar to that when the lymphokines were added before infection. That is, in PP-induced cells no parasites were visible by 72 h after the addition of the SCF. This clearly indicates that the SCF is influencing the fate of organisms free in the cytoplasmic matrix.

### Discussion

Since the studies of Mackaness (8-11) were carried out, it has been recognized that the host resistance to facultative intracellular bacteria is expressed by means of a cell-mediated reaction involving sensitized T cells (12, 13) and mononuclear phagocytes. These experiments conducted under *in vivo* conditions were associated with striking reductions in the number of viable organisms residing in the liver and spleen, as the result of preinfection or the adoptive transfer of sensitized spleen cells. Attempts at reproducing these results under *in vitro* conditions met with less success (14-17). Employing the guinea-pig model, Simon and Sheagren (15, 16) and Fowles et al. (17) were able to demonstrate transient bacteriostatic effects in the presence of macrophages, sensitized lymphocytes, or lymphocyte products. This system, however, presented a number of difficulties including the extracellular multiplication of *Listeria monocytogenes* and the progressive loss of guinea-pig macrophages upon cultivation. Certain of these difficulties were obviated in the more recent

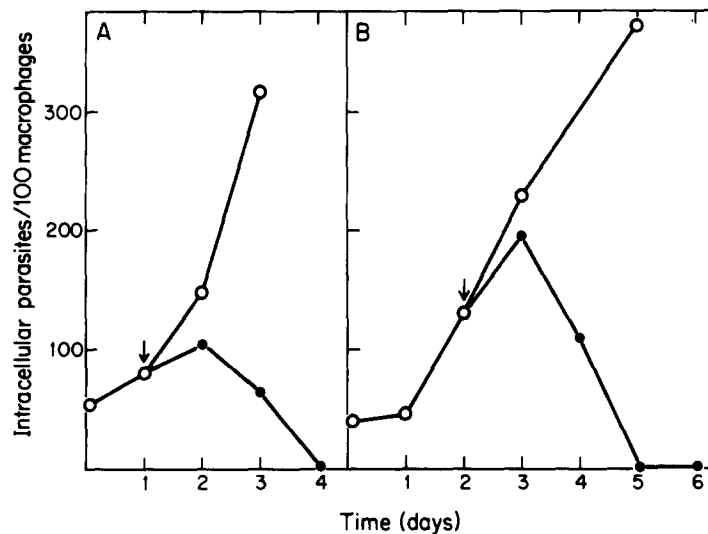


FIG. 8. Effect of time of addition of active SCF on the trypanocidal activity of macrophages. Macrophages were harvested from PP-stimulated mice and cultivated for 24 h in  $D_2$ HIFBS. Cells were then exposed to purified trypomastigotes at a 2:1 multiplicity for 2½ h, extracellular parasites were removed by washing, and cells were fed either medium alone (○) or medium + 25% active SCF (●) at 24 h (A) or 48 h (B) after infection. Arrows indicate the time of supernate addition.

studies employing murine and human monocytes and macrophages, by examining their parasitization with *Toxoplasma gondii* and *T. cruzi* (18–22). Nevertheless, under the best conditions the end result was either inhibition of growth or a short-lived reduction in viable organisms.

The experiments we report are therefore rather unique in the literature of cell-mediated immunity. Utilizing reagents in a completely *in vitro* environment it has been possible to induce the microbicidal activity of macrophages to kill the obligate intracellular parasite *T. cruzi*. This property could be evoked in unstimulated, resident cells as well as in inflammatory macrophages, and it led to the total disappearance of intracellular parasites within a period of 48–72 h. Two conditions were necessary to insure this state of activation. The first was the availability of an active spleen factor which was generated by the addition of microbial antigen to spleen cells from a previously infected animal. This material was present in the Millipore-filtered supernatant fluids of such incubation mixtures. The generation of the SCF required immunological specificity between antigen and sensitized spleen cells; however, once generated, it nonspecifically activated macrophage populations for an intracellular kill, i.e., SCF generated from a BCG interaction activated macrophages to kill *T. cruzi*. Since similar products have been employed to enhance the macrophage kill of tumor cells (23), it is possible that SCF yields a population of macrophages which is primed for both intracellular and extracellular cytotoxic events.

