TRYPANOSOMA CRUZI: MODIFICATION OF MACROPHAGE FUNCTION DURING INFECTION*

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Trypanosoma cruzi, the etiological agent of Chagas' disease, produces widespread infections in man and animals, parasitizing a variety of cell types, including those of the myocardium. In this and other sites, accumulation of lymphocytes and macrophages are common (1), thus leading a number of investigators to suggest a role for cell-mediated immunity in resistance to the parasite. The presence of delayed hypersensitivity in humans (2) and experimental animals (3) as well as inhibition of leukocyte migration (4) are consistent with this concept. In addition, suggestions that partial protection can be conferred by transfer of spleen cells from immune donors (5, 6) and the resistance of peritoneal macrophages to in vitro parasitization after infection with either *T. cruzi* or BCG are in keeping with a cellular mechanism (7).

Prior work from this laboratory has defined conditions whereby trypomastigotes may be separated from mixed culture forms of *T. cruzi* (8). Epimastigotes taken up by resident, unstimulated macrophages are promptly destroyed, whereas trypomastigotes survive quantitatively and replicate in the cytoplasm of both normal and thioglycollate-induced macrophages (9).

In this paper we examine the property of macrophages obtained from animals infected with both *T. cruzi* and BCG, first in terms of their microbicidal activity against trypomastigotes of *T. cruzi* and second in terms of other parameters of macrophage activation, such as secretion of plasminogen activator and phagocytosis mediated by the C3 receptor.

Materials and Methods

Parasites. The Y strain of *T. cruzi* was obtained from Dr. S. C. Correa, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, and the Tulahuén strain from Dr. B. Bloom, Albert Einstein School of Medicine, Bronx, N. Y. The parasites were grown in Tobie's medium and passed weekly (10). Parasites were harvested from 8-day-old cultures, washed five times in ice-cold phosphate-buffered saline (PBS)[1] (Dulbecco's; Grand Island Biological Co., Grand Island, N. Y.) at 750 *g* for

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[1] *Abbreviations used in this paper:* AT, acid treated; CM, conditioned medium; D10, Dulbecco's medium containing 10% heat-inactivated bovine serum; D20STI, Dulbecco's medium containing 20% heat-inactivated bovine serum and 60 μg/ml soybean trypsin inhibitor: E(IgM)C, sheep erythrocytes coated with anti-sheep erythrocyte IgM and complement; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HKT, heat-killed trypanosomes; H37Ra, strain H37Ra of *Mycobacterium tuberculosis*; PBS, phosphate-buffered saline; PMNs, polymorphonuclear leukocytes; PP, proteose-peptone; PPD, purified protein derivative of tuberculin; STI, soybean trypsin inhibitor.

15 min, resuspended in ice-cold PBS or Dulbecco's modified Eagle's medium (GIBCO) and counted in a hemocytometer with a 40 × objective. The cultures of Y strain contained 85% epimastigotes and 15% trypomastigotes. The Tulahuén strain contained 95% epimastigotes. Purified trypomastigotes were obtained as previously described (8).

Cell Culture

NORMAL MACROPHAGES. Mouse peritoneal macrophages were obtained from Swiss mice maintained at The Rockefeller University, and C57BL/6J, A/J, and C3H/HeJ mice obtained from The Jackson Laboratory, Bar Harbor, Maine. Cells were harvested according to the methods of Cohn and Benson (11) and plated either on 13-mm round cover slips in Dulbecco's plus 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (D10), or in ^{125}I-fibrin-coated 16-mm Linbro plates (Linbro Chemical Co., New Haven, Conn.) in Dulbecco's plus 20% heat-inactivated FBS, 60 μg/ml soybean trypsin inhibitor (STI, fraction VI; Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.), to suppress fibrinolysis, and antibiotics, as above (D20STI). The average yield of cells obtained from normal mice was 5×10^6, of which 45–50% were macrophages. Serial dilutions of cells were plated for most experiments, ranging from 1×10^5 to 10^6 cells per well.

IMMUNE MACROPHAGES. Immune macrophages were obtained from animals previously infected intraperitoneally with 5×10^6 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.). Animals were kept in large plastic cages, usually six per cage. Controls were kept under the same conditions for the same period of time. Cells were harvested, at different times after the infection, from the peritoneal cavity with or without an intraperitoneal challenge with the following agents: (*a*) heat-killed trypanosomes (HKT), 5×10^6 culture forms of the Y or Tulahuén strain, heated at 80°C for 10 min, in PBS. (*b*) *Mycobacterium tuberculosis*, strain H37Ra, (lyophilized; Difco Laboratories, Detroit, Mich.). Stock solutions were sonicated, 2 mg/ml in PBS plus 0.1% bovine serum albumin and 400 μg in 1 ml PBS injected per mouse. (*c*) Purified protein derivative of tuberculin (PPD) (Connaught Medical Research Laboratory, Willowdale, Ontario, Canada), 50 μg in 1 ml PBS. (*d*) Proteose-peptone (PP) (Difco Laboratories), 1 ml of a 1% solution.

Cells were cultivated as described above for normal macrophages for 24 h before assays were started. Serial dilutions of cells were plated for most experiments, ranging from 10^5 to 10^6 cells per well. Average yields were: 10×10^6 cells per mouse for control challenged animals and $15–20 \times 10^6$ for immune, boosted animals from which 70–75% were macrophages and 20–25% polymorphonuclear leukocytes (PMNs) at day 2. Immune resident cells were 10×10^6 per mouse, from which 45–50% were macrophages, 45–50% lymphocytes, and about 5% PMNs.

Assays

FIBRINOLYSIS ASSAY. Fibrinolysis was assayed on ^{125}I-fibrin-coated 16-mm Linbro plates as described elsewhere (12). The plates contained 10 μg fibrinogen/cm^2 and 5×10^5 trypsin-releasable cpm. Peritoneal cells were plated on ^{125}I-fibrin-coated plates in D20STI. 2 h later nonadherent cells were removed by washing the plates three times with Hanks' balanced salt solution (HBSS; GIBCO); the adherent cells were cultivated for 24 h in D20STI, washed three times in HBSS to remove inhibitors and dead PMNs, and the assay started by adding 0.5 ml Dulbecco's plus 5% acid-treated (AT) dog serum. At this time, the cultures were devoid of granulocytes, as determined by microscopic observation. Cultures were monitored at 2 h and 24 h to insure that no fibrinolysis had occurred before starting the assay. 50- to 100-μl samples were withdrawn and assayed for release of radioactivity in a Packard Gamma Counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Cells were plated in duplicate or in serial dilutions for all the assays. Results are expressed as percent of the total radioactivity releasable/4 h/10^6 cells.

Plasminogen activator in conditioned medium (CM) was measured by collecting 50- to 100-μl samples of cells kept in serum-free medium with 0.2% lactalbumin hydrolysate for 48 h, and measuring radioactivity released in the presence and absence of 2 μg dog plasminogen on labeled fibrin plates. Plates contained 95,000 cpm trypsin-releasable ^{125}I-fibrin; 1 U = 10% of total radioactivity releasable/4 h. For measurement in cell lysates cells were washed twice with HBSS, scraped from the dish in 0.5–1 ml of 0.1% Triton X-100, and stored at −20°C, as described elsewhere (12, 13). Protein was measured by the method of Lowry (14) using egg lysozyme as a standard.

LYSOZYME ASSAY. Lysozyme secretion was measured in CM of cells kept in D10 for 48 h, from the initial rate of lysis of a suspension of *Micrococcus lysodeikticus* (spray-dried; Miles Laboratories, Inc., Miles Research Div.) using a recording spectrophotometer (15).

Reagents

AT DOG SERUM. AT dog serum was prepared by adjusting the pH of dog serum (GIBCO) to 2 with 2 N HCl in isotonic saline; after 30 min at room temperature, the pH was adjusted to 7.4 with 2 N NaOH in isotonic saline and filter sterilized (12).

PLASMINOGEN. Plasminogen was purified from dog serum by affinity chromatography on lysine-Sepharose according to the method of Deutsch and Mertz (16).

Preparation of Sensitized Erythrocytes. Sheep erythrocytes coated with IgM and complement [E(IgM)C] and the assay of ingestion were performed as described previously (17, 18).

Trypomastigote-Killing Assay. 50 μl of a suspension of purified trypomastigotes in Dulbecco's were added to macrophage monolayers on 13-mm round cover slips, at a 1:1 or 1:2 parasite per cell ratio, and incubated for 90–180 min at 37°C. At the end of the exposure period, cover slips were washed extensively to remove all extracellular parasites and either fixed for microscopic observation or replenished with complete medium (D10), and incubation continued at 37°C for the desired time. Percentage of macrophages infected, number of parasites per macrophage, and total cell number were counted in Giemsa-stained samples as previously described (9).

Results

The Trypanocidal Activity of Macrophages

We have previously reported that resident mouse peritoneal macrophages or macrophages obtained from thioglycollate-stimulated animals fail to kill the trypomastigotes of *T. cruzi*, whereas epimastigotes are promptly destroyed within their phagolysosomes (9). This prompted us to examine the role of acquired, cell-mediated immunity, elucidated in other systems in the destruction of intracellular parasites. For this purpose, we tested the ability of macrophages from *T. cruzi*-infected mice to deal with trypomastigotes in vitro. In addition, animals were also infected with BCG to examine the specificity of the acquired immunity. Finally, a comparison was made between the resident cells of infected animals and those evoked by a secondary intraperitoneal challenge with either HKT, H37Ra antigen, or a nonspecific irritant in the form of PP. These results are outlined in Table I.

Resident macrophages from animals infected with *T. cruzi* showed no trypanocidal activity, when compared to macrophages from noninfected controls. However, when these animals, infected with *T. cruzi* for 2–6 wk, were challenged intraperitoneally with HKT, their macrophages were able to destroy approximately 70% of the ingested trypomastigotes. Similarly, macrophages from *T. cruzi*-infected animals, challenged either with the unrelated antigen H37Ra or a nonspecific stimulant, were also able to kill equal numbers of organisms. The injection of HKT, H37Ra, or PP into noninfected animals was without influence on their microbicidal activity. Animals infected with BCG and challenged either with HKT or H37Ra antigens expressed a lower, but significant, trypanocidal activity compared with cells from animals infected with live trypanosomes.

