

## ACTIVATION OF MACROPHAGES IN VIVO AND IN VITRO

### Correlation between Hydrogen Peroxide Release and Killing of *Trypanosoma cruzi*\*

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The biochemical basis of the enhanced anti-microbial function of activated macrophages is unknown. The dramatic consequences of activation, both in vivo and in vitro, are well illustrated in the case of mouse peritoneal macrophages infected with *Trypanosoma cruzi*, the agent of Chagas' disease. Trypomastigotes are phagocytized via a protease-sensitive structure and enclosed in a phagocytic vacuole (1). In resident peritoneal macrophages, or macrophages elicited by certain inflammatory agents, trypomastigotes escape from the phagosome, multiply in the cytoplasm, and destroy the phagocyte (1, 2). In contrast, immunization of the mouse with viable *T. cruzi* or Bacille Calmette-Guérin (BCG),<sup>1</sup> followed by intraperitoneal boosting with the homologous antigen, results in peritoneal macrophages that kill the trypomastigotes (3). Spleen cells from immunized mice, when challenged in vitro with the homologous antigen, release factor(s) (SCF) which alter the course of trypanosomal infection in resident or inflammatory macrophages in vitro (2). Depending on the time of exposure of the macrophages to SCF, trypomastigotes either remain in the vacuoles, or are recaptured from the cytoplasm into vacuoles. In either case, the parasites are killed.

Treatment of mice with BCG or *Corynebacterium parvum*, but not thioglycollate broth or proteose-peptone, markedly enhances the ability of their peritoneal macrophages to release hydrogen peroxide (4, 5). When suitably triggered, such macrophages release enough H<sub>2</sub>O<sub>2</sub> to lyse tumor cells in vitro (5, 6). In view of the known importance of H<sub>2</sub>O<sub>2</sub> in the anti-microbial functions of granulocytes (7), we wished to explore the ability of macrophages to release H<sub>2</sub>O<sub>2</sub> as a biochemical correlate of their level of anti-microbial function. We found that macrophages could be reversibly activated by incubation in SCF so that they became capable of releasing greatly

\* Supported by grants CA-22090 from the National Cancer Institute and GA-HS-7716 from the Rockefeller Foundation.

‡ Scholar of the Leukemia Society of America.

§ Recipient of a Research Career Development Award in Geographic Medicine from the Rockefeller Foundation.

<sup>1</sup> Abbreviations used in this paper: BCG, Bacille Calmette-Guérin; CB, peritoneal macrophages from nonimmunized (control) mice injected 2 d before harvest with heat-killed *T. cruzi*; D5, Dulbecco's modification of Eagle's medium, containing 5% heat-inactivated FBS (56 deg C, 30 min), 100 U/ml penicillin, and 100 µg/ml streptomycin; FBS, fetal bovine serum; HMPS, hexose monophosphate shunt; IB, peritoneal macrophages from mice immunized with viable *T. cruzi* and boosted with heat-killed *T. cruzi*; KRPG, Krebs-Ringer phosphate buffer with 5.5 mM glucose; PMA, phorbol myristate acetate; PP, peritoneal macrophages from mice injected with proteose-peptone; PPD, purified protein derivative of tubercle bacilli; Res, resident peritoneal macrophages; SCF, spleen cell factor(s).

increased amounts of  $H_2O_2$ . The ability to release  $H_2O_2$  and the ability to kill trypanosomes were closely correlated under a variety of experimental conditions.

### Materials and Methods

**Immunization of Mice and Preparation of Spleen-Cell Supernates.** Female NCS mice (Rockefeller University, N. Y.), C3H/HeJ or C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, Maine), or C3H/He mice (Charles River Breeding Laboratories, Wilmington, Mass.) were infected intraperitoneally with  $5 \times 10^6$  viable culture forms of *T. cruzi*, or intravenously with  $2-6 \times 10^7$  viable Pasteur type BCG, as described (3). For preparation of spleen cell factor(s) (SCF), spleen cells were removed 2-5 wk later and cultured for 48 h with the homologous antigen (heat-killed *T. cruzi* or purified protein derivative [PPD]), and supernates collected as reported (2). Control supernates were prepared in the same manner from spleens of unimmunized mice.

**Culture of Macrophages.** Mice were immunized as above and boosted 3 wk later with an i.p. injection of homologous antigen ( $5 \times 10^6$  heat-killed *T. cruzi* or 50  $\mu$ g of PPD in 1 ml of phosphate-buffered saline). 2 d thereafter, their peritoneal macrophages (immune-boosted, or IB cells) were collected as reported (3). Macrophages were also collected from control mice given  $5 \times 10^6$  heat-killed *T. cruzi* 2 d before (CB cells), from mice given 1 ml of 1% proteose-peptone (Difco Laboratories, Detroit, Mich.) 4 d before (PP cells), or from normal mice (resident cells).  $1 \times 10^6-2 \times 10^6$  peritoneal cells were plated on 13 x 27 x 0.15-mm glass cover slips (Bellco Glass, Inc., Vineland, N. J.) in 0.2 ml of Dulbecco's modification of Eagle's medium containing 5% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N. Y.), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (D5). The cover slips were cleaned by soaking in 70% ethanol (prepared with water double-distilled in glass), dipping in 95% ethanol, and flaming. After 2-4 h of incubation in 7%  $CO_2$  in air, the cover slips were rinsed by agitation in three containers of Hanks' balanced salt solution or 0.9% saline, and placed in 35-mm plastic culture dishes (Nunc, Roskilde, Denmark) with 1 ml of D5 alone, or 1 ml of D5 containing dilutions of experimental or control spleen cell supernates. Each day, the medium was replaced with 1 ml of fresh medium of the same type.

