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Cells within the Vascular System Capable of Mediating Trypanocidal Activity In Vitro

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Cure of *Trypanosoma musculi* infection involves an effector mechanism mediated by immunoglobulin G2a antibody, C3, and an unidentified effector cell. In the present study, experiments were designed to identify the cell(s) within the vascular system that may be responsible for cure of trypanosomiasis. The ability of various cell populations to mediate killing of trypanosomes in the presence of C3 and immune plasma (IP) was tested in vitro. Blood-derived platelets or leukocytes or Bio-Gel-elicited macrophages or neutrophils were incubated at various concentrations with *T. musculi*, C3, and IP diluted up to 1 in 8. Trypanocidal activity was dependent upon the presence and concentration of IP and on the number of cells in the wells. Macrophages, neutrophils, and platelets were shown to kill with different potencies. With a 2:1 cell-to-parasite ratio, both macrophages and neutrophils reduced parasite numbers by 2 log, while platelets at a 40:1 ratio mediated a 1 log decrease. In addition, even in the absence of C3, the phagocytes were capable of killing trypanosomes while platelet trypanocidal activity was abrogated. The time course of trypanocidal activity was monitored for macrophages and neutrophils. The number of parasites decreased by 0.5 log by 4 h and 1 to 2 log by 8 h and by 20 h was reduced to zero. Cultured monolayers of endothelial cells were also tested for trypanocidal activity and shown to kill the parasites in the presence of IP and C3. The level of trypanocidal activity was dependent on the concentration of IP.

Trypanosoma musculi is a protozoan parasite which causes a self-limiting, 3-week infection in mice. The course of parasitemia is composed of an initial growth phase, a plateau phase, and an elimination phase, the last signifying the development of specific immunity by the host (29). The mechanism of elimination of parasites from the circulation has not been fully characterized as yet. In normocomplementemic mice, a trypanolytic mechanism mediated by the late-acting complement components is responsible for killing at least some of the parasites (8). Another pathway apparently exists, however, which is crucial for trypanosome elimination in C5-deficient mouse strains (4). Previous studies in our laboratory have established that three components are essential for this pathway, namely, specific immunoglobulin G2a antibodies, complement component C3, and an effector cell which has not been identified (32–34).

In an effort to elucidate the identity of the effector cell, recent experiments were carried out in which mice were immunodeprived of one or more cell types and assessed for their ability to eliminate sensitized parasites (12). When mice were rendered leukopenic by irradiation and depleted of platelets by antiplatelet antibody, trypanocidal activity was still present in vivo, indicating that leukocytes (WBC) were probably not the primary effector cell. Kupffer cells also did not appear to play a major role in curing infection, since in mice immunocompromised by pretreatment with silica or in which the hepatic circulation had been occluded, trypanosomes were still eliminated from the circulation. Thus, it appeared that either sufficient cells remained in the vascular system of the immunocompromised mice to effect killing or another cell population was responsible. Such a cell would be radioresistant and silica resistant, and it was suggested that the endothelial cell constituted a possible candidate (12). This cell is abundant in the vascular sinuses and small blood vessels, where a large number of *T. musculi* are located (2, 3).

From the in vivo experiments, therefore, it was apparent that a variety of cells existed, none of which could be ruled out definitively as effector cells in the immune elimination of the trypanosomes. Furthermore, a role for the endothelial cell had been suggested. Although it had been shown previously by others that macrophages can kill *T. musculi* in vitro in the presence of immune plasma (IP) (6, 30), the other cell types in question had not been investigated in this manner. The purpose of the present experiments, therefore, was to examine in vitro the ability of WBC, platelets, and endothelial cells to kill *T. musculi* in the presence of IP. In this way, the potential ability of each cell type to act as an effector cell in the in vivo elimination of *T. musculi* could be assessed.

MATERIALS AND METHODS

Animals, parasites, and enumeration. Retired female DBA/2 breeders (Harlan Sprague Dawley Inc., Indianapolis, Ind.) were used as a source for the isolation of different cell populations and in the preparation of normal mouse plasma as a fresh source of C3. Six- to ten-week-old A/J mice (Jackson Laboratories, Bar Harbor, Maine) were used for passage of *T. musculi* and for the various preparations of plasma. Thus, all sources of cells and plasma used were from C5-deficient mouse strains. Parasites were passaged and enumerated as previously described (32).

Plasma preparation. IP (plasma from a cured mouse), normal mouse plasma, and cobra venom factor (CVF)-treated IP were prepared from methods described elsewhere (25, 32).

Purification of *T. musculi*. Bloodstream forms of *T. musculi* were separated from blood collected from the retro-orbital sinus of infected A/J mice (9 to 12 days postinocula-

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tion) and partially purified by differential centrifugation by a method previously described (33). The trypanosomes were then separated from contaminating WBC in a DEAE-cellulose column (13). The purified preparation of trypanosomes obtained was suspended in RPMI 1640 with L-glutamine (GIBCO Laboratories Life Technologies Inc., Grand Island, N.Y.), 5% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, Utah), and gentamicin sulfate (Schering Canada Inc., Pointe Claire, Quebec, Canada) and adjusted to a concentration of 5×10^7 trypanosomes per ml.