Resident peritoneal macrophages from *T. cruzi*- or BCG-infected animals, harvested without a previous secondary challenge, displayed a trypanostatic, rather than trypanocidal activity, as can be seen in Figs. 1 A and 1 B. The intracellular parasites multiply at a slower rate in these cells. It is apparent

TABLE I
*The Influence of Infection and Challenge on the Trypanocidal Activity of Cultivated Macrophages**

Infection	Challenge	Parasites/100 macrophages at 24 h (% initial value)‡	No. infected cells at 24 h (% initial value)‡
wk			
Control	–	118	104
	HKT	97	81
	PP	102	100
T. cruzi			
3	–	97	95
2	HKT	28	21
3	HKT	35	19
4	HKT	37	22
6	HKT	33	23
6	H37	22	18
4	PP	35	44
BCG			
3	–	92	82
3	HKT	72	53
3	H37	53	42

* Swiss mice were infected intraperitoneally with 5×10^6 live culture forms of *T. cruzi* Y strain, and at different times thereafter challenged intraperitoneally with HKT (5×10^6), PP (1 ml of a 1% solution), and H37Ra (400 μg in 1 ml PBS). Cells were harvested 2 days later, nonadherent cells removed by washing three times in HBSS, and macrophages cultivated for 24 h in D10 before infection.

‡ Cells were exposed to purified trypomastigotes at a 1:1 or 1:2 parasite/cell multiplicity for 3 h; under these conditions about 30–35% of the original macrophage population is infected. Values represent numbers at (T 24 h/T 3 h) × 100.

that even *T. cruzi* infection and specific antigenic challenge yielded cell populations which could not completely inactivate the phagocytized inoculum. The surviving 30–40% of the ingested inoculum multiplied at a slower rate in these cells. However, after 72 h of cultivation, the remaining trypomastigotes in these cells, as well as the trypomastigotes in the immune resident cells, were multiplying at a normal rate and were able to parasitize uninfected cells in the monolayer.

We concluded from these experiments that the peritoneal macrophages from infected animals express significant trypanocidal activity only after a secondary challenge. In contrast, resident cells from immune animals, obtained without a secondary challenge express a trypanostatic, rather than trypanocidal activity. Employing these systems, other parameters of macrophage stimulation were next examined.

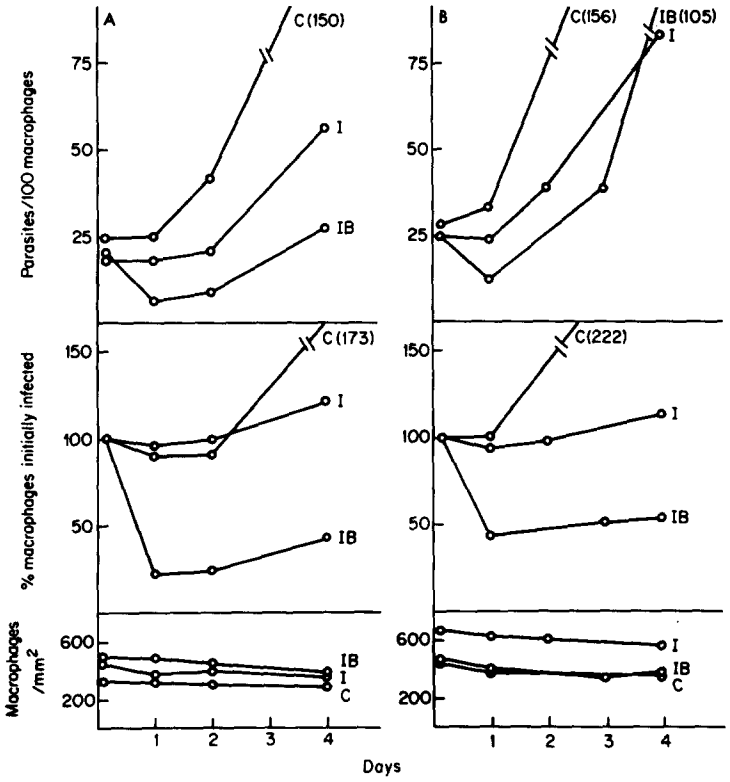

FIG. 1. The behavior of trypomastigotes of *T. cruzi* in macrophages from (A) *T. cruzi*- and (B) BCG-infected mice. Peritoneal macrophages from *T. cruzi*- or BCG-infected Swiss mice were harvested 3 wk after infection without (I) or with (IB) an intraperitoneal challenge with the respective antigen, 5×10^6 HKT or 400 μg H37Ra. 24-h explanted macrophages were exposed to purified trypomastigotes at a 1:1 multiplicity for 3 h. Infection and microscopic evaluation were done as described in the Materials and Methods. Control cells (C) are resident peritoneal macrophages from noninfected mice.

Macrophage Fibrinolytic Activity After Infection and Challenge with T. cruzi

Prior work had indicated that a plasminogen activator was secreted by macrophages activated by either thioglycollate medium (12) or endotoxin followed by particle ingestion (13). It was therefore of interest to investigate the role of infection with intracellular parasites on the secretion of this product. Fig. 2 A shows representative data on the role of infection and challenge with *T. cruzi* on the fibrinolytic activity of cultivated mouse peritoneal macrophages.