**Assay for  $H_2O_2$  Release.** The oxidation of scopoletin by  $H_2O_2$ , catalyzed by horseradish peroxidase, was followed fluorometrically, as described (4, 8). Cover slips were rinsed in three beakers of 0.9% saline and placed diagonally in  $1 \times 1 \times 4.5$  cm quartz cuvettes containing 3 ml of Krebs-Ringer phosphate buffer with 5.5 mM glucose (KRPB) (4), 2.5-10 nmol of scopoletin, and 3-6 purpurogallin U of horseradish peroxidase in the temperature-controlled compartment (37°C) of the fluorometer. The close fit of the cover slip made repositioning unnecessary after mixing. Phorbol myristate acetate (PMA) (Consolidated Midland Co., Brewster, N. Y.) was added in dimethyl sulfoxide to give 10-100 ng/ml PMA and 0.0033%-0.033% dimethylsulfoxide (vol/vol). Readings were taken at regular intervals of 2 to 10 min, immediately after mixing by inversion. Additional scopoletin was added as necessary. Matched cover slips were rinsed in the same manner and stained in Diff-Quik (Harleco, Hartman-Leddon, Philadelphia, Pa.) to determine the percentage of granulocytes. This averaged  $0.97 \pm 0.31\%$  (SEM) after 2-4 h of adherence of IB cells. After 24 h of adherence, granulocytes were undetectable on rinsed cover slips of all cell types used. Other matched slips were rinsed, dried, placed in 1 ml of 0.5 N NaOH at 4°C overnight, and used to determine the protein content of the monolayers by the method of Lowry et al. (9) with a bovine serum albumin standard.  $H_2O_2$  released by different cell populations, cells cultured in different types or concentrations of spleen cell supernates, or cells cultured for different lengths of time, was expressed in terms of the amount of adherent cell protein under the same conditions.

**Assay of Trypanocidal Activity.** Infection of macrophages adherent to glass cover slips, and microscopic enumeration of intracellular trypomastigotes, were performed by the previous methods (2).

**Determination of Sensitivity of Trypanosomes to  $H_2O_2$ .** Trypomastigotes were purified from 4-wk cultures of *T. cruzi* as described (10). Unseparated cultures were used for epimastigotes and contained 70-85% of this form. Both preparations were suspended separately in KRPB at  $5 \times 10^6-10 \times 10^6$  per ml. Various dilutions of glucose oxidase (type V, Sigma Chemical Co., St. Louis, Mo.) were added to 1 ml of the suspension. The cultures were incubated for 1 h at 37°C in air and washed by centrifugation. Viability of the parasites was assessed by determining the

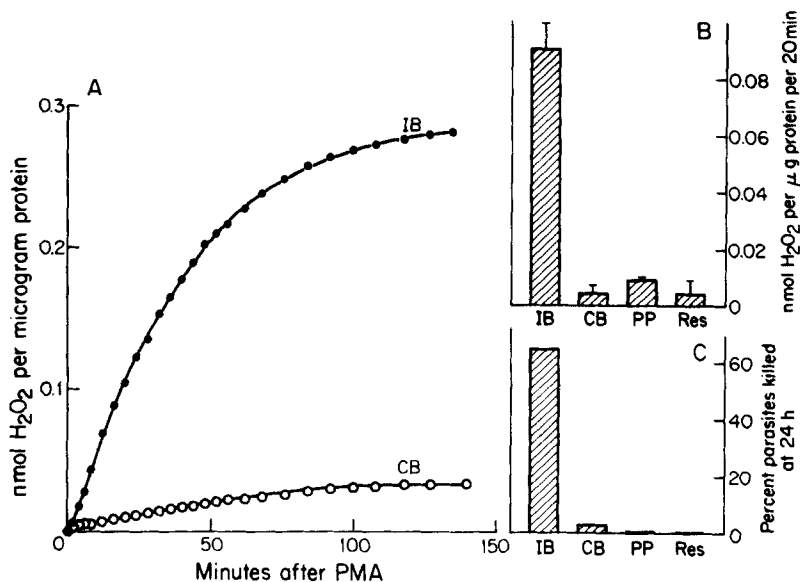


FIG. 1. H<sub>2</sub>O<sub>2</sub> release and trypanocidal activity of freshly explanted macrophages after various treatments of the cell donors. Macrophages were adherent to glass cover slips for 2–4 h before the start of the assays. Panel A: time-course of H<sub>2</sub>O<sub>2</sub> release after the addition of 100 ng/ml PMA to macrophages from mice immunized with viable *T. cruzi* and boosted with heat-killed *T. cruzi* (IB) or from control mice given the boosting injection alone (CB). Results are expressed in terms of the adherent cell protein on matched cover slips. Panel B: initial rates of H<sub>2</sub>O<sub>2</sub> release after addition of 100 ng/ml of PMA to macrophages from mice which were immunized and boosted (IB) or only boosted (CB) as in panel A, or given proteose-peptone (PP), or not treated (resident peritoneal macrophages [Res]). Means  $\pm$  SEM for three experiments with each cell type, except two experiments with Res cells. Panel C: percent reduction of intracellular trypanosomes 24 h after infection of freshly plated macrophages from mice treated as in panel B. Mean of two experiments.

percentage of motile forms in a hemocytometer. Nonmotile forms proceeded to lyse within 60 min in the hemocytometer, although motile forms remained intact. The enzymatic activity of glucose oxidase was assayed as reported (6).

## Results

**Correlation between H<sub>2</sub>O<sub>2</sub>-Releasing Capacity and Trypanocidal Activity of Freshly Explanted Macrophages.** When mice were immunized with viable *T. cruzi* and boosted i.p. with heat-killed *T. cruzi*, their peritoneal macrophages (IB cells) had a markedly increased capacity to release H<sub>2</sub>O<sub>2</sub> in response to PMA, compared to macrophages from control mice given the boosting injection alone (CB cells) (Fig. 1). As shown in Fig. 1 A, H<sub>2</sub>O<sub>2</sub> release continued from IB cells for 2 h after the addition of PMA, yielding 12.9 nmol of H<sub>2</sub>O<sub>2</sub> from the cover slip, whereas the cover slip of CB cells released a total of 1.1 nmol. In three such experiments, the total amount of H<sub>2</sub>O<sub>2</sub> detected from IB cells was  $0.24 \pm 0.02$  (SEM) nmol per  $\mu$ g adherent cell protein, from CB cells, it was  $0.01 \pm 0.01$  nmol per  $\mu$ g cell protein. In Fig. 1 B, the initial rates of H<sub>2</sub>O<sub>2</sub> release are compared for adherent macrophages 2–4 h after harvest from IB mice, CB mice, mice given proteose-peptone (PP cells), or normal mice (resident cells). When considered together with Fig. 1 C, it is evident that only the macrophages with an enhanced capacity to release H<sub>2</sub>O<sub>2</sub> were capable of killing trypanosomes.

**Correlation between H<sub>2</sub>O<sub>2</sub>-Releasing Capacity and Trypanocidal Activity after Incubation in**

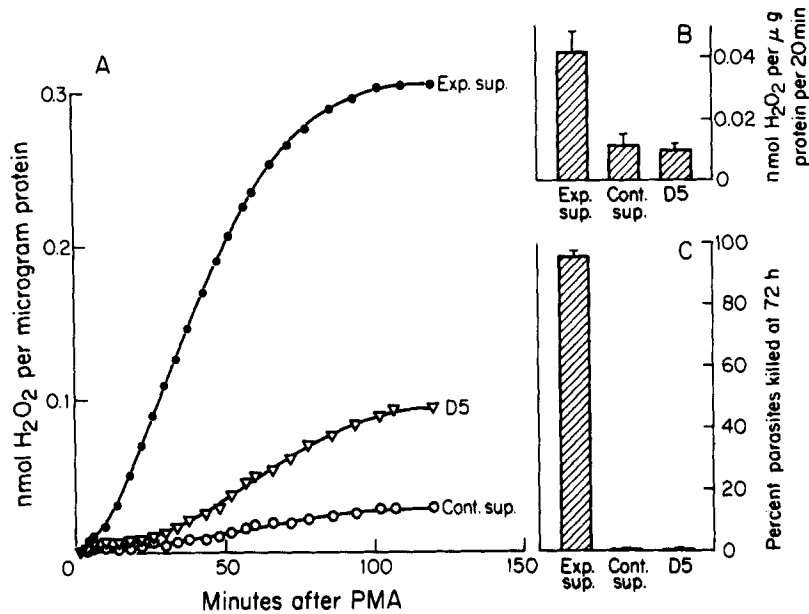


FIG. 2. H<sub>2</sub>O<sub>2</sub> release and trypanocidal activity of macrophages after 3 d of culture in SCF and control media. Panel A: time-course of H<sub>2</sub>O<sub>2</sub> release after the addition of 100 ng/ml PMA to CB macrophages incubated in 12.5% of supernate of spleen cells from *T. cruzi*-immunized mice cultured with heat-killed *T. cruzi* (exp. sup.), 12.5% of supernate of normal spleen cells cultured with heat-killed *T. cruzi* (cont. sup.), or D5 alone. Panel B: initial rates of H<sub>2</sub>O<sub>2</sub> release after the addition of 100 ng/ml PMA to PP, CB, or Res macrophages incubated for 3–4 d in D5 alone or in 6–25% of experimental or control supernates prepared from spleens of normal mice or mice immunized with either *T. cruzi* or BCG and challenged with the homologous antigen. Mean  $\pm$  SEM for eight experiments. Panel C: percent reduction in intracellular trypanosomes 48 h after infection of macrophages. The macrophages were cultured for 24 h before infection. Thus the results pertain to 72 h of culture, in similar media as for panel B. Mean  $\pm$  SEM for eight experiments with PP cells. The initial numbers of parasites per 100 macrophages (mean  $\pm$  SEM) were  $53.1 \pm 3.7$  (exp. sup.),  $53.3 \pm 3.2$  (control sup.), and  $50.5 \pm 3.2$  (D5).

*SCF.* Resident, CB, or PP macrophages acquired the capacity to release substantial amounts of H<sub>2</sub>O<sub>2</sub> in response to PMA after 3 d of incubation in supernates from spleen cells of *T. cruzi*-immunized or BCG-immunized mice cultured with the homologous antigen (experimental supernate, Fig. 2). No such increase in H<sub>2</sub>O<sub>2</sub>-releasing capacity followed incubation in control supernates (from normal spleen cells cultured with antigen) or medium alone (D5) (Fig. 2). The H<sub>2</sub>O<sub>2</sub> release from macrophages activated in vitro with SCF resembled that from freshly harvested IB cells, in that it lasted from 1.5 to 2.5 h, and when followed to completion, was as copious as that from IB cells (compare Figs. 1 A and 2 A). However, the initial rate of release of H<sub>2</sub>O<sub>2</sub> after in vitro activation tended to be about one-half that of IB cells (Figs. 1 B and 2 B). Fig. 2 C indicates that resident or inflammatory macrophages incubated in experimental supernates (containing SCF) acquired the ability to kill intracellular trypomastigotes, although macrophages cultured in control supernates or in medium alone did not, confirming a previous report (2).

*Time-Course of Activation in SCF.* PP or resident macrophages incubated in SCF acquired an enhanced capacity to release H<sub>2</sub>O<sub>2</sub> after 2 d in vitro, and this capacity increased further by the 3rd d (Fig. 3 A). This finding correlated with the onset of

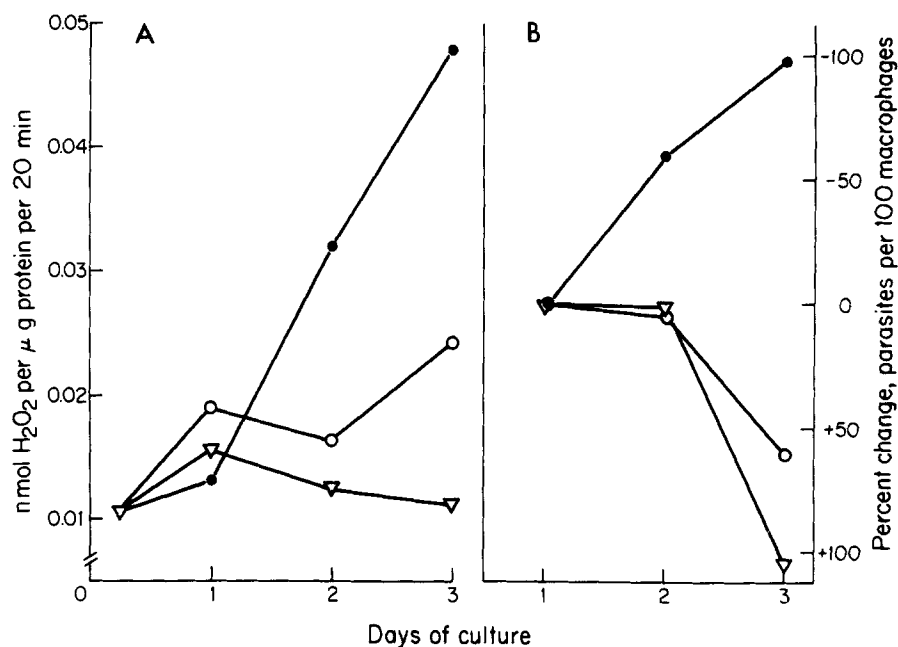


FIG. 3. Time-course of in vitro activation for  $H_2O_2$  release and trypanocidal activity. PP macrophages were incubated for the indicated times in D5 (triangles), D5 containing 25% of supernate of spleen cells from BCG-immunized mice cultured with PPD (closed circles), or D5 containing 25% of supernate of normal spleen cells cultured with PPD (open circles). Panel A: the initial rate of  $H_2O_2$  release after the addition of 100 ng/ml PMA is expressed in terms of adherent cell protein on matched slips. Panel B: the trypanocidal assay was performed with other samples of the same cells and media as in panel A. The macrophages were infected after 24 h of culture. They took up from 55 to 59 parasites per 100 cells after a 3-h exposure to a 2:1 multiplicity of trypomastigotes. The percent change in this number over the next 2 d is indicated (positive percent change reflects intracellular replication; negative percent change reflects intracellular killing of trypanosomes).

trypanocidal activity in other cover slips of the same cells (Fig. 3 B). In the trypanocidal assay, the macrophages were cultured in test media for 24 h before infection. Trypanocidal activity of macrophages incubated in the experimental supernate was detectable 1 d after infection (after 2 d of incubation) and was more marked by the 2nd d of infection (3 d of incubation). In contrast, macrophages incubated in control supernate or medium alone supported the replication of trypanosomes and were eventually destroyed (Fig. 3 B).

*Effect of Concentration of SCF.* As shown in Fig. 4, the ability of macrophages to release  $H_2O_2$  in response to PMA after 3 d of incubation in various concentrations of SCF-containing supernate increased over the range from 0.1 to 6.25%, and was no greater at 25%. In earlier studies, induction of trypanocidal activity was maximal after incubation in 12.5% of SCF-containing supernate (2).

*Loss of Activation of IB Cells During Culture In Vitro and Its Maintenance with SCF.* When IB cells were allowed to adhere, rinsed thoroughly to remove lymphocytes, and incubated for 3 d in D5 or control supernates, their capacity to release  $H_2O_2$  in response to PMA fell to the level of CB cells (Table I). Incubation in SCF reduced this decline by onehalf (Table I). It was observed previously that the trypanocidal activity of IB cells waned over 3 d in culture, but could be maintained by incubation

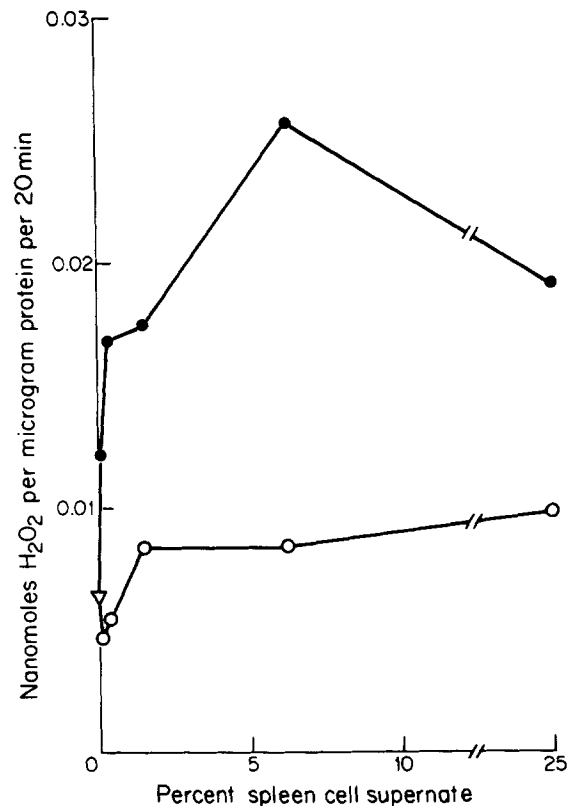


FIG. 4. Effect of concentration of SCF on H<sub>2</sub>O<sub>2</sub> release. PP macrophages were incubated for 2 d in the indicated concentrations of supernates of spleen cells from *T. cruzi*-immunized mice cultured with heat-killed *T. cruzi* (closed circles), or supernates of normal spleen cells cultured with heat-killed *T. cruzi* (open circles) or with D5 alone (triangle). The initial rate of H<sub>2</sub>O<sub>2</sub> release after the addition of 100 ng/ml PMA is expressed in terms of the adherent cell protein at each concentration of supernates. Mean of two experiments.

in SCF (2). When cover slips were not rinsed until just before the H<sub>2</sub>O<sub>2</sub> assay, so that nonadherent peritoneal cells remained in the culture, the H<sub>2</sub>O<sub>2</sub>-releasing capacity of the adherent cells was maintained for up to 4 d in vitro in medium alone (not shown).

**Reversal of In Vitro Activation By Removal of SCF.** PP macrophages were activated by 4 d of incubation in SCF, and then placed in D5. Over the next 2 d, their capacity to release H<sub>2</sub>O<sub>2</sub> in response to PMA returned to the level of freshly harvested PP cells (Table II). It was previously observed that removal of SCF from macrophages before complete sterilization of an infection of *T. cruzi* resulted in replication of the remaining trypanosomes (2).

**Adherent Cell Proteins.** Because the ability of mouse peritoneal macrophages to release superoxide anion in response to PMA appeared to be dependent on cell plating density (11), and because changes in adherent cell protein have been noted during culture of macrophages in lymphocyte mediators (12), it was important to conduct the H<sub>2</sub>O<sub>2</sub> assays at approximately equal plating densities, and to take into account any differences in cell protein which might emerge during culture. In nine experiments, the adherent protein for all cell types on day 0 averaged  $26.9 \pm 3.0 \mu\text{g}$  per

TABLE I  
*Loss of H<sub>2</sub>O<sub>2</sub>-Releasing Capacity of In Vivo-Activated Macrophages after a 3-d Incubation in Media Lacking SCF*

Exp.	Day of assay	Rate of H <sub>2</sub> O <sub>2</sub> release* after culture of cells‡ in:		
		D5§	Control supernate	Experimental supernate¶
A	0	0.104	—	—
	3	0.016	0.008	0.050
B	0	0.071	—	—
	3	0.006	0.002	0.032

\* Nanomoles H<sub>2</sub>O<sub>2</sub> per microgram adherent cell protein, released in 20 min after the addition of 100 ng/ml PMA.

‡ IB macrophages, from mice immunized with viable *T. cruzi* and boosted with heat-killed *T. cruzi*.

§ Dulbecco's modification of Eagle's medium with 5% FBS.

|| D5 containing 12.5% (exp. A) or 25% (exp. B) of supernate of spleen cells from normal mice cultured with heat-killed *T. cruzi*.

¶ D5 containing 12.5% (exp. A) or 25% (exp. B) of supernate of spleen cells from *T. cruzi*-immunized mice cultured with heat-killed *T. cruzi*.

TABLE II  
*Loss of H<sub>2</sub>O<sub>2</sub>-Releasing Capacity of In Vitro-Activated Macrophages by Removal of SCF*

Media during first 4 d of culture (all changed to D5* on 4th d)	Total H <sub>2</sub> O <sub>2</sub> release‡ from cells§ on day:		
	4	5	6
Experimental supernate	0.149	0.079	0.016
Control supernate¶	0.012	0.000	0.006
D5	0.000	0.000	0.000

\* D5, Dulbecco's modification of Eagle's medium with 5% FBS.

‡ Nanomoles H<sub>2</sub>O<sub>2</sub> per microgram adherent cell protein released in 146 min after the addition of 100 ng/ml PMA.

§ Macrophages harvested from mice injected with proteose-peptone.

|| D5 containing 12.5% of supernate of spleen cells from *T. cruzi*-immunized mice cultured with heat-killed *T. cruzi*.

¶ D5 containing 12.5% of supernate of spleen cells from normal mice cultured with heat-killed *T. cruzi*.

coverslip. On day 3, the adherent cell protein in experimental supernate averaged  $138 \pm 15\%$  of the value for day 0; for control supernates, it averaged  $131 \pm 19\%$ ; for medium alone, it averaged  $81 \pm 13\%$ .

*Sensitivity of Trypanosomes to H<sub>2</sub>O<sub>2</sub>.* When culture forms of trypanosomes were exposed to an appropriate dilution of glucose oxidase in KRPG for 1 h, the epimastigotes lost motility and subsequently lysed. 50% lysis occurred when the flux of H<sub>2</sub>O<sub>2</sub> measured 6.5 nmol/ml per min (Fig. 5). At that level, the occasional trypomastigotes present in the mixture showed no signs of damage. At the end of the incubation

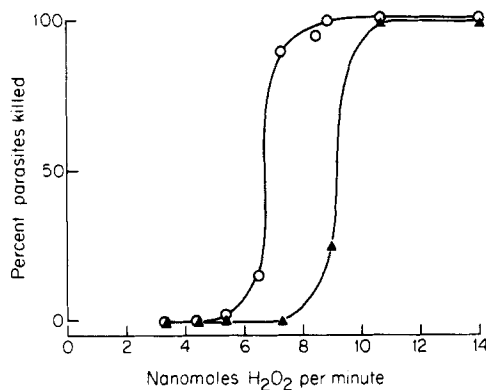


FIG. 5. Sensitivity of trypanosomes to  $H_2O_2$ . Purified trypomastigotes (triangles) or unseparated culture forms rich in epimastigotes (circles) were incubated separately at  $37^\circ C$  for 1 h in 1 ml of KRPG containing various dilutions of glucose oxidase. The rate of  $H_2O_2$  production by glucose oxidase was determined at the same time under the same conditions by the fluorometric assay for scopoletin oxidation. The viability of trypanosomes was assessed from their motility in a hemocytometer chamber.

period, the  $H_2O_2$  would be expected to be  $3.9 \times 10^{-4}$  M, if there were no loss. When purified suspensions of trypomastigotes were similarly tested, 50% of them were immobilized and subsequently lysed by a flux of  $H_2O_2$  measuring 9.4 nmol/ml per min (Fig. 5), corresponding to  $5.6 \times 10^{-4}$  M at the end of the incubation.

### Discussion

These studies indicated a close correlation between the ability of macrophages to release  $H_2O_2$  in response to PMA and their ability to kill intracellular trypomastigotes of *T. cruzi*. The two functions increased in parallel when the macrophages were activated by immunizing and boosting the mice in vivo, or by culture in spleen cell factors in vitro. Both functions subsided after removing immune lymphocytes or SCF. A similar dose of SCF induced trypanocidal activity and  $H_2O_2$  release, and did so over the same period of culture.