The second factor necessary to induce and maintain the trypanocidal activity of macrophages is the daily addition of fresh SCF. If not present at concentrations of 10–25%, macrophages lose their ability to inactivate trypanosomes. This finding may explain the loss of microbicidal activity exhibited by macrophages activated *in vivo* when explanted to a culture system in the absence of sensitized

lymphoid cells. The fact that SCF can restore the microbicidal activity of this population suggests that similar factor(s) play a role in the intact host. If this is the case and the system is under similar controls, then the continuous stimulation of lymphoid cells with antigen should play an important role in organ clearance.

The cellular origin of SCF is at present unclear, since the spleen contains a variety of lymphoid and myeloid elements. There are, however, suggestions that both bone marrow- and thymus-derived lymphocytes may play a role in its generation. Both Con A and LPS can evoke the production of SCF when incubated with the spleen cells from uninfected animals. Furthermore, preliminary experiments utilizing an anti- $\theta$  serum and complement leads to a marked inhibition of the ability of sensitized spleen cells to liberate SCF in response to antigen.

The more complex intracellular life cycle of *T. cruzi* makes its study both a help and a hindrance in the understanding of the microbicidal event. Two factors must be taken into consideration. The first is the site at which the organism is killed, and the second is the mechanism of killing. It was known from previous work (1) that trypomastigotes rapidly escape the confines of the phagosome and replicate, as amastigotes, in the cytoplasm of normal macrophage. In macrophages activated *in vivo* or exposed to active SCF for 24 h before infection, the partially digested organisms are now within the vascular apparatus. This might suggest that SCF or similar *in vivo* products prevent the trypomastigotes from escaping from the phagocytic vacuole. Other possibilities are suggested from experiments in which the organisms are first allowed to escape into the cytoplasm and the cell is then exposed to active SCF. Under these conditions, trypomastigotes are again found within membrane-bound organelles in a disrupted state. This implies either that trypanosomes are first killed in the cytoplasm and then autophagocytized by the cell, or that autophagy occurs first and the organism is killed within the vacuole. In the latter case, SCF could interact with the organism in the vacuole, whereas in the former, some induced metabolic cytoplasmic event would have to be implicated. At this time we are ignorant of the mechanisms, but we are involved in an examination of the role of oxygen intermediates in the process.

### Summary

Normal, resident and inflammatory mouse peritoneal macrophages can be induced to display microbicidal activity against trypomastigotes of *Trypanosoma cruzi* by exposure to products from antigen-pulsed, sensitized spleen cell populations. Optimal macrophage microbicidal activity was achieved by constant exposure and daily renewal of the spleen cell factors. Macrophages obtained after an intraperitoneal injection of mild inflammatory agents were rapidly induced, displaying trypanocidal activity 24 h after exposure to the active spleen cell factor(s), and by 48 h, parasites were no longer observed. Resident peritoneal macrophages required 24 h longer for activation. Removal of the factor(s) before achieving complete disappearance of intracellular parasites led to resumed growth of the surviving organisms. The spleen cell factor(s) is effective when added either before or after exposure of the macrophages to

trypomastigotes, and does not itself alter parasite viability. Dilution of the factor(s) up to 1:16 still results in significant trypanocidal activity.

In vivo activated cells, obtained after a specific secondary challenge of animals infected with *T. cruzi* or Bacille Calmette-Guérin, lose their trypanocidal activity under in vitro conditions. This loss of activity can be prevented or restored by the addition of the active spleen cell factor(s). Induction of trypanocidal activity is also obtained with products from Concanavalin A- or lipopolysaccharide-stimulated normal spleen cells.

We thank Ms. Marianne Polimeni, Chawanee Juangbhanich, and Judy Adams for their excellent technical assistance, and Ms. Grace Silvestri for typing the manuscript.