Cell enumeration. Total cell (macrophage, neutrophil, WBC, and platelet) counts were performed by standard techniques.

In vitro trypanocytolysis assay. In order to determine the ability of a cell population to mediate killing of trypanosomes in vitro, a standard assay was set up. Aliquots (100 μl) of cell suspensions at various concentrations were plated out into 96-well Costar tissue culture plates (Fisher Scientific, Nepean, Ontario, Canada), and to each well were added 25 μl of IP, 25 μl of normal mouse plasma (C3 source), and 10 μl of purified trypanosomes. A time zero count was done in which the total number of trypanosomes in each well was estimated by enumerating the number of viable trypanosomes in a 5-μl sample by using a wet smear (32). If parasite numbers were $>10^5$/ml, the cell suspension was diluted (1:10, 1:50, or 1:100) with 0.2% formol-saline and enumerated with a hemocytometer. The plates were then incubated at 37°C in 5% CO_2 for up to 18 to 20 h, and the number of viable trypanosomes was enumerated as before.

Preparation of platelets. DBA/2 mice were sacrificed by asphyxiation with CO_2 and bled by cardiac puncture with heparinized syringes. The blood was pooled, mixed with 4% dextran (Sigma Chemical Co., St. Louis, Mo.) in saline or in RPMI 1640 with L-glutamine and brought to a final volume of 50 ml. The mixture was centrifuged at room temperature (RT) at $170 \times g$ for 15 min. The resulting pellet contained erythrocytes and WBC, while platelets and WBC were present in the supernatant. The supernatant was collected, and the pellet was rewashed with 4% dextran under the same conditions. The supernatants from each wash were pooled, and the pellets were kept separately for WBC purification as described later.

To obtain the platelets, a 1:1 (vol/vol) amount of normal saline was added to the supernatant and this was spun at RT at $170 \times g$ for 15 min. After centrifugation the supernatant was removed by gently using a Pasteur pipette and checked for WBC contamination. If WBC were still present, the procedure was repeated. The resulting pellets were pooled with the 4% dextran pellets and saved for further WBC purification. The remaining pure platelet suspension (with less than 0.1% WBC contamination) was enumerated and pelleted by centrifugation at RT at $1,000 \times g$ for 30 min. The cells were resuspended at a concentration of 2×10^7 cells per ml in RPMI 1640 with L-glutamine, 5% FCS, and gentamicin and used in the in vitro assay. Further dilutions of the cells were made as necessary.

Preparation of WBC. WBC were separated from erythrocytes in the pellets obtained during platelet purification. The pellet was resuspended in a small volume of medium, and the contaminating erythrocytes were lysed with Tris-ammonium chloride. A 10:1 ratio of lysis buffer (0.17 M Tris [Sigma], 0.16 M NH_4Cl [Fisher], pH 7.2) to cell sample volume was incubated at 37°C for 10 min with gentle mixing. The mixture was centrifuged at RT at $275 \times g$ for 5 min, and the supernatant (containing lysed erythrocytes) was removed. The WBC were washed three times with Hanks buffered salt solution (GIBCO) at $275 \times g$ for 10 min. The purified cells were resuspended in RPMI 1640 with L-glutamine, 5% FCS, and gentamicin at a concentration of 2×10^7 cells per ml.

Preparation of endothelial cells. Cultured monolayers of endothelial cells were derived from mouse aorta by the method described by McGuire and Orkin (16). Briefly, 50 μl of Matrigel (Collaborative Laboratories Inc., Bedford, Mass.) was added at 4°C to the wells of a 24-well Costar cluster plate (Fisher). Gelation of Matrigel was induced by adding 0.5 ml of RPMI 1640 with L-glutamine to each well and incubating the plate at 37°C for 1 h. Mice were sacrificed by CO_2 asphyxiation, and their aortas were removed under sterile conditions and rinsed in three individual baths of Hanks buffered salt solution. Each vessel was gently cleaned of periadventitial fat and cut into rings (1 to 2 mm in width), and the explants were transferred to the Matrigel substratum. A small volume (200 μl) of enriched medium (RPMI 1640 with L-glutamine, 10% FCS, 50 μl endothelial cell growth supplement [Collaborative Laboratories], gentamicin) was added to keep the gel surface moist. The explants were removed within 1 week, leaving behind large outgrowths of endothelial cells on the surface of the substratum. The cells were grown to confluence and enzymatically removed for passage by incubating with a small amount of 2% dispase (Collaborative Research) in calcium- and magnesium-free Hanks buffered salt solution at 37°C for up to 5 min. An equivalent volume of complete serum-containing medium was added to the cultures to inhibit further enzymatic action, and the cell suspension was then collected and spun at RT at $275 \times g$ for 10 min. The cell pellet was resuspended in complete medium at a concentration of 10^5 cells per ml, and 500 μl per well was dispensed into plastic 24-well Costar tissue culture dishes. All subsequent cell cultures were routinely subcultured by treatment with 0.05% trypsin–0.02% EDTA (GIBCO) under the same conditions as used for the dispase treatment.

Characterization of endothelial cells. Cultured endothelial cells were characterized by morphological identification and by immunostaining with antibodies specific for von Willebrand factor. Cells were passaged onto Lab-Tek eight-well chamber slides (Miles Laboratories, Inc., Naperville, Ill.) and allowed to grow to near confluence. The culture medium was removed, and the cells were fixed and permeabilized with periodate-lysine-paraformaldehyde fixative adapted from the formula of McLean and Nakane (15). The cells were then washed three times with phosphate-buffered saline (PBS), pH 7.2, and stored at 4°C until staining could be done.

The cells were stained according to manufacturer's instructions by using the Zymed Streptadvidin-Biotin System (Zymed Laboratories Inc., South San Francisco, Calif.) reagents with minor modifications. Briefly, the cells were blocked with 2% bovine serum albumin (Sigma) in PBS, pH 7.5, for 15 min at RT. All subsequent incubations were followed by a PBS wash and carried out as follows: (i) serum block reagent for 10 min at RT; (ii) 100 μl of mouse anti-von Willebrand antibody (Chemicon International Inc., El Segundo, Calif.) diluted 1:20 in PBS for 30 min at 37°C; (iii) biotinylated second antibody reagent for 10 min at RT; (iv) peroxidase blocking solution (H_2O_2-methanol, 1:10) for 10 min at RT; (v) enzyme conjugate reagent in PBS for 5 min at RT; (vi) substrate chromogen mix in double-distilled H_2O for 5 min at 37°C. The cell chamber and gasket were removed, and the slides were washed with water and air dried. The cells were observed under a light microscope.

Preparation of neutrophils and macrophages. Neutrophils

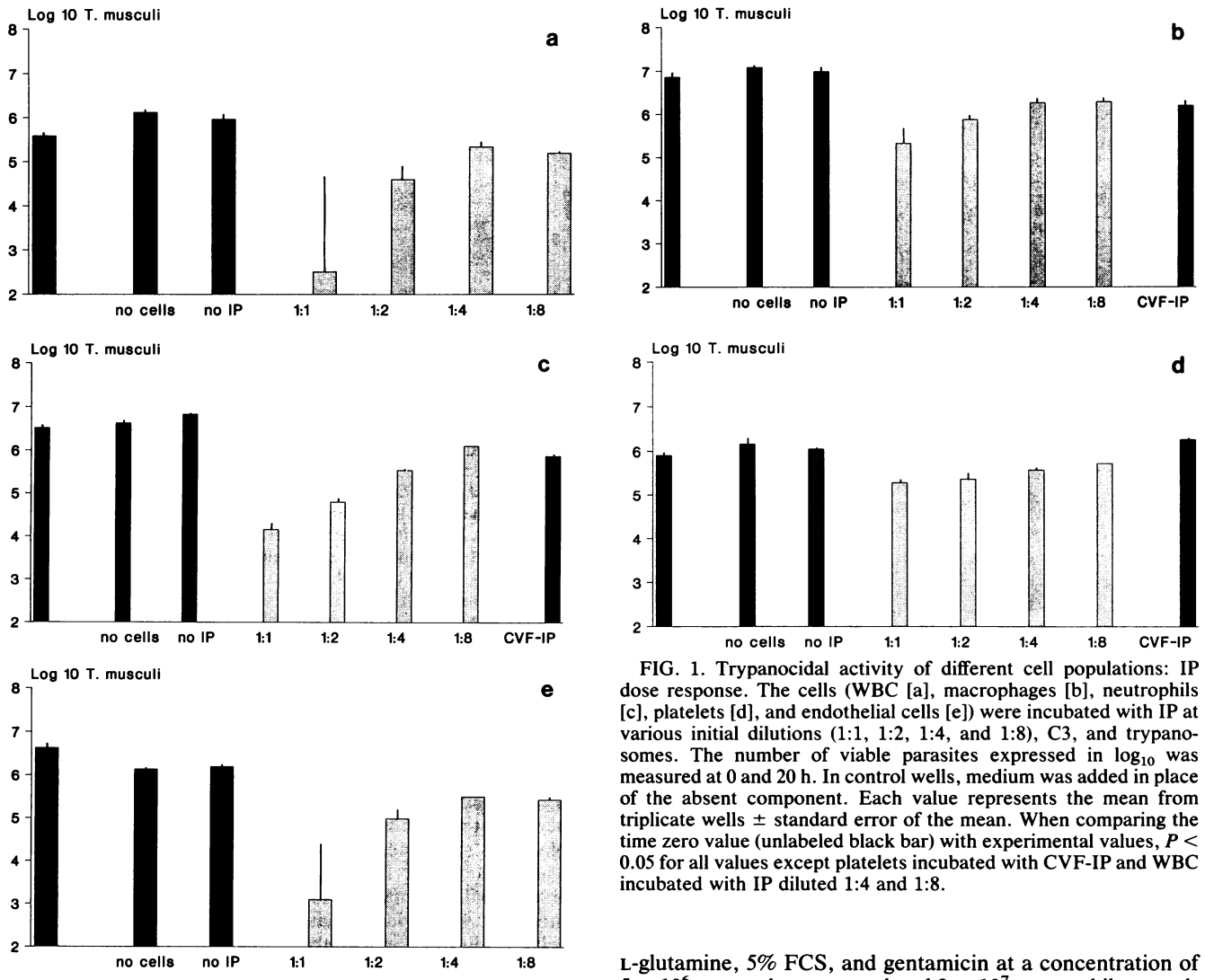


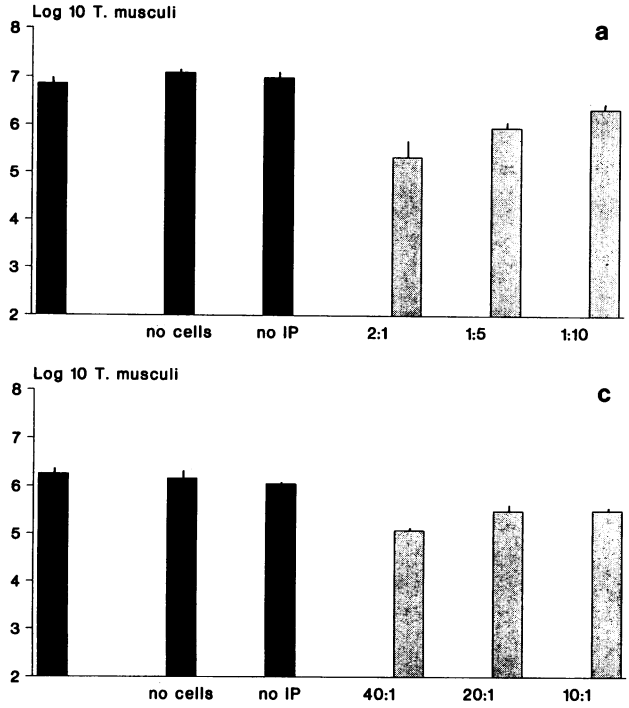
FIG. 1. Trypanocidal activity of different cell populations: IP dose response. The cells (WBC [a], macrophages [b], neutrophils [c], platelets [d], and endothelial cells [e]) were incubated with IP at various initial dilutions (1:1, 1:2, 1:4, and 1:8), C3, and trypanosomes. The number of viable parasites expressed in $\log_{10}$ was measured at 0 and 20 h. In control wells, medium was added in place of the absent component. Each value represents the mean from triplicate wells ± standard error of the mean. When comparing the time zero value (unlabeled black bar) with experimental values, $P < 0.05$ for all values except platelets incubated with CVF-IP and WBC incubated with IP diluted 1:4 and 1:8.

and macrophages were obtained from a subcutaneous inflammation induced by injecting a suspension of polyacrylamide beads into a subcutaneous air pocket (5). More specifically, 30 to 35 mg of beads (Bio-Gel P300; Bio-Rad Laboratories, Richmond, Calif.) was suspended in 100 ml of PBS, pH 7.2, and heated at 90°C for 10 min. The beads were autoclaved, and 40 ml of sterile PBS was added to resuspend the gel. The suspension was washed three times with PBS at RT at 275 × *g* for 10 min. After the last wash, the beads were centrifuged at 25 × *g* for 2 min and the supernatant was removed.

Mice were anesthetized by CO_2, and 5 ml of air was injected subcutaneously in the region of the lower back. Four milliliters of the bead suspension was injected into the air pocket. After 18 h (neutrophils) or 4 days (macrophages), the inflammatory cells were harvested. The mice were sacrificed, and their lower backs were shaved to relocate the original site of injection. Ten milliliters of RPMI 1640 with L-glutamine was injected into the lesion at the site, and the pocket was rinsed three to four times to remove as many cells as possible. The cells were separated from the beads by passaging the suspension through a tissue sieve, washing the cells in medium, and resuspending them in RPMI 1640 with L-glutamine, 5% FCS, and gentamicin at a concentration of 5×10^6 macrophages per ml and 2×10^7 neutrophils per ml. Neutrophils were checked for purity (>85%). Since the macrophages were contaminated with neutrophils, this cell suspension was plated out into 96-well plates 2 to 24 h before commencing the in vitro assay and then washed three times with fresh medium, and then the well volumes were readjusted to 100 μl.

RESULTS

Endothelial cell identification and characterization. Murine aorta explants were cultured on Matrigel and gave rise to endothelial cell outgrowths within 1 week of culture. The shape and cellular organization of the outgrowths emerged in an organized manner. The cells extended outward first as elongated chains and cords. Once cell confluence was reached, the cultures took on a cobbled morphology characteristic of endothelial cells. In order to specifically identify the endothelial cells as such, permeabilized cultures were immunostained with anti-von Willebrand antibody. The presence of this factor was observed by light microscopy and revealed by the light red staining of the cultures. The biotinylated antibody staining was present throughout the cell and was especially localized in the perinuclear region. In contrast, in control cells such as fetal liver stromal cells and