No significant increase in fibrinolytic activity was found in macrophages harvested from mice infected 5 wk previously with *T. cruzi* (I). However, a marked increase in fibrinolytic activity was found when the infected animals had received an intraperitoneal challenge with HKT 3 days before harvesting. These cells (IB) showed a 7- to 15-fold increase over the unstimulated controls (C). The resident immune macrophages (I) did show a two- to threefold increase

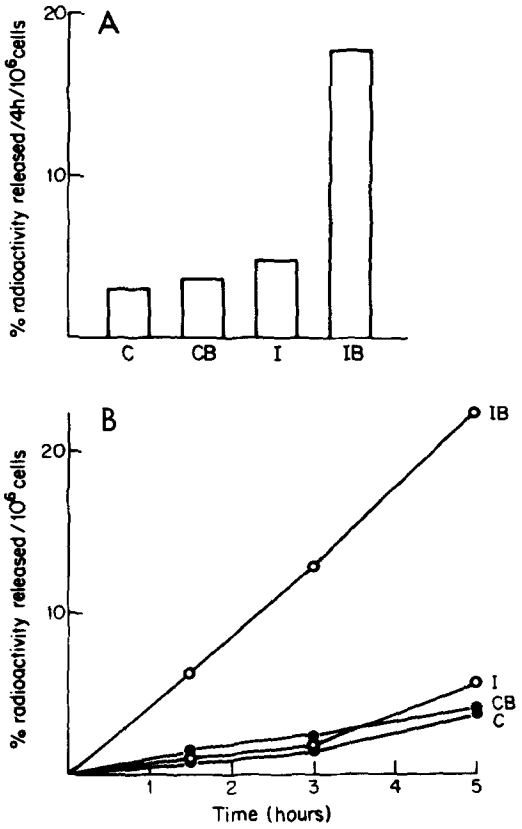

FIG. 2. Effect of infection and challenge with *T. cruzi* on the fibrinolytic activity of cultivated mouse macrophages. Mice were infected 5 wk previously with 5×10^6 live culture forms of *T. cruzi*. Resident peritoneal cells were harvested from infected (I) and control mice (C), and from infected (IB) and control (CB) mice which were challenged intraperitoneally 3 days before with 5×10^6 HKT (A). A time-course of fibrinolysis is shown in the lower graph (B).

in fibrinolytic activity in some experiments, but after the challenge this activity was always enhanced an additional four- to fivefold. In contrast, the intraperitoneal injection of HKT into noninfected controls (CB) yielded only an insignificant response although a similar amount of phagocytizable material was introduced into the cavity. Fig. 2 B illustrates the time-course of fibrinolysis, employing cells from the same sources, and shows the prompt and linear reaction of infected and boosted populations (IB).

The appearance of these activated macrophages in the peritoneal cavity after a secondary challenge requires a previous infection (Table II). The injection 3 wk before challenge of 5×10^6 HKT is not sufficient to generate macrophages with increased fibrinolytic activity. In contrast, infection with the same number of viable trypanosomes, followed 3 wk later by the intraperitoneal challenge with HKT yields macrophages which display enhanced fibrinolytic activity.

The Nature and Intracellular Localization of the Fibrinolytic Activity. The nature and distribution of the fibrinolytic activity of these activated macro-

TABLE II
*Fibrinolytic Activity of Macrophages Obtained from Animals Receiving Viable or Heat-Killed Trypanosomes**

Infection	Challenge	Plasminogen activator, % radioactivity released/4 h/10^6 cells
Control	–	1.0
	HKT	1.1
HKT	–	0.9
	HKT	1.8
T. cruzi	–	3.6
	HKT	12.2

*Mice were either given 5×10^6 live culture forms of *T. cruzi*, or 5×10^6 heat-killed culture forms by the intraperitoneal route. 3 wk later they were challenged intraperitoneally with 5×10^6 HKT and cells harvested 2 days later.

TABLE III
*The Nature and Localization of the Fibrinolytic Activity of Cultivated Macrophages**

Treatment		Plasminogen activator‡			
Infection	Challenge	Conditioned medium	Cell lysate	Total	sp act
			U/dish		*U/mg cell protein*
Control	–	6.2 (0.25)	1.8 (<0.1)	8.0	106
	+	5.5 (0.03)	1.2 (<0.1)	6.7	130
T. cruzi	–	20.0 (0.1)	6.7 (<0.1)	27.0	480
	+	88.0 (0.31)	23.0 (<0.1)	111.0	1860

* Mice were infected intraperitoneally with 5×10^6 culture forms of *T. cruzi* and boosted 2 wk later with 5×10^6 HKT. Peritoneal cells were harvested 2 days after the boost and macrophages cultivated for 2 days in medium containing FBS. The cultures were then washed and incubated for 2 days in serum-free medium.

