EFFECT OF NORMAL AND ACTIVATED HUMAN MACROPHAGES ON *TOXOPLASMA GONDII**

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(Received for publication 11 December 1973)

Cell-mediated immunity (CMI)[1] has been shown in experimental animal models to play a major role in resistance against many of the opportunistic pathogens producing disease in immunosuppressed patients (1). Although monocytes and macrophages have been shown to be the major effector cells of CMI against many of these pathogens in animals, relatively little is known of the microbicidal capacity of human monocytes and macrophages for these organisms. In addition to the problem of infection in the immunosuppressed host, the concept of the monocyte derivation of macrophages (2) and the development of better techniques for human white blood cell separation and in vitro culture have been instrumental in providing the impetus for investigations into the microbicidal capacities of human macrophages (3–12). We chose to study the effect of human macrophages on the obligate intracellular protozoan *Toxoplasma gondii* because this organism is an increasing cause of morbidity and mortality in immunosuppressed patients and because CMI has been demonstrated to play a major role in host defense against this parasite in experimental animal models (13–17).

Materials and Methods

Antibody Determination.—The Sabin-Feldman dye test (DT) was performed as described by Frenkel and Jacobs (18) and the titer is reported as the initial dilution of the serum.

Volunteers.—All volunteers were in good health and on no medications. Their ages ranged from 18 to 45 years and all were males. Individuals with titers of less than 1:2 were considered to be DT negative. Those positive in the DT were known to have chronic (latent) toxoplasmosis with stable titers ranging from 1:32 to 1:512.

Macrophage Cultures.—100-150 ml of heparinized (10 U/ml) peripheral venous blood was drawn from each of 12 volunteers (8 were DT negative, 4 were DT positive). Mononuclear

* Supported by grant AI-04717 from the National Institutes of Health and a grant from the John A. Hartford Foundation, Inc.

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[1] *Abbreviations used in this paper:* CM, control macrophages; CMI, cell-mediated immunity; DT, dye test; HBSS, Hanks' balanced salt solution; SK-SD, Streptokinase-Streptodornase; SM, stimulated macrophages; TLA, Toxoplasma lysate antigen.

cells were obtained as previously described (19). The cells were suspended in tissue culture medium 199 (M199, Grand Island Biological Co., Grand Island, N. Y.) containing antibiotics (100 U penicillin/ml, 100 μg streptomycin/ml) and 40% autologous human serum (M199 + 40% AS). The total number of mononuclear cells (monocytes and lