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*

By RODNEY HOFF

(From the Department of Tropical Public Health, Harvard University School of Public Health, Boston, Massachusetts 02115)

Resistance to infection with *Trypanosoma cruzi*, the causative agent of Chagas' disease, may be mediated by cellular immunity (1). Lesions of the disease are characterized by infiltration with lymphocytes and macrophages (2), and patients have delayed skin hypersensitivity to antigens of *T. cruzi* (3). In experimental animals, immunity can be transferred with spleen cells from immune animals (4) but not with immune serum (1). That macrophages play a major role in the defense mechanism to *T. cruzi* is suggested by studies in vivo indicating that organisms are destroyed in the macrophages of immune mice while they survive and reproduce in macrophages of nonimmune mice (5).

The present study describes the resistance to *T. cruzi* of cultured macrophages from mice immune to the homologous agent, and also of macrophages from mice immunized with BCG or *Listeria*. However, BCG- or *Listeria*-immunized mice were not protected against challenge in vivo with *T. cruzi*.

Materials and Methods

Animals. Inbred C3H/StCrl strain female mice, purchased from Charles River Breeding Laboratories, North Wilmington, Mass., weighed 14–16 g when used.

Organisms and Immunizations. The House 510 strain (H510)[1] of *T. cruzi* from Costa Rica (6), supplied by Dr. F. A. Neva, National Institute of Allergy and Infectious Disease, was maintained by serial blood passage in C3H mice and in Novy, MacNeal and Nicolle's biphasic medium (NNN). Before use this strain was cultured in F29 liquid medium (7) at 35°C for 48 to 72 h at which time 5–15% of the organisms were trypomastigotes and the remainder largely epimastigotes. Parasites were washed twice with Hanks' solution and counted in a Neubauer hemocytometer.

Mice were immunized against *T. cruzi* by intraperitoneal injection of 10^5 H510 *T. cruzi*. After 60 days, when the infection became chronic, these immune mice were either challenged with *T. cruzi* or they provided the source of macrophages for in vitro cultures and the source of immune serum.

Listeria monocytogenes was obtained from Dr. J. R. David, Robert B. Brigham Hospital, Boston, Mass. Mice were immunized by a sublethal intravenous injection of 5×10^4 bacteria 6 days before challenge with *T. cruzi* or collection of macrophages (8). Mice were immunized with BCG (Glaxo-strain BCG Vaccine, Eli Lilly & Co., Indianapolis, Ind.) by injecting them with 4×10^5 to $9 \times$

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; H510, House 510 strain of *T. cruzi* from Costa Rica; NNN, Novy, MacNeil and Nicolle's biphasic medium; PE cells, peritoneal exudate cells.

10^5 mycobacteria intraperitoneally 18 days and again 3 days before challenge with *T. cruzi* or collection of macrophages.

Challenge of Mice with T. cruzi. Mice immunized with H510 *T. cruzi*, BCG, or *Listeria* were challenged by two methods. For sublethal challenge, mice were injected with 5×10^5 H510 culture forms grown in F29 as above. The course of the infection in control and challenged mice was then assessed by quantifying parasitemia in blood samples lysed with isotonic ammonium chloride solution (9). For lethal challenge, mice were injected intraperitoneally with 10^4 blood-stream trypomastigotes of a Colombian strain of *T. cruzi* (10). This strain was maintained by serial blood passage in Charles River CD-1 mice from which inoculum for challenging immunized C3H mice was obtained on the 12th day of infection.

Immune and Control Sera. Sera collected from *T. cruzi*-immune mice, and control sera from normal mice, were inactivated at 56°C for 30 min before use.

Preparation of Peritoneal Macrophage Cultures. Mice were exsanguinated and 3–4 ml of Hanks' solution (containing 10 U of heparin per ml) were injected intraperitoneally. Peritoneal exudate, withdrawn with a Pasteur pipet, was pooled from several mice. Peritoneal exudate cells (PE cells) were centrifuged (250 *g*) and were resuspended in Medium 199 (Microbiological Associates, Baltimore, Md.) containing 15% fetal calf serum (FCS) and antibiotics (100 U penicillin and 100 μg of streptomycin per ml). PE cells were counted in a hemocytometer and the suspension was adjusted to desired concentration. About 45–50% of the PE cells were macrophages as assessed in Giemsa-stained smears. To assay resistance to *T. cruzi*, 1×10^6 to 2×10^6 PE cells, suspended in 0.75 ml medium, were added to tissue culture chamber-slides (4-chamber, Lab Tek Products, Div. Miles Laboratories, Naperville, Ill.). To assay resistance to *Listeria*, 5×10^6 PE cells, suspended in 2.0 ml of medium without antibiotics, were plated in 35-mm diameter plastic tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Cultures were incubated at 37°C in 5% CO_2 and air. Nonadherent cells were removed with two changes of medium 1–2 h after cultures were prepared. The cultures were used 18–24 h later.