‡ 50- to 100-μl samples were assayed in duplicate ± 2 μg dog plasminogen on fibrin plates containing 95,000 cpm trypsin-releasable ^{125}I-fibrin. 1 U, 10% of total radioactivity releasable/4 h. Results in parentheses were obtained in the absence of added plasminogen.

phages was next examined. Table III confirmed that fibrinolysis was induced in macrophages from immune challenged mice and was strictly dependent upon the presence of added plasminogen. In addition, the plasminogen-dependent product was largely (70–80%) found in the extracellular medium in cells from both immune and immune-challenged animals. These findings strongly suggest the secretion of a plasminogen activator. Although not illustrated, lysozyme levels were only twofold higher in culture fluids from infected and challenged mice than in noninfected controls.

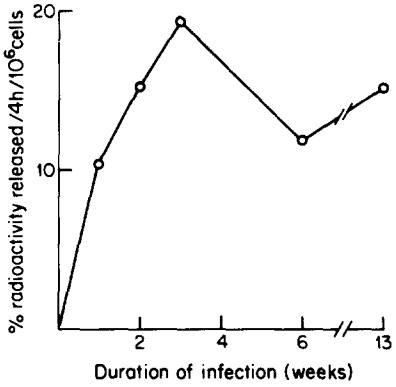

FIG. 3. Time-course of the appearance of macrophages with enhanced fibrinolytic activity elicited by an intraperitoneal challenge with HKT, during the course of an infection with *T. cruzi*. Cells were harvested 2 days after intraperitoneal challenge.

Factors Modifying the Secretion of Plasminogen Activator by Macrophages. A detailed examination was next conducted on the in vivo events which modified the expression of fibrinolysis by explanted macrophages.

THE DURATION OF INFECTION BEFORE CHALLENGE. Mice were given a sublethal infection and examined over the course of 13 wk. At intervals from 1 to 13 wk after infection the animals were challenged intraperitoneally with HKT, and the peritoneal cells harvested after 2 days and explanted in vitro. Fig. 3 demonstrates the rapid elevation of fibrinolytic activity, reaching a maximum 3 wk after infection and persisting at somewhat lower levels for over 3 mo. The fibrinolytic activity of the resident peritoneal macrophages from infected animals remained unaltered during this period. Spleen size increased 1.5- to 3-fold and, in general, paralleled the fibrinolytic activity.

THE INTRAPERITONEAL CHALLENGE-DOSE AND TIME OF HARVESTING. The effect of various amounts of HKT on the fibrinolytic activity of macrophages obtained from control and *T. cruzi*-infected animals is seen in Table IV. No significant alteration in fibrinolysis occurs in cells obtained from control mice. In contrast, macrophages from infected animals, obtained 2 days after challenge, expressed four- to sixfold higher activity when injected with 5×10^6 HKT organisms. Significant stimulation is also seen with 1×10^6 HKT but not with 5×10^5 organisms.

A number of experiments were performed to evaluate the fibrinolytic activity of macrophages obtained 2–4 days after an intraperitoneal challenge with 5×10^6 HKT. Cells obtained from noninfected mice exhibited low and constant levels of activity 2, 3, and 4 days postchallenge (3.3% cpm released/4 h/10^6 cells). Infected animals, however, exhibited a sharp maximum 2 days after challenge (18.9% cpm released/4 h/10^6 cells), decreasing progressively on days 3 and 4. This somewhat surprising reduction in fibrinolytic activity on the 3rd and 4th days was associated with poor macrophage viability and the presence of extremely vacuolated cells containing phagocytized debris. It should also be noted that considerable numbers of granulocytes are present in the 2-day exudates. These cells are, however, removed by in vitro cultivation (see Materials and Methods) and do not contribute to the macrophage response.

TABLE IV
*Effect of Challenge Size on the Fibrinolytic Activity of Cultivated Macrophages from Mice Infected with T. Cruzi**

Treatment		Plasminogen activator, % radioactivity released/4 h/10^6 cells
Infection	Dose of boost	
Control	–	2.1
	5×10^5 HKT	1.9
	1×10^6 HKT	1.7
	5×10^6 HKT	1.1
T. cruzi	–	3.6
	5×10^5 HKT	2.7
	1×10^6 HKT	8.1
	5×10^6 HKT	12.2

* Mice were infected intraperitoneally with 5×10^6 live culture forms of *T. cruzi*, boosted 3 wk later with HKT and the peritoneal cells harvested 2 days later.

THE SECRETION OF PLASMINOGEN ACTIVATOR BY OTHER MOUSE STRAINS. Comparative studies were performed with other strains of mice which differed in their susceptibility to infection with *T. cruzi*. In both the A/J (highly susceptible) and C57BL/6J (less susceptible) similar levels of fibrinolysis were observed in infected animals which received an intraperitoneal challenge with HKT.

THE IMMUNOLOGICAL SPECIFICITY OF THE PLASMINOGEN ACTIVATOR RESPONSE. To establish the specificity of the secondary challenge on the induction of plasminogen activator secretion, a comparison was made with cells obtained either from BCG- or *T. cruzi*-infected animals and noninfected controls challenged intraperitoneally with HKT and PPD. In addition, a non-specific inflammatory agent in the form of PP was included for the intraperitoneal challenge of both infected and noninfected groups of animals. The results of such experiments are shown in Table V.