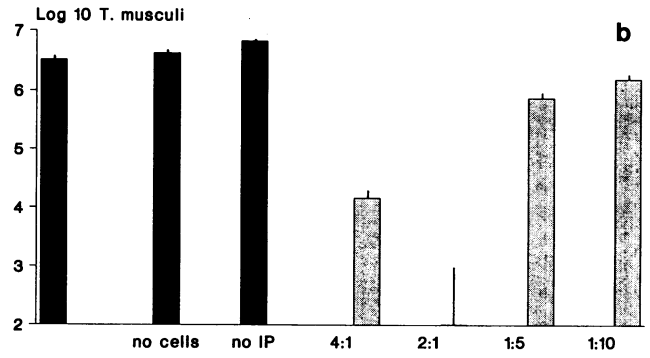


FIG. 2. Trypanocidal activity of different cell populations with various cell-to-parasite ratios (indicated in the figure). Cells (macrophages [a], neutrophils [b], and platelets [c]) were plated at various concentrations and incubated with IP, C3, and parasites. The number of viable trypanosomes expressed in $\log_{10}$ was measured at 0 and 20 h. In control wells, medium was added in place of the absent component. Each value represents the mean from triplicate wells ± 1 standard error of the mean. In all experiments except that with neutrophils, when comparing the time zero value (unlabeled black bar) with experimental values, $P < 0.05$ for all values. When comparing the time zero value with experimental values for neutrophils, $P < 0.05$ for 4:1 and 2:1 cell-to-parasite ratios.

L cells (both of which grow into cellular monolayers), the presence of von Willebrand factor was not observed (data not shown).

Trypanocytolytic activity of different cell populations in vitro: IP dose response. In order to examine the potential ability of WBC to mediate killing of *T. musculi* in vitro, the WBC were incubated with all of the components necessary for trypanocidal activity: C3, IP, and parasites (Fig. 1a). The degree of cytotoxic activity was assessed by measuring the number of viable parasites (expressed in $\log_{10}$) remaining in the well after 20 h. It was found that the WBC were able to kill the trypanosomes. A significant decrease of 3 log in *T. musculi* was observed in wells in which undiluted IP was added. The trypanocidal activity was dose dependent. In wells containing IP at lower concentrations (1:2, 1:4, and 1:8), the cells were less effective in killing the parasites. In control wells containing either no cells or no IP, the number of viable trypanosomes did not decrease. A small rise in parasite numbers was observed instead.

Macrophages were also found to have trypanocidal activity (Fig. 1b). The macrophages obtained by the injection of Bio-Gel are derived from recruited blood monocytes and thus constitute a pure population of inflammatory macrophages. These would be likely to be the macrophages encountered by *T. musculi* in the extravascular spaces and, to some extent, in the blood. At the end of the assay, a substantial decrease in parasite numbers in the wells containing IP at 1:1 and 1:2 dilutions was observed. IP diluted 1:4 and 1:8 was less effective in enabling the macrophages to kill the trypanosomes. The number of parasites remaining in control wells was high. In order to assess the requirement for C3, CVF-treated IP was used in one set of wells. Trypanocidal activity by the macrophages was observed, but it was less effective than when normal IP was used. The number of trypanosomes killed in these wells was comparable to the values obtained when macrophages were incubated with IP at 1:4 and 1:8 dilutions.

Similar results were observed with neutrophils (Fig. 1c). After 20 h, the cells had killed trypanosomes in the presence of IP. A significant decrease of 2 log in the *T. musculi* number was observed again and the trypanocidal activity was reduced when the concentration of IP was lowered. As before, in control wells (no neutrophils or no IP), the trypanosome numbers were not affected. When the IP had been treated with CVF, trypanocidal activity by the neutrophils was still observed but was lower than when normal IP was used. Thus, like macrophages, neutrophils were still able to kill the trypanosomes under these conditions, although it was evident that the presence of C3 enabled the cells to kill more efficiently.

Platelets exhibited trypanocidal activity in the presence of C3 and IP (Fig. 1d). A noticeable decrease in viable organisms could be seen in wells containing undiluted IP. At lower concentrations of IP, the cells were less well able to mediate killing. In control wells with no cells or no IP, the trypanosome number remained high and rose slightly from the time zero count. Trypanocidal activity of platelets is dependent on the presence of C3, since the cells were not able to kill the parasites when CVF-treated IP was used in the wells.

Murine endothelial cell monolayers were examined for their ability to mediate trypanocidal activity in vitro (Fig. 1e). After a 20-h incubation, parasite numbers decreased and the level of trypanocidal activity corresponded in a dose-dependent manner with the various concentrations of IP. The cells incubated with undiluted IP were able to bring about a 3-log decrease in parasite numbers. The standard error of this experimental value was large because one well had a parasite count of zero.

Trypanocytolytic activity of different cell populations in vitro: effect of various cell-to-parasite ratios. In the next set of experiments, the effect of various cell-to-parasite ratios on the trypanocytolytic activity of the various cell types was investigated (Fig. 2). In these assays, different concentrations of cell suspensions were plated with a constant number of trypanosomes in the presence of C3 and undiluted IP.

Macrophages were plated with trypanosomes at 2:1, 1:5,

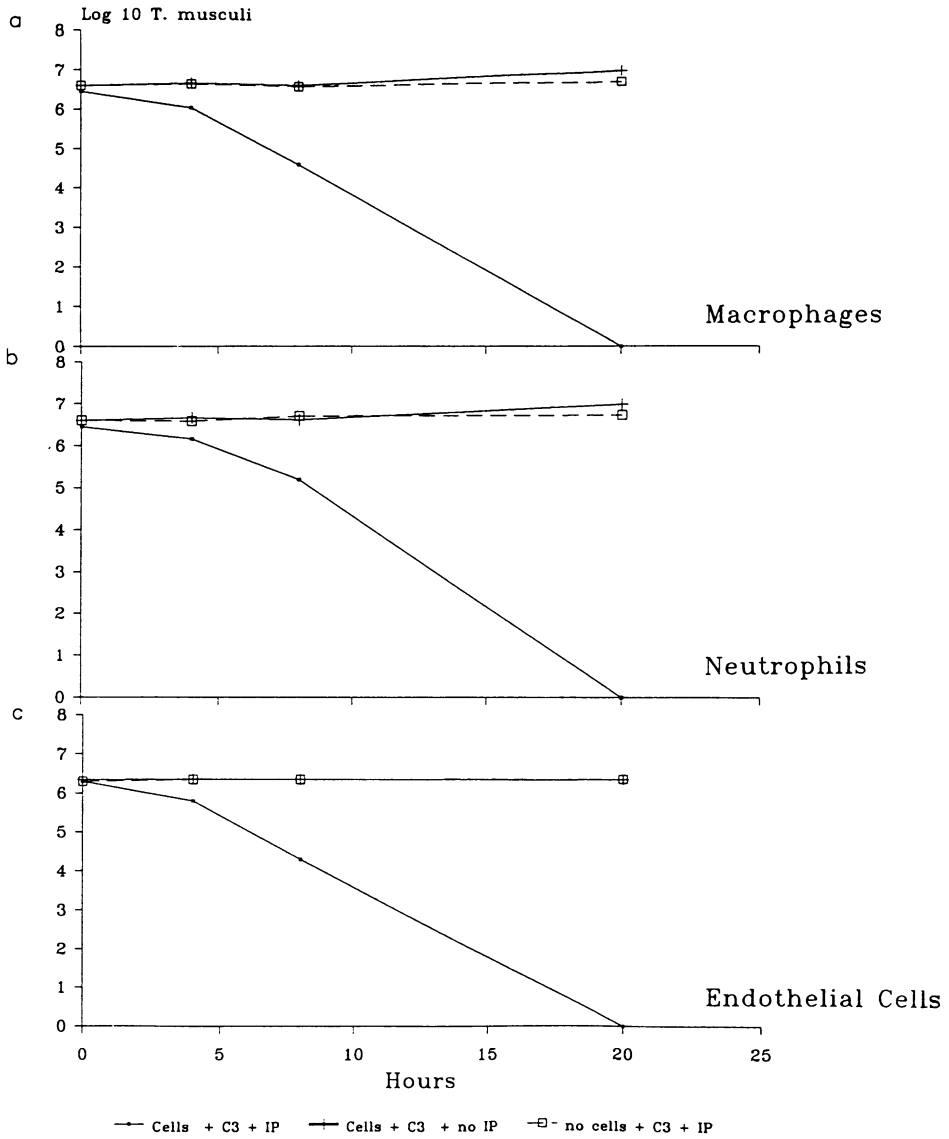


FIG. 3. Kinetics of trypanocytolysis. Macrophages, neutrophils, or endothelial cells were incubated with IP, C3, and parasites. The number of viable trypanosomes expressed in $\log_{10}$ was measured at 0, 4, 8, and 20 h. In control wells, medium was added in place of the absent component. Each value represents the mean from triplicate wells ± 1 standard error of the mean. When comparing the time zero value with experimental values, $P < 0.05$ for all values.

and 1:10 cell-to-parasite ratios. At the start of the assay, the $\log_{10}$ number of parasites in the well was 6.9. After 20 h, a graded level of trypanocidal activity was observed, with a maximum (2 log) decrease in the number of parasites in wells containing cells at the highest ratio (2:1).

The number of neutrophils in each well also determined the number of parasites killed. The cells were plated at 4:1, 2:1, 1:5, and 1:10 in relation to parasites. At the end of the assay, the number of parasites in wells with the highest concentration of cells decreased by 2 log. Although in wells containing cells at 2:1 ratios the killing appeared more efficient than in wells with cells at the 4:1 ratio, this was not statistically significant, because of a large standard error.

The trypanocidal activity of platelets was examined when cell-to-parasite ratios were tested at 40:1, 20:1, and 10:1. After 20 h, trypanocidal activity was greatest in the wells containing the highest concentration of cells and the parasite number was reduced by 1 log. Trypanocidal activity was minimal when the cell concentration was further diluted.

Kinetics of trypanocidal activity. The course of trypanocidal activity of macrophages (Fig. 3a), neutrophils (Fig. 3b), and endothelial cells (Fig. 3c) was then monitored throughout a 20-h incubation period. For the macrophages at time zero, wells containing cells, C3, and IP had a trypanosome number of approximately $\log_{10}$ 6.5. Controls wells which lacked either cells or IP also had trypanosome counts of the same value ($\log_{10}$ 6.5) at time zero. After 4 h, parasite numbers in the experimental wells had decreased to $\log_{10}$ 6.1, and after 8 h of incubation, trypanocidal activity by the macrophage resulted in a further reduction in parasite numbers to $\log_{10}$ 4.5. By 20 h, parasite numbers were reduced to zero. However, there were no changes observed in any control wells, and the number of trypanosomes remained essentially identical to the values obtained at time zero.

Trypanocidal activity of neutrophils had similar kinetics. At time zero, wells containing C3 and IP had an initial parasite number of approximately $\log_{10}$ 6.5. After 4 h, parasite numbers decreased slightly to $\log_{10}$ 6.1. A significant reduction in trypanosome number to the value of $\log_{10}$ 5.5 was observed after 8 h, and by the end of the assay, parasite killing was complete. In control wells, the number of *T. musculi* in the wells did not vary from $\log_{10}$ 6.5 at all time points.

The kinetics of trypanocidal activity of endothelial cells was also carried out. At the beginning of the assay, wells containing all the components had a parasite count of approximately $\log_{10}$ 6.3. After 4 h, the number of parasites was reduced to $\log_{10}$ 5.8 and by 8 h had fallen to $\log_{10}$ 4.3. All of the parasites had been killed by the end of the assay. In control wells, the number of trypanosomes did not vary from the value of $\log_{10}$ 6.3 obtained at time zero.

DISCUSSION

The objective of the present experiments was to determine the capability of cells within the vascular system to mediate trypanocidal activity in the presence of IP in vitro. As outlined earlier, the identity of the effector cell population(s) responsible for curing parasitemia in vivo is still unclear. Thus, the blood cells normally encountered by the trypanosomes in the circulation and extravascular spaces and the endothelial cells that line the blood vessels were selected for examination in this study. (Kupffer cells, unfortunately, could not be included.) It was found that WBC, platelets, macrophages, neutrophils, and cultures of endothelial cells were all capable of mediating parasite killing in vitro. Since this has been shown previously only for macrophages (6, 30), the present findings are interesting and quite novel. They do not allow us to rule out a particular cell population as the potential effector cell in vivo, but rather show that all of the cell types examined have this potential. To what extent each or all of them are effective in resolving parasitemia during the course of an infection remains an open question.

The experimental results shown in Fig. 1 and 2 showed decreases in parasite numbers by 1 to 3 log, whereas in Fig. 3 the results showed complete killing. This difference may be explained by the fact that a different batch of IP was used in the latter experiments. This IP could have been more potent, thus enabling the cells to kill the trypanosomes more effectively. Another reason could be the limitation of our method of enumeration at low parasite counts. At parasite counts of $\geq 10^6$, the sample was diluted and hemocytometer counts were done. When parasite numbers were $\geq 10^4$, samples were counted on wet smears. On a wet smear, detection of one parasite results in the $\log_{10}$ value of 4. By doing triplicate samples, $\log_{10}$ mean values of 3 may be obtained if one well has a zero count. Thus, the difference between $\log_{10}$ 3 and $\log_{10}$ 0 is not of great relevance.

The trypanocidal activity observed in our experiments is unlikely to be due to an in vitro artifact. For one thing, control cultures in which IP was absent failed to exhibit any trypanocidal activity. Furthermore, cultures of spleen cells were also tested in the assay for trypanocidal activity (data not shown). Unlike the other cell populations, spleen cells did not kill the trypanosomes; instead, an increase in the number of parasites was observed. (This increase is not an unexpected result, since spleen cells are often used to enhance trypanosome multiplication in vitro [1].) The validity of our observations is further substantiated by the dose-response and kinetics data. Thus, when either the concentration of IP or the cell-to-parasite ratio in the cultures was increased, typical dose responses were observed (Fig. 1 and 2). Similarly, the kinetics curves obtained with neutrophils and macrophages showed that trypanocidal activity increased with time over a period of 20 h (Fig. 3). Mammalian cell viability remained high at 4 and 8 h even though a significant number of parasites had been killed during this period. Indeed, all of the different cultures were always examined at the end of the assay (18 to 20 h), and very little cell death was seen, except in the endothelial cell cultures (discussed later). All of these findings, therefore, would argue against the possibility that the trypanocidal activity measured in the assays was due to a nonspecific destruction of the trypanosomes brought about, for example, by cytotoxins released from damaged effector cells.

The level of trypanocidal activity exhibited by macrophages, neutrophils, and platelets on a per-cell basis can be compared (Fig. 2). For macrophages, a cell-to-parasite ratio of 2:1 is necessary for the number of trypanosomes to be decreased by 2 log. Neutrophils are equally effective, since a 2:1 ratio is needed to achieve similar results. Platelets, however, require a 40:1 ratio of cells to parasites to mediate a 1 log killing. On the basis of these results, it could be inferred that the phagocytes, which are larger than platelets and better equipped to kill foreign target cells, would be more effective in killing *T. musculi* and curing infection in vivo. This observation, however, is subject to further interpretation. In considering the situation within the vascular system, circulating platelets are abundant in the mouse ($\sim 10^9$/ml) whereas polymorphonuclear WBC and mononuclear phagocytes are far less frequent ($\sim 5 \times 10^5$/ml and $\sim 2 \times 10^5$/ml, respectively), so that, in fact, the platelet population could be the more effective one in eliminating the parasites within the bloodstream. The macrophages and neutrophils could play their most important role in destroying trypanosomes that enter the extravascular spaces such as the peritoneal cavity (when the concentration of antibodies reaches a high-enough level to be effective in these areas).