Assay of Macrophage Resistance to T. cruzi. Macrophage cultures were exposed to 0.75×10^6 to 2.0×10^6 H510 *T. cruzi* grown in F29. After 24 h of exposure, extracellular parasites were removed with two changes of medium. At specified intervals thereafter replicate cultures were fixed with Zenker's solution and stained with buffered Giemsa. Macrophage density was indexed by counting cells in 10 ocular grids (7 mm $\times$ 7 mm), using a 40$\times$ objective and a calibrated mechanical stage to locate identical coordinates in each chamber. Intact parasites found in 250 or 500 macrophages were counted, using a 100$\times$ objective, and the percent of infected cells and the mean number of *T. cruzi* per 100 macrophages were calculated.

Assay of Macrophage Resistance to Listeria. Intracellular bacteriostasis of *Listeria* was assessed by modifying the method of Fowles et al. (11). The freeze-thaw lysis with distilled water was repeated three times to release intracellular bacteria for counting, and the amount of macrophage cell-protein was then estimated. Viability of *Listeria* was not significantly reduced by the modified procedure.

Results

In Vitro Culture of Macrophages from T. cruzi-Immune Mice. Within 15–30 min of incubation in culture, macrophages from mice immunized with *T. cruzi* ("immune" macrophages) spread rapidly and circumferentially on glass (Fig. 1B). By contrast, macrophages from normal mice ("normal" macrophages) began spreading only after 3 h, and did not spread fully until the next day. Immune macrophages were also more adherent to glass in the culture chambers. Thus, to obtain equivalent numbers of normal and immune macrophages in the cultures (i.e., varying less than 10%), twice as many normal PE cells (2.0×10^6) as immune PE cells (1.0×10^6) were plated. Nearly 100% of adherent PE cells remaining in cultures after 24 h were macrophages as judged by their morphology and uptake of India ink or starch particles. Although peritoneal exudates from immune mice contained free trypomastigotes at the time of collection, macrophages cultured overnight from these mice were parasite-free.