Received for publication 30 March 1978

### References

1. Nogueira, N., and Z. Cohn. 1976. *Trypanosoma cruzi*: mechanism of entry and intracellular fate in mammalian cells. *J. Exp. Med.* 143:1402.
2. Nogueira, N., S. Gordon, and Z. Cohn. 1977. *Trypanosoma cruzi*: modification of macrophage function during infection. *J. Exp. Med.* 146:157.
3. Nogueira, N., S. Gordon, and Z. Cohn. 1977. *Trypanosoma cruzi*: the immunological induction of macrophage plasminogen activator requires thymus-derived lymphocytes. *J. Exp. Med.* 146:172.
4. Fernandes, J. F., and O. Castellani. 1966. Growth characteristics and chemical composition of *Trypanosoma cruzi*. *Exp. Parasitol.* 18:195.
5. Nogueira, N., C. Bianco, and Z. Cohn. 1975. Studies on the selective lysis and purification of *Trypanosoma cruzi*. *J. Exp. Med.* 142:224.
6. Munthe-Kaas, A. C., and P. O. Seglen. 1974. The use of metrizamide as a gradient medium for isopycnic separation of rat liver cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 43:252.
7. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* 121:153.
8. Mackaness, G. B. 1962. Cellular resistance to infection. *J. Exp. Med.* 116:381.
9. Mackaness, G. B. 1964. The immunological basis of acquired cellular resistance. *J. Exp. Med.* 120:105.
10. Mackaness, G. B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity *in vivo*. *J. Exp. Med.* 129:973.
11. Mackaness, G. B. 1970. The monocyte in cellular immunity. *Semin. Hematol.* 7:172.
12. Lane, F. C., and E. R. Unanue. 1972. Requirement of thymus (T) lymphocytes for resistance to listeriosis. *J. Exp. Med.* 135:1104.
13. North, R. J. 1973. Importance of thymus-derived lymphocytes in cell-mediated immunity to infection. *Cell. Immunol.* 7:166.
14. Krahenbuhl, J. L., and J. S. Remington. 1971. *In vitro* induction of nonspecific resistance in macrophages by specifically sensitized lymphocytes. *Infect. Immun.* 4:337.
15. Simon, H. B., and J. N. Sheagren. 1971. Cellular immunity *in vitro*. *J. Exp. Med.* 133:1377.
16. Simon, H. B., and J. N. Sheagren. 1972. Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. *Cell. Immunol.* 4:163.
17. Fowles, R. E., I. M. Fajardo, J. L. Leibowitch, and J. R. David. 1973. The enhancement of macrophage bacteriostasis by products of activated lymphocytes. *J. Exp. Med.* 138:952.

18. Hirsch, J. G., T. C. Jones, and L. Len. 1974. Interactions *in vitro* between *Toxoplasma gondii* and mouse cells. Parasites in the immunized host; mechanisms of survival. *Ciba Found. Symp.* 25:205.
19. Anderson, S. E. Jr., and J. S. Remington. 1974. Effect of normal and activated human macrophages on *Toxoplasma gondii*. *J. Exp. Med.* 139:1154.
20. Borges, J. S., and W. D. Johnson, Jr. 1975. Inhibition of multiplication of *Toxoplasma gondii* by human monocytes exposed to T-lymphocyte products. *J. Exp. Med.* 141:483.
21. Anderson, S. E., S. Bautista, and J. S. Remington. 1976. Induction of resistance to *Toxoplasma gondii* in human macrophages by soluble lymphocyte products. *J. Immunol.* 117:381.
22. Williams, D. M., and J. S. Remington. 1977. Effect of human monocytes and macrophages on *Trypanosoma cruzi*. *Immunology* 32:19.
23. Ruco, L. P., and M. S. Meltzer. 1977. Macrophage activation for tumor cytotoxicity; induction of tumoricidal macrophages by supernatants of PPD-stimulated *Bacillus Calmette-Guérin*-immune spleen cell cultures. *J. Immunol.* 119:889.