From the results with CVF-IP, it was observed that platelets require the presence of C3 in the cultures in order to mediate trypanocidal activity, whereas neutrophils and macrophages can kill a certain proportion of trypanosomes in the absence of this factor, albeit less effectively than when C3 is present (Fig. 1). In all probability, the density of Fc receptors on the platelet is lower than that on the phagocytes, so that in the case of the platelet, C3 becomes a critical ligand for strengthening the binding between this cell and the trypanosome.

The involvement of platelets in cell-mediated cytolysis is an intriguing phenomenon. A role for platelets in *T. musculi* infection has been documented previously, but the studies were done with C5-sufficient mice in which trypanosome destruction was also occurring through the lytic pathway mediated by the late-acting complement components (28). In a series of studies by others, it has been shown that platelets are able to kill schistosome larvae in the presence of specific antibody of the immunoglobulin E class (10, 11). T-cell regulatory factors apparently govern this effector mechanism (18). Platelet-mediated antibody-dependent cellular cytotoxicity with erythrocyte target cells has also been reported (27). These earlier findings, that platelets can act as effector cells in an antibody-mediated target cell lysis, thus substantiate the present observation.

As outlined previously, evidence from our earlier studies led us to propose a role for the endothelial cell in mediating trypanosome elimination. The fact that endothelial cells

have both Fc and C3 receptors lends support to the idea (9, 22). The results presented in this paper demonstrate the ability of these cells to exhibit trypanocidal activity in vitro. The cytotoxic destruction of the parasites by the endothelial cell was comparable to that shown by the other cells examined, and the IP dependency of this process was also observed. This is a novel phenomenon, since the immunoeffector ability of endothelial cells has not been described previously for a parasitic infection. The ability of endothelial cells to bind (19), phagocytose, and, in some instances, kill a variety of bacteria has been reported previously (20, 21, 23), suggesting that these cells can act as a line of defense against bacterial infection.

The endothelial cell cultures used in the experiments were verified as such by morphological characterization and by the presence of von Willebrand factor (which is restricted to these cells) (31). Although the monolayer was disturbed at the end of the assay, the destruction of the parasites was unlikely to be due to factors released during endothelial cell disruption. When the kinetics of this assay was determined (Fig. 3c), the data were similar to those found with other cells. Thus, an initial 0.5 log decrease was observed by 4 h, and at 8 h, a significant 2 log reduction of trypanosome numbers occurred. At these time points, the cultures were examined and the monolayers were found to be intact. The destruction of *T. musculi* was therefore not a bystander effect due to endothelial cell lysis. In addition, when performing the endothelial cell assays, we included control cultures containing lymph node cells, L cells, or spleen cells (data not shown) and found that none of the other cells were able to mediate parasite destruction.

The manner in which the cells mediate trypanocidal activity is unknown. It is well established that small organisms may be ingested by phagocytosis. In the case of macrophages, neutrophils, and perhaps endothelial cells, this is a likely mechanism for trypanosome destruction. It was demonstrated by Ferrante (6) that in the presence of immune serum, *T. musculi* was phagocytosed and killed intracellularly within phagocytic vacuoles of mouse macrophages. Vincendeau et al. (30) also showed phagocytosis of opsonized *T. musculi* by mouse macrophages and identified immunoglobulins G1 and G2a as the subclasses of specific antibodies that were involved in this process. *T. musculi* is a large protozoan organism, however, and cells such as platelets cannot kill by this method. Samarawickrema and Howell (24) observed interactions between peritoneal cells and *T. musculi* and suggested that phagocytosis plays an insignificant part in parasite elimination. They proposed that the effector mechanism is extracellular and dependent on diffusible substances. For macrophages, these may include lysozyme, hydrolases, proteinases, oxygen metabolites, and prostaglandins. However, for platelets, Slezak et al. (26) have shown that the biological mechanism is unlikely to involve granular cytolysin, perforin, proteases, and oxygen metabolites. Indeed, a novel cytotoxic mechanism associated with the platelet membrane appears to be responsible.

It has been known for many years that endothelium-derived relaxing factor mediates the control of vascular tone and platelet aggregation. In 1987, this factor was identified to be the nitrogen oxide molecule, nitric oxide (NO) (17). The role of NO as a potent microbicidal mechanism against a variety of targets, such as bacteria, fungi, tumor cells, and protozoan and helminth parasites, has been demonstrated in recent years (7, 14). Its inducibility in a number of cell types, including endothelial cells, WBC, macrophages, neutrophils, and Kupffer cells, following cytokine stimulation has an important implication for host defense mechanisms during infection. In our experiments, we have shown that a variety of cells of the vascular system, including endothelial cells, have the in vitro potential to kill *T. musculi*. It is likely that NO could be an important killer molecule involved in the mediation of *T. musculi* clearance in vivo by these cells.

In summary, there is evidence that any or all of the cell populations examined in this study could be responsible for the elimination of *T. musculi* in vivo. One could propose, for example, that the trypanosomes become trapped in small blood vessels by adherence to endothelial cells and, following this, the platelets, WBC, and endothelial cells act in concert to kill the parasites and resolve the infection.

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