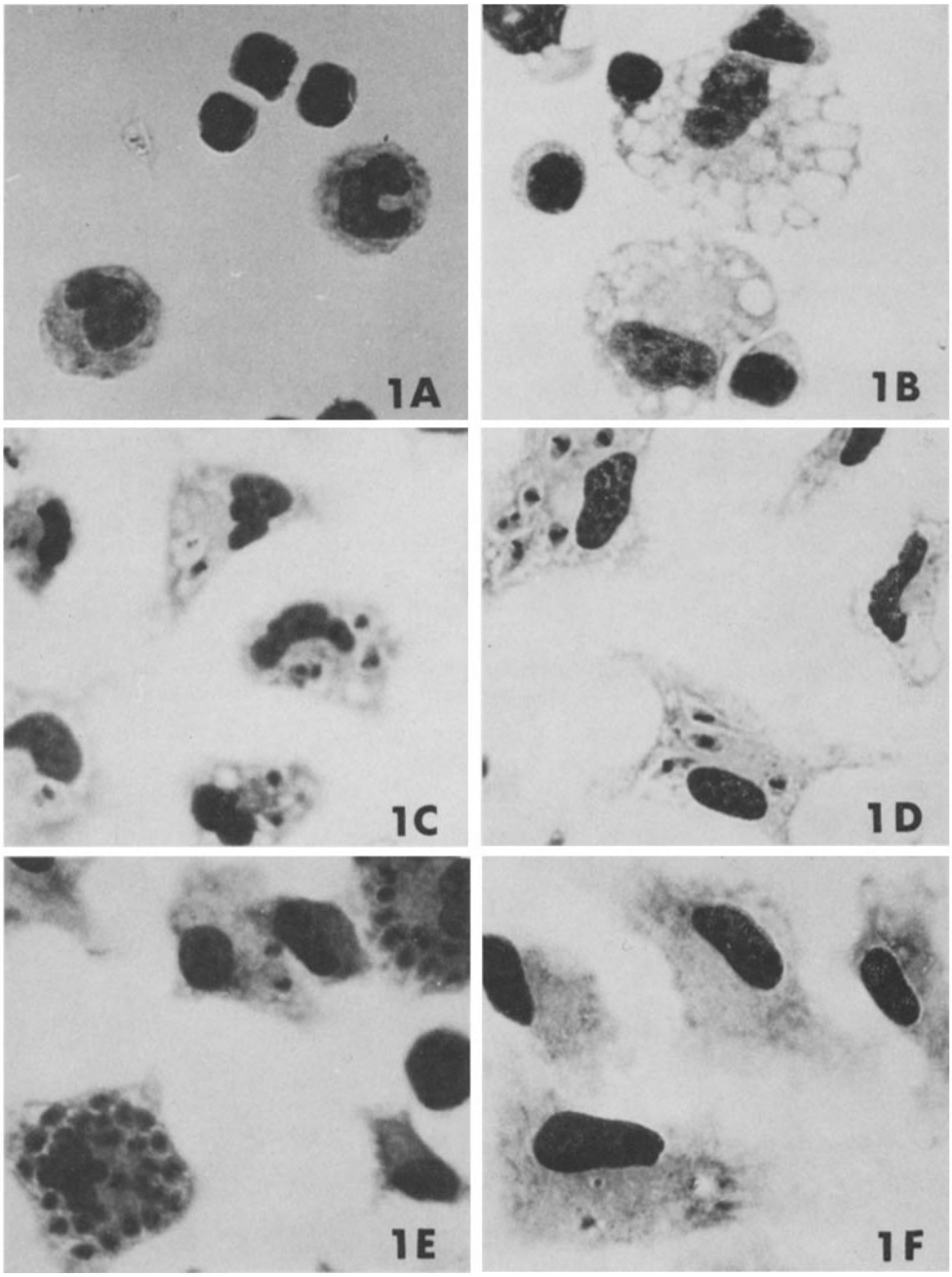

FIG. 1. Macrophages from normal and *T. cruzi*-immune mice:(A) Normal macrophages after 30-min incubation. (B) Immune macrophage after 30-min incubation. (C) Normal macrophages after 24-h exposure to *T. cruzi*. (D) Immune macrophage after 24-h exposure to *T. cruzi*. (E) Growth of *T. cruzi* in normal macrophages after 72-h incubation. (F) Absence of parasites in immune macrophages after 72-h incubation.

Challenge of Normal and Immune Macrophage Cultures with T. cruzi. Inoculating a relatively large number of parasites caused a high loss of normal macrophages. However, when the inocula contained less than five parasites per adherent macrophage, fewer cells (less than 10%) were lost from the monolayer. The number of organisms taken up by macrophages then depended on exposure time. Using an inoculum containing two parasites per adherent cell, the number of organisms taken up and the percentage of infected macrophages increased gradually during the first 24-h exposure period. At the end of the exposure period, intracellular parasites in normal macrophages appeared morphologically intact, whereas many organisms in immune macrophages appeared to be degenerating.

In the assay, to assess cellular uptake, intracellular parasites were counted after the 24-h exposure period, and to assess survival or reproduction they were counted after 48 and 72 h. Results were consistent in five replicate experiments, and representative findings are shown in Fig. 2. Uptake of parasites differed little in replicate cultures within an experiment but varied from one experiment to another. After 48 h the number of intracellular organisms and the percent of infected cells declined markedly in immune macrophages and less so in normal macrophages. Thereafter, in normal macrophages the surviving parasites began reproducing as amastigotes and after 72 h some cells contained up to 40 organisms (Fig. 1 E). At this time, the intracellular amastigotes began transforming into trypomastigotes, and host-cell lysis ensued. In contrast, the number of intracellular parasites in immune macrophages remained low, and from 48 to 72 h only occasional lightly parasitized cells were found (Fig. 1 F). Because disappearance of *T. cruzi* in immune macrophages could be attributed to initial