The intraperitoneal administration of PPD, HKT, or PP into noninfected controls resulted in cells with low levels of fibrinolytic activity, similar to those found in control, noninjected animals. *T. cruzi*-infected animals challenged with HKT or BCG-infected animals challenged with PPD yielded cells which exhibited a 10- to 15-fold increase in fibrinolytic activity. In contrast, intraperitoneal challenge with the unrelated antigen or nonspecific irritant yielded a less dramatic (threefold) but definite increase in activity. The strength of the response was therefore closely related to the specificity of the challenge antigen as well as the previous infected state of the animals.

Although the majority of the experiments were conducted with animals infected with the Y strain of *T. cruzi*, it was noted that intraperitoneal challenge with heat-killed organisms of either the Tulahuén or Y strains gave similar results. This indicates a close antigenic relationship between these two strains of *T. cruzi*.

Infection and C-Mediated Phagocytosis

Prior studies had demonstrated that the ability of macrophages to ingest

TABLE V
*Specificity of Challenge on the Fibrinolytic Activity of Cultivated Macrophages from Mice Infected with BCG and T. Cruzi**

Treatment		Plasminogen activator, % radioactivity released/4 h/10^6 cells
Infection	Challenge	
Control	–	1.8
	PPD	1.6
	HKT	2.5
	PP	1.9
T. cruzi	–	2.1
	PPD	6.9
	HKT	23.9
	PP	5.6
BCG	–	1.3
	PPD	18.3
	HKT	3.8
	PP	2.4

* Mice were infected i.v. with 2–6 × 10^7 viable BCG or i.p. with 5 × 10^6 culture forms of *T. cruzi*. 3 wk later, the animals were challenged i.p. with either 50 μg PPD, 5 × 10^6 HKT, or 1 ml of a 1% solution of proteose peptone. Peritoneal cells were harvested 2 days later and cultivated for 24 h in D20STI before assayed.

E(IgM)C was related to their degree of "activation" or stimulation (17, 18). To examine this under conditions of chronic infection we employed both *T. cruzi* and BCG and compared the level of C-mediated ingestion in infected and challenged animals. Table VI outlines the results obtained. The expression of phagocytosis mediated by the C receptor is displayed nonspecifically by immune cells with or without challenge, as well as by cells from noninfected animals challenged intraperitoneally with the same inflammatory agents.

Discussion

We present data indicating that a sublethal infection with *T. cruzi* or BCG in mice, followed by an intraperitoneal challenge with the respective antigen, induces the appearance of macrophages which display microbicidal activity against trypomastigotes of *T. cruzi*. These activated macrophages also secrete large amounts of plasminogen activator, exhibit rapid spreading on glass or plastic surfaces, and ingest C-coated erythrocytes.

The trypanocidal activity displayed by these activated macrophages is not totally efficient, and a small percentage of the phagocytized trypomastigote inoculum survives and multiplies after 72 h. If the cultures are followed for longer periods of time, the infection spreads to the previously resistant cells. This could be due either to a loss of the microbicidal and microbistatic properties of the macrophages upon cultivation, or, less likely, to the survival of a resistant population of parasites.

A previous report by Hoff (7) has indicated that BCG- and *T. cruzi*-immune macrophages were microbicidal in vitro for culture forms of *T. cruzi*. In this

TABLE VI
Complement-Mediated Ingestion by Peritoneal Macrophages

Infection	Challenge	E(IgM)C ingestion, %*
Control	–	5
	HKT	31
	PPD	35
	PP	28
BCG	–	51
	HKT	47
	PPD	54
	PP	Not done
T. cruzi	–	24
	HKT	76
	PPD	33
	PP	20

* Cells were kept for 24 h in Dulbecco's medium containing 2% FBS after explanting, washed three times in HBSS, and incubated for 60 min in 1 ml Dulbecco's plus 0.1 ml 1% E(IgM)C. Attachment and uptake of E(IgM) was absent in all of the above groups. E(IgM)C ingestion expressed as percent of macrophages ingesting >3 sheep erythrocytes.

system, the *T. cruzi*-immune cells were resident peritoneal cells obtained without a secondary challenge. In our experience, such cells do not display microbicidal activity against trypomastigotes, but only a microbistatic effect which delays the growth of the parasites. The difference in the two reports may be explained by two factors. In Hoff's system, the challenging inoculum consisted of total culture forms which contained only 5–15% trypomastigotes, and at least 85% epimastigotes. We have previously shown that epimastigotes are destroyed by normal macrophages. In addition, the cells were not followed longer than 72 h. Therefore, it is likely that the apparent microbicidal activity was only reflecting intracellular killing of epimastigotes and inhibition of growth of the few trypomastigotes in the ingested inoculum.

Infections caused by facultative intracellular bacteria have been shown to provoke an immune response associated with delayed-type hypersensitivity, not transmissible by serum, and resulting in the formation of immunologically committed lymphoid cells and activation of macrophages (19). Thymus-derived lymphocytes have been implicated as the cell population carrying the specificity of the response (20, 21) and macrophages can be activated by them to become effectors which can kill a variety of immunologically unrelated organisms (22). This may help us to explain the observation that macrophages obtained from BCG-infected animals have significant microbicidal activity against *T. cruzi* trypomastigotes and is in general consistent with the data of Mackaness for bacterial (23), and Hoff (7) and Mauel and Behin (24) for protozoan systems. In other protozoan systems, authors have stressed the specificity for the microbicidal or microbistatic properties of macrophages (25, 26).