Numerous changes in structure and function, involving both secretory and endocytic events, have been observed during the stimulation or activation of macrophages (13–15). However, no biochemical process has been shown to correlate closely with macrophage microbicidal capacity (13). Either the correlation has not been attempted, or the property under study has been altered in a similar manner by inflammatory agents without the induction of microbicidal capacity. Moreover, with many of the biochemical features of macrophage stimulation or activation, it has not been evident how the property in question might be directly responsible for the killing of a microbe. In contrast, the ability of macrophages to release  $H_2O_2$  not only correlates with their trypanocidal activity, but may in large part explain it.

The hypothesis was put forward many years ago that macrophage production of  $H_2O_2$  might be a biochemical basis for acquired cellular immunity. Earlier workers observed that variations in the virulence of macrophage-parasitizing bacteria appeared to be linked to their resistance to  $H_2O_2$ . Thus, a positive correlation between virulence and catalase activity obtained for different isolates of *Mycobacterium tuberculosis hominis* (16), *Pasteurella pestis* (17), and *Brucella* (18, 19). Isolates of *M. tuberculosis*



displayed an inverse relation between virulence and sensitivity to  $H_2O_2$  in vitro (20, 21). Although catalase-deficient isolates of *M. tuberculosis* produced attenuated infection in guinea pigs, they replicated as well as catalase-positive isolates during the first few days of primary exposure (22). To explain such observations, Coleman and Middlebrook wrote in 1956, “. . . if the hypothesis presented here is correct, the steady-state concentration of hydrogen peroxide presumed to be in the cytoplasm of the phagocytes which engulf tubercle bacilli in the animal with established tuberculosis should be higher than that inside phagocytes of normal hosts during the first phase of infection.” (22).

In support of a similar hypothesis, we have tried to measure both the sensitivity of trypanosomes to  $H_2O_2$ , and the ability of macrophages to release it. Direct comparison of these two sets of figures is difficult, but leads to several points of interest. There appeared to be a sharp threshold separating trypanocidal and nontrypanocidal rates of  $H_2O_2$  generation by glucose oxidase. This raises the possibility that activation of macrophages to release  $H_2O_2$  may need to attain a critical level before the cells become trypanocidal. Thus, macrophages with relatively small differences in  $H_2O_2$ -releasing capacity might display large differences in trypanocidal activity. There were levels of  $H_2O_2$  generation that killed all epimastigotes but no trypomastigotes. This may be reflected in the ability of resident and inflammatory peritoneal macrophages to kill intracellular epimastigotes while failing to kill trypomastigotes (1). On the other hand, the curves for sensitivity to  $H_2O_2$  of epimastigotes and trypomastigotes were displaced from each other by only a small amount. Thus, other factors may also play a role in the differential susceptibility of these two forms to killing by macrophages.

The data in this study do not indicate directly whether activated macrophages produce a high enough concentration of  $H_2O_2$  to account for their trypanocidal activity. Specifically, we do not know how much  $H_2O_2$  is made during or after the attachment and ingestion of trypanosomes, the rate at which it is catabolized, or the volume in which it is distributed. Furthermore, the scopoletin assay only indicates a lower limit for the amount of  $H_2O_2$  produced by macrophages (4, 23). If the  $H_2O_2$  detected in the medium in Figs. 1 and 2 ( $\approx 30$  nmol from  $10^6$  cells) were accumulated within the cytoplasm of the macrophages in response to a trypanosomal infection, its concentration would theoretically be 76 mM,<sup>2</sup> or over 100 LD<sub>100s</sub> for trypomastigotes. If the same amount of  $H_2O_2$  accumulated in phagolysosomes, its concentration might in theory be more than 1,400 LD<sub>100s</sub>.<sup>3</sup> If one considers only the fluid phase surrounding the parasite within the phagolysosomes, the concentration of  $H_2O_2$  would presumably be higher still. Thus it seems that an activated macrophage has the capacity to generate a trypanocidal concentration of  $H_2O_2$ , if there were sufficient stimulus for its release and means for its accumulation in the vicinity of the parasite.

The sensitivity of various trypanosomes to reagent  $H_2O_2$  has been studied repeatedly over the last 40 yr (25–28), although there are few data pertaining to *T. cruzi*. For example, Fulton and Spooner observed a 50% inhibition of the respiration of *Trypanosoma rhodesiense* by  $2.86 \times 10^{-4}$  M  $H_2O_2$  (27), a value similar to the estimated LD<sub>50s</sub> in the present report.

<sup>2</sup> Considering the cell volume to be  $395 \mu\text{m}^3$  (24).

<sup>3</sup> Assuming one phagosome per macrophage, containing one trypomastigote, as a sphere of diameter 4  $\mu\text{m}$ .

The ability to destroy trypanosomes through oxidative means has been clearly demonstrated by another approach. Several laboratories have used naphthoquinones to augment the endogenous production of reactive metabolites of oxygen by the parasites (29–31). Such treatment resulted in peroxidation of trypanosomal lipids (32) and lysis of the parasites (29, 32). It would be of interest to examine the effect of trypanocidal naphthoquinones on the oxidative metabolism and anti-trypanosomal capacity of resident, inflammatory, and activated macrophages.

Our focus on  $H_2O_2$  does not discount the possible role of other trypanocidal mechanisms, including additional reactive metabolites of oxygen, such as singlet oxygen, hydroxyl radical, and superoxide anion, which may be produced by macrophages along with  $H_2O_2$ . Increased reduction of nitroblue tetrazolium, probably as a result of superoxide (33), was observed when guinea pig macrophages were incubated in lymphocyte mediators (34, 35, and unpublished observations of C. Nathan, H. Remold, and J. R. David). However, macrophages elicited with thioglycollate broth were not trypanocidal (1); such cells have been found to release substantial amounts of superoxide (11) but little  $H_2O_2$  (4) in response to PMA.

Increased oxidation of glucose through the hexose monophosphate shunt (HMPS) after incubation of macrophages in lymphocyte mediators was one of the first biochemical changes detected in activated macrophages (12). It was surmised that enhanced HMPS activity might indicate increased production of microbicidal  $H_2O_2$  (12). The present findings support that view. With guinea pig peritoneal macrophages, augmentation of HMPS activity required 2–3 d of culture, was dependent on the dose of lymphocyte mediators, and could be reversed by removal of the mediators (12, 36), all of which are similar to the results with  $H_2O_2$  release. The HMPS is closely linked to  $H_2O_2$  metabolism in leukocytes, providing electrons both for the reduction of molecular oxygen to superoxide, and for the detoxification of  $H_2O_2$  via the glutathione cycle (37).

There is an apparent discrepancy, however, in that extracellular release of  $H_2O_2$  from mouse peritoneal macrophages is not observed without the addition of a triggering agent, such as a phagocytic particle (4), although enhanced HMPS activity appears during incubation of guinea pig (12) or mouse peritoneal macrophages (38) or human blood monocytes (39) in lymphocyte mediators, without either the addition or formation of phagocytic particles (12). However, the oxidation of glucose-1-C increases even more markedly in such macrophages when phagocytic particles are added (J. R. David, personal communication). Macrophages activated *in vivo* with BCG or *Listeria* also have an elevated oxidation of glucose-1-C, which increases still further with phagocytosis (40–42). Thus, it is possible that the increased HMPS activity seen in activated macrophages before the addition of particles, reflects enhanced base line synthesis and catabolism of  $H_2O_2$ , without extracellular release. A triggering agent may lead to greater production of  $H_2O_2$ , and in some cases to its secretion.

It is worth emphasizing, however, that HMPS activity, reflecting both the synthesis and catabolism of  $H_2O_2$ , need not correlate with the amount of  $H_2O_2$  secreted (43, 44). For example, mouse peritoneal macrophages elicited with peptone displayed markedly enhanced glucose-1-C oxidation at rest and during phagocytosis (45). In our hands, such cells released little  $H_2O_2$  in response to PMA, and were not trypanocidal.

Macrophage-activating or migration-inhibiting factors produced by lymphoid cells of guinea pig, mouse, and man have been partially characterized (46–52). Work is underway to characterize SCF and to learn whether the substances inducing trypanocidal activity and H<sub>2</sub>O<sub>2</sub>-releasing capacity are the same.

### Summary

As reported previously, mouse peritoneal macrophages could be activated to kill intracellular trypomastigotes of *Trypanosoma cruzi*, the agent of Chagas' disease, in either of two ways: by immunizing and boosting the mice (3), or by culturing resident or inflammatory macrophages in spleen cell factor(s) (SCF) in vitro (2). Macrophages activated in vivo became less trypanocidal with time in culture, and cells activated in vitro lost trypanocidal capacity when SCF was removed (2).

In the present study, the ability of macrophages to release H<sub>2</sub>O<sub>2</sub> in response to phorbol myristate acetate (PMA) could be induced in vivo and in vitro, and reversed in vitro, in a manner correlating closely with changes in trypanocidal activity. Macrophages could be activated in vitro with SCF in a time-dependent and dose-dependent fashion, so that they released as much H<sub>2</sub>O<sub>2</sub> as macrophages activated in vivo. The sensitivity of epimastigotes and trypomastigotes to enzymatically generated H<sub>2</sub>O<sub>2</sub> suggested that the generation of H<sub>2</sub>O<sub>2</sub> by activated macrophages could be a plausible explanation for their trypanocidal activity.

Of the biochemical correlates of macrophage activation reported to date, increased ability to release H<sub>2</sub>O<sub>2</sub> seems most closely allied to enhanced capacity to kill an intracellular pathogen.

We are grateful to Ms. Linda Brukner for excellent technical assistance.

Received for publication 5 February 1979.

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