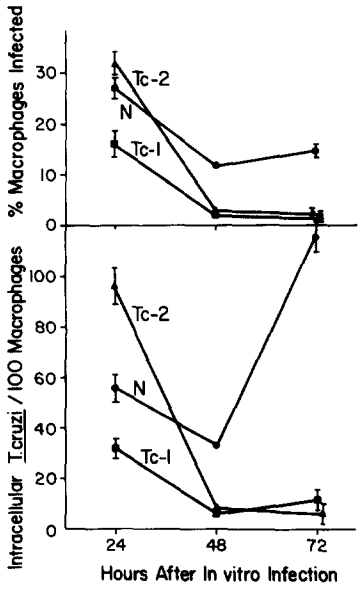

FIG. 2. Resistance to *T. cruzi* by macrophages from specifically immune mice. Normal macrophages (N) and immune macrophages (Tc-1) were exposed to 1×10^6 organisms. Other cultures of immune macrophages (Tc-2) were exposed to 2×10^6 organisms. Values are shown as means ± SE of triplicate cultures.

uptake of fewer parasites, cultures of immune macrophages were exposed to heavier inocula (Fig. 2, Tc-2). More parasites were then taken up by the immune macrophages than by controls. Nevertheless, again after 48 and 72 h the number of intracellular *T. cruzi* declined markedly in the immune cells.

Time-Course Development of Macrophage Resistance to T. cruzi. In vitro resistance to *T. cruzi* by macrophages from infected mice first appeared in those collected 21 days after infection, and persisted for 70 days or more (Fig. 3). Macrophages collected 7 days postinfection did not spread rapidly and were not resistant to challenge in the in vitro assay. Although some macrophages ($<10\%$) appeared activated at 14 days postinfection, the cultured cells were not resistant to challenge in vitro.

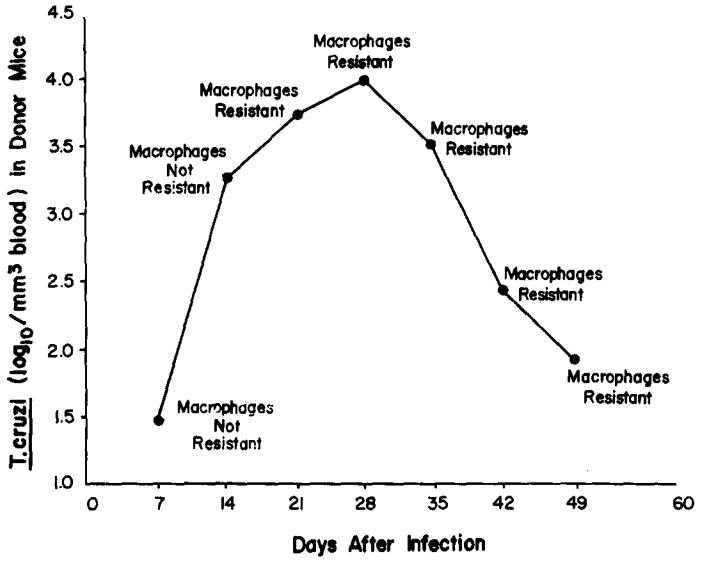

FIG. 3. Time-course development of macrophage resistance to *T. cruzi*. 30 mice were injected with *T. cruzi*. Starting at 7 days postinfection, and at weekly intervals thereafter, parasitemia was measured in 5 mice and their PE cells were harvested and assayed for resistance to *T. cruzi* in vitro. Macrophages from infected mice were judged resistant when intracellular *T. cruzi* decreased to low numbers, in contrast to their increase in normal macrophages.

Specificity of In Vitro Macrophage Resistance to T. cruzi. The in vitro specificity of activated macrophages was explored by challenging activated macrophages derived from mice infected with BCG or *Listeria*. Macrophages from these mice spread rapidly on glass. BCG-activated macrophages showed the same degree of resistance to *T. cruzi* as did *T. cruzi*-activated macrophages. As with specifically activated macrophages, BCG-activated macrophages took up fewer parasites than normal macrophages. In the experiment summarized in Fig. 4, greater uptake was achieved by doubling the inoculum and by substituting normal mouse serum for FCS in the exposure medium. Even with greater initial uptake of parasites, the BCG-activated macrophages were able to contain the infection.

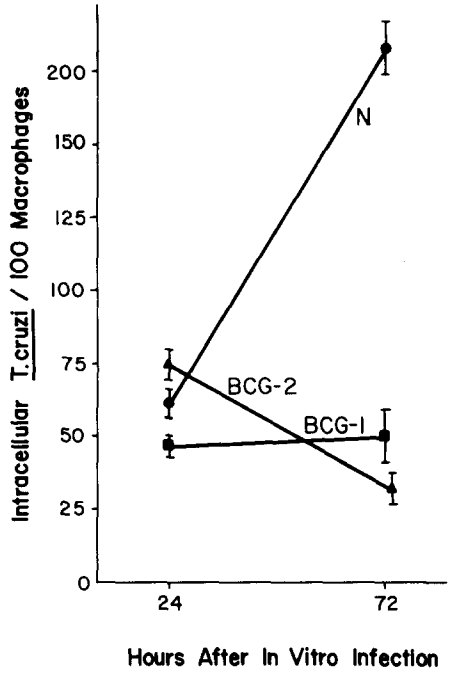

FIG. 4. Nonspecific in vitro resistance to *T. cruzi* by macrophages from mice immunized with BCG. Control macrophages (N) and BCG-activated macrophages (BCG-1) were exposed to 10^6 parasites suspended in medium containing 15% normal mouse serum. Other cultures of BCG-activated macrophages (BCG-2) were exposed to 2×10^6 parasites in the same medium. Values shown are means ± SE of six replicate cultures. Note that BCG-2-activated macrophages displayed enhanced resistance to *T. cruzi* even though they took up more parasites initially than did normal controls.

Macrophages activated by *Listeria* infection were less resistant to *T. cruzi* challenge than were macrophages activated by *T. cruzi* or BCG infection. After 48 h incubation in some experiments, surviving parasites in the *Listeria*-activated macrophages appeared to reproduce.

Enhanced Macrophage Resistance to Listeria by T. cruzi-Activated Macrophages. This experiment was designed to confirm that *Listeria*-activated macrophages used in the previous experiment were indeed resistant to *Listeria*, and to explore whether *T. cruzi*-activated macrophages were resistant to *Listeria*. Both the *Listeria*- and the *T. cruzi*-activated macrophages were significantly more bacteriostatic than were normal macrophages (Fig. 5).

Effect of Cytophilic Antibody on In Vitro Infection of Macrophages with T. cruzi. Cultures of normal macrophages and of macrophages from *T. cruzi*- and BCG-immune mice were washed twice with medium and incubated for 1 h with 0.5 ml of medium containing 15% normal or immune mouse serum to bind cytophilic antibodies. After incubation the macrophage cultures were washed again, and infected with *T. cruzi* suspended in medium with 15% A-Gamma globulin FCS instead of FCS. After a 24-h exposure, control macrophages preincubated with normal mouse serum took up nearly all parasites inoculated. However, *T. cruzi*- and BCG-activated macrophages, also preincubated with

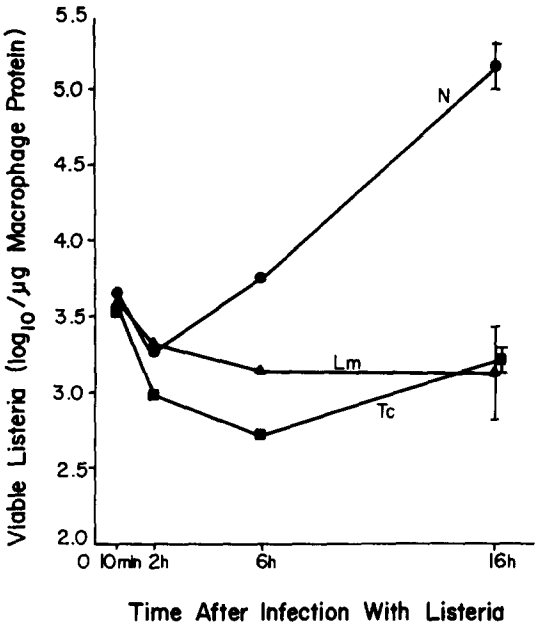

FIG. 5. Enhanced resistance to *Listeria* in vitro by macrophages from *T. cruzi*- (Tc) and *Listeria*- (Lm) immune mice. Cultures were exposed for 10 min to 2.7×10^7 bacteria. The number of intracellular bacteria was assessed immediately after exposure and 2, 6, and 16 h later. Values shown are means ± SE of triplicate cultures. After 16 h both *T. cruzi*- and *Listeria*-activated macrophages contained significantly fewer bacteria than did normal macrophages ($P < 0.001$).