In keeping with the prior observations of others, viable organisms were

necessary to provide a persisting antigenic load, and immunization with HKT was ineffective in modifying cell-mediated immunity. In contrast to these other bactericidal systems, a secondary intraperitoneal challenge was required with either *T. cruzi* or BCG infections to yield trypanocidal macrophages. The conversion of a trypanostatic to a trypanocidal effect after the secondary challenge of infected animals may depend on at least two factors. First, the intraperitoneal influx of a larger population of sensitized lymphocytes which can in turn alter macrophage function (27). Second, the influence of products generated during the local inflammatory process, which might influence both lymphocytes and macrophages. The presence of lymphokines, persisting antigen, and inflammatory products may explain the apparent lack of antigenic specificity elicited by the secondary challenge on the trypanocidal activity.

Macrophages activated either through the generation of an inflammatory environment or through microbial infection display other properties, such as rapid spreading on glass or plastic surfaces (28), increase in lysosomal hydrolases (11), metabolic perturbations, tumoricidal effects (29, 30), and the ingestion of C-coated erythrocytes (18). Inflammatory macrophages induced with thioglycollate broth have been shown to secrete large amounts of neutral proteases, such as plasminogen activator (12), elastase (31), and collagenase (32). Cells from *T. cruzi*- and BCG-infected animals, harvested after a secondary challenge, also secrete enhanced amounts of plasminogen activator. Both the increased secretory activity and trypanocidal activity have a parallel time-course. Increased secretion of plasminogen activator was found as early as 1 wk after infection and challenge, and microbicidal activity was present from the 2nd wk on. Peak activity in both cases was found at 3 wk, when spleen size was also very enlarged. However, these two properties are probably not related since thioglycollate-activated macrophages secrete large amounts of plasminogen activator but do not display trypanocidal activity.

The induction of plasminogen activator secretion under the conditions employed in this study showed a definite immunological specificity in keeping with the trypanocidal results. These findings could not be explained on the basis of particle ingestion since a soluble antigen in the form of PPD elicits the response in BCG-infected animals. One explanation for these data is a role for products of specifically sensitized lymphoid elements. This possibility has been examined and is presented in the accompanying publication.

The cells obtained after a specific, secondary antigenic challenge also exhibited a number of other properties. Rapid, symmetrical spreading leads to flattened cells with intensely ruffled plasma membrane and numerous pinocytic vesicles. This appearance was maintained in culture for many days, even in the absence of serum in the medium. If cells were harvested 2 days after challenge, their appearance was as that described above. However, harvesting at 3 and 4 days led to a population which was heavily vacuolated and which died rapidly upon in vitro cultivation. The nature of this cytotoxic effect is unknown. Another attribute is their ability to ingest E(IgM)C via the C receptor. This property, described initially with thioglycollate-induced populations does not depend upon a specific antigenic challenge and is expressed by noninfected animals challenged with all the agents employed. It would seem that this may

be the result of mediators generated during an inflammatory event and is in keeping with the prior observations on macrophage spreading in which C components and coagulation factors play significant roles (33).

Summary

Infection of mice with *Trypanosoma cruzi* and subsequent intraperitoneal challenge with heat-killed trypanosomes elicits peritoneal macrophages which display in vitro microbicidal activity against trypomastigotes of *T. cruzi*. These cells also display other activated properties including rapid spreading, intense membrane activity, secretion of high levels of plasminogen activator, and ingestion mediated by the C3 receptor.

An intravenous infection with BCG, followed by an intraperitoneal challenge with mycobacterial antigens brings about macrophages with similar properties. These criteria of macrophage activation were compared in normal and BCG- or *T. cruzi*-immune mice, with or without an intraperitoneal challenge with specific or unrelated antigens. Trypanocidal activity is displayed by both BCG- and *T. cruzi*-immune macrophages after intraperitoneal challenge with either antigen. Resident-immune macrophages from both *T. cruzi*- and BCG-infected mice show a trypanostatic, rather than trypanocidal activity. Macrophages from noninfected mice, challenged with the same antigens, show neither trypanostatic nor trypanocidal activity.

Increased secretion of plasminogen activator shows a definite immunological specificity. Challenge with the specific antigen induces the appearance of macrophages secreting high levels of plasminogen activator, while unrelated antigens induce much smaller levels. Noninfected mice challenged with the same antigens do not display any enhancement in secretion. In contrast, increased spreading and phagocytosis mediated by the complement receptor are also displayed by cells from noninfected mice challenged with any of the agents tested.