normal serum, took up less than half as many *T. cruzi* as did controls, and many extracellular parasites were left in the supernatant medium. In contrast, when preincubated with immune serum, the activated macrophages took up nearly all the inoculum and contained as many intracellular parasites as did control macrophages (Fig. 6). Cytophilic antibody did not, however, render normal macrophages resistant to infection. In other experiments, direct opsonization of the inoculum with immune serum caused agglutination, thereby affecting the distribution of intracellular infection in the monolayer and making evaluation difficult.

Challenge of Listeria- and BCG-Immune Mice with T. cruzi. Since nonspecifically activated macrophages resist infection in vitro with *T. cruzi*, mice immunized with BCG or *Listeria* were challenged with *T. cruzi* to determine whether either immunization was protective in vivo. Parasitemia in BCG-immune mice or in *Listeria*-immune mice, when challenged with H510 *T. cruzi*, followed a course similar to that in control mice; however, in mice immunized and challenged with *T. cruzi*, parasitemia was low (Fig. 7). In another experiment, opsonizing the inoculum with immune serum failed to protect control and BCG-immune mice.

Since challenge with H510 was nonlethal, immune mice were also challenged with the lethal Colombian strain. BCG immunization failed to prevent or significantly delay mortality in mice challenged with this strain, while mice

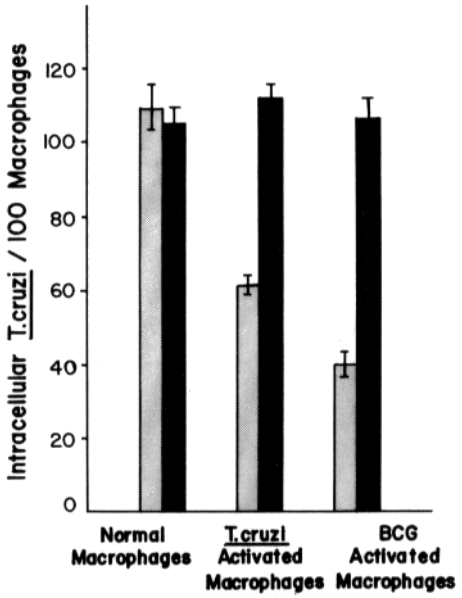

FIG. 6. Effect of immune mouse serum on the uptake of *T. cruzi* by macrophages during 24 h of exposure to 1.5×10^6 parasites. Light bars indicate uptake by macrophages preincubated with normal mouse serum; dark bars indicate uptake by macrophages preincubated with immune mouse serum. Values are means ± SE of six replicate cultures.

immunized by infection with H510 *T. cruzi* survived challenge with the lethal strain (Fig. 8).

Discussion

The results indicate that peritoneal macrophages from mice with induced resistance to *T. cruzi* have an enhanced capacity to kill *T. cruzi* in vitro. Macrophage resistance, which developed in about 3 wk after mice were infected with *T. cruzi*, also occurred in macrophages from BCG-immunized mice. In contrast to these findings in vitro, immunizing mice with BCG or *Listeria* did not protect them against challenge with *T. cruzi*.

Controversy exists concerning the ability of *T. cruzi* to reproduce in macrophage cultures. Uptake in vitro and subsequent reproduction of *T. cruzi* in macrophages has been reported by others (12–14). Dvorak et al. (15), on the other hand, observed no division of *T. cruzi* after infection of mouse peritoneal macrophages with either epimastigotes or trypomastigotes. These authors point out that parasite survival in multiply-infected macrophages might misleadingly be interpreted as parasite reproduction. In the present studies, dividing intracellular amastigotes were observed after 48 h. Although multiple infections were common, the number of intracellular parasites per cell increased while the density of macrophages remained constant, strongly suggesting parasite multiplication. Besides the increase in numbers, the appearance of extracellular trypomastigotes in the supernatant medium after 72 h suggests the completion of the intracellular reproductive cycle as described by Behbehani (12).

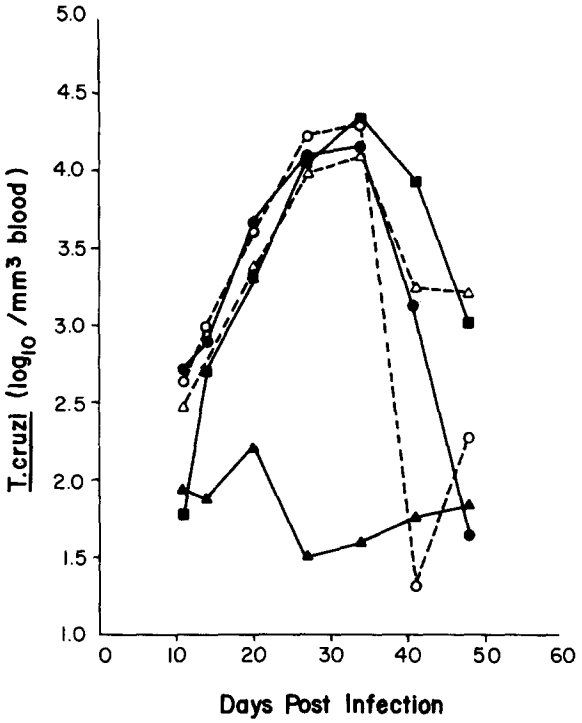

FIG. 7. Challenge of BCG-, *Listeria*- and *T. cruzi*-immune mice with *T. cruzi*. Mice were injected intraperitoneally with 5×10^6 culture forms of *T. cruzi*. The course of parasitemia in normal mice (●—●), BCG-immune mice (■—■), *Listeria*-immune mice (O- - -O), *Listeria*-hyperimmune mice (Δ- - -Δ), and *T. cruzi*-immune mice (▲—▲) is shown. Mean parasitema of five mice per group is shown. Note that only specifically immune mice were resistant to *T. cruzi* challenge even though peritoneal macrophages from BCG- and *Listeria*-immune mice were resistant to in vitro infection.

In the present in vitro studies, macrophages from specifically immunized mice killed intracellular *T. cruzi* while the organism survived and grew in normal macrophages. This finding is consistent with the in vivo findings of Taliaferro and Pizzi (5) who observed destruction of virulent blood forms of *T. cruzi* in the macrophages of immune C3H mice. Likewise, in vitro killing of other intracellular protozoa by macrophages from specifically immune hosts has been reported (16–18).

After 24 h exposure, fewer *T. cruzi* were present in BCG- and *T. cruzi*-immune macrophages than in normal macrophages. This finding parallels that noted by Miller and Twohy (18) using *Leishmania donovani*. These authors suggest that decreased susceptibility of immune macrophages to infection may represent a mechanism of resistance to *Leishmania*. Our results show that resistance to *T. cruzi* penetration was nonspecific since both *T. cruzi*- and BCG-immune macrophages, exposed to equivalent inocula, took up fewer organisms than did control macrophages. Other possible explanations for the observed difference in uptake of *T. cruzi* may be their early intracellular death and digestion by

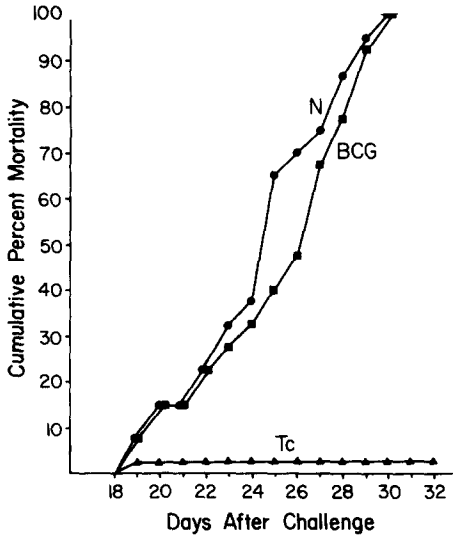

FIG. 8. Cumulative mortality among normal and BCG- or *T. cruzi*-immune C3H/HcJ mice (40 per group) challenged with 10^4 blood-stream forms of Columbian-strain *T. cruzi*. Graph key: normal (N, ●—●), BCG-immune (BCG, ■—■), and *T. cruzi*-immune (Tc, ▲—▲) mice.

immune macrophages, or rapid reproduction of the parasites in normal macrophages.

Efforts were made to ensure that the number of normal macrophages in experimental cultures equalled or exceeded that of immune macrophages, and to ensure that immune macrophages took up equal or greater numbers of *T. cruzi* than did control macrophages. Thus, enhanced killing could be attributed to properties of immune macrophages rather than to a decreased parasite uptake or to greater macrophage numbers.

Enhanced killing of *T. cruzi* by macrophages derived from *T. cruzi*-infected mice appeared during the 3rd week of infection when parasitemia was still increasing. This corresponds in time to the first successful transfer of immunity with spleen cells in rats infected with *T. cruzi* (4). Thus, there is evidence in vivo and in vitro that cellular immunity may function early in the natural history of the infection to protect the host; this contrasts with the much later appearance of partially protective humoral antibody demonstrated using serum passively transferred from rats (19).

Mackaness (20) showed that macrophages become activated by specific immunological mechanisms, but once activated could kill organisms unrelated to the original immunogen. Nonspecific bacterial resistance by activated macrophages has been produced by infection with *Toxoplasma* (21). In the present studies, macrophages from *T. cruzi*-immune mice were nonspecifically resistant to infection with *Listeria* in vitro, and macrophages from BCG-immune mice were nonspecifically resistant to infection with *T. cruzi* in vitro. Tanowitz et al. (13) in similar experiments observed that macrophages from BCG-immunized mice were more resistant to destruction by *T. cruzi* than were normal macrophages. The above contrasts with recent findings of others who did not find comparable nonspecific resistance to *Toxoplasma*, *Besnoitia*, and *Leishmania* by

nonspecifically activated macrophages (17, 22). These authors suggest that macrophage resistance to these intracellular protozoa has specific qualities and is dependent on the host-parasite combination.

The incorporation of specific antibody greatly increased the uptake of *T. cruzi* by macrophages but did not interfere with their intracellular replication in normal macrophages. Similar interactions of antibody, parasites, and macrophages have been reported by others (18, 23, 24).

The surprising finding of this study is that BCG-immune mice, whose peritoneal macrophages strongly resisted *T. cruzi* infection in vitro, were not protected against in vivo challenge, i.e., there was no difference in parasitemia or mortality between control and BCG-immunized mice challenged with sublethal or lethal injections of *T. cruzi*. Mice immunized and boosted with BCG are resistant to *Listeria* challenge for more than 8 wk (25), a period longer than that of an acute *T. cruzi* infection in mice. Hanson[2] was also unable to demonstrate protection in BCG-immunized mice challenged with *T. cruzi*. The above results suggest that nonspecific activation of macrophages alone is insufficient completely to protect mice against *T. cruzi* even when the parasites are opsonized. Even if all infected parasites were to be taken up by activated macrophages, some organisms must survive and initiate infection.

Obviously, therefore, in *T. cruzi*-immune mice other cellular or humoral factors must function, in addition to nonspecifically activated macrophages, to produce the specific immunity demonstrated in Figs. 7 and 8. It has been shown, for example, that specifically sensitized lymphocytes are required for arming macrophages to kill *Toxoplasma* and *Besnoitia* (17). *T. cruzi* invades various connective tissue cells and there may escape intracellular destruction by activated macrophages. It may be that specifically sensitized lymphocytes are required to call forth and focus the macrophage reaction on the parasite, or that specifically sensitized lymphocytes or their products in combination with antibody are required for an immunity to *T. cruzi*. This study points out the need to combine in vitro and in vivo experiments when investigating mechanisms of immunity to intracellular parasites.

Summary

Peritoneal macrophages from *T. cruzi*-immune mice were resistant to infection in vitro with culture forms of the parasite. Macrophage resistance appeared in infected mice about 21 days postinfection when parasitemia was still rising. Resistance in vitro was nonspecific since macrophages from BCG-immune mice were resistant to *T. cruzi*, and since macrophages from *T. cruzi*-immune mice were resistant to infection in vitro with *Listeria*. Despite the findings in vitro, mice immunized with BCG or *Listeria* were not resistant to challenge with *T. cruzi* even when the parasites were opsonized.

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