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References

1. Torres, C. M. 1930. Patogenia de la miocarditis crónica en la enfermedad de Chagas. Sociedad Argentina de Patologia Regional del Norte, Quinta Reunión, Buenos Aires. 2:902.
2. Tschudi, E. I., D. F. Anziano, and A. P. Dalmasso. 1972. Lymphocyte transformation in Changes disease. *Infect. Immun.* 6:905.
3. Gonzalez Cappa, S. M., G. A. Schmunis, O. C. Traversa, J. F. Yanofsky, and A. S. Parodi. 1968. Complement fixation tests, skin tests, and experimental immunization with antigens of *Trypanosoma cruzi* prepared under pressure. *Am. J. Trop. Med. Hyg.* 17:709.
4. Yanofsky, J. F., and E. Albado. 1972. Humoral and cellular responses to *Trypanosoma cruzi* infection. *J. Immunol.* 109:1159.
5. Roberson, E. L., and W. L. Hanson. 1974. Transfer of Immunity to *T. cruzi*. *Trans. R. Soc. Trop. Med. Hyg.* 68:388.

6. Kuhn, R. E., and S. K. Duram. 1975. The onset of immune protection in acute experimental Chagas' disease in C3H(He) mice. *Int. J. Parasitol.* 5:241.
7. Hoff, R. 1975. Killing in vitro of *Trypanosoma cruzi* by macrophages from mice immunized with *T. cruzi* or BCG, and absence of cross-immunity on challenge in vivo. *J. Exp. Med.* 142:299.
8. Nogueira, N., C. Bianco, and Z. Cohn. 1975. Studies on the selective lysis and purification of *Trypanosoma cruzi*. *J. Exp. Med.* 142:224.
9. Nogueira, N., and Z. Cohn. 1976. *Trypanosoma cruzi*: mechanism of entry and intracellular fate in mammalian cells. *J. Exp. Med.* 143:1402.
10. Tobie, E. J., T. von Brand, and B. Mehlman. 1950. Cultural and physiological observations on *Trypanosoma rhodesiense* and *Trypanosoma gambiense*. *J. Parasitol.* 36:48.
11. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* 121:153.
12. Unkeless, J. C., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* 139:834.
13. Gordon, S., J. C. Unkeless, and Z. Cohn. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis. Evidence for a two-stage process. *J. Exp. Med.* 140:995.
14. Lowry, O. H., N. T. Rosenbrough, A. L. Farr, and R. L. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265.
15. Gordon, S., J. Todd, and Z. Cohn. 1974. In vitro synthesis and secretion of lysozyme by mononuclear phagocytes. *J. Exp. Med.* 139:1228.
16. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen: purification from human plasma by affinity chromatography. *Science (Wash. D. C.).* 170:1095.
17. Griffin, F. M., C. Bianco, and S. C. Silverstein. 1975. Characterization of the macrophage receptor for complement and demonstration of its functional independence from the receptor for the Fc portion of immunoglobulin. *J. Exp. Med.* 141:1269.
18. Bianco, C., F. M. Griffin, and S. C. Silverstein. 1975. Studies on the macrophage complement receptor. Alteration of receptor function upon macrophage activation. *J. Exp. Med.* 141:1278.
19. Mackaness, G. B. 1970. The monocyte in cellular immunity. *Semin. Hematol.* 7:172.
20. Lane, F. C., and E. R. Unanue. 1972. Requirement of thymus (T) lymphocytes for resistance to listeriosis. *J. Exp. Med.* 135:1104.
21. North, R. J. 1973. Importance of thymus-derived lymphocytes in cell-mediated immunity to infection. *Cell. Immunol.* 7:166.
22. Mackaness, G. B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. *J. Exp. Med.* 129:973.
23. Mackaness, G. B. 1964. The immunological basis of acquired cellular resistance. *J. Exp. Med.* 120:105.
24. Mauel, J., and R. Behin. 1974. Cell-mediated and humoral immunity to protozoan infections (with special reference to leishmaniasis). *Transplant. Rev.* 19:121.
25. 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.* 25:205.
26. Hoff, R. L., and J. K. Frenkel. 1974. Cell-mediated immunity against Besnoitia or Toxoplasma in specifically and cross-immunized hamsters and in cultures. *J. Exp. Med.* 139:560.
27. McGregor, D. D., and F. T. Koster. 1971. The mediator of cellular immunity. IV. Cooperation between lymphocytes and mononuclear phagocytes. *Cell. Immunol.* 2:317.

28. Blanden, R. V. 1968. Modification of macrophage function. *J. Reticuloendothel. Soc.* 5:179.
29. Germain, R. N., R. M. Williams, and B. Benacerraf. 1975. Specific and non-specific anti-tumor immunity. II. Macrophage-mediated non-specific effector activity induced by BCG and similar agents. *J. Natl. Cancer Inst.* 54:709.
30. Hibbs, J. B., L. H. Lambert, and J. S. Remington. 1972. *In vitro* non-immunological destruction of cells with abnormal growth characteristics by adjuvant activated macrophages. *Proc. Soc. Exp. Biol. Med.* 139:1049.
31. Werb, Z., and S. Gordon. 1975. Elastase secretion by mouse macrophages. Characterization and regulation. *J. Exp. Med.* 142:361.
32. Werb, Z., and S. Gordon. 1975. Secretion of a specific collagenase by mouse macrophages. *J. Exp. Med.* 142:346.
33. Bianco, C., A. Eden, and Z. Cohn. 1976. The induction of macrophage spreading: role of coagulation factors and the complement system. *J. Exp. Med.* 144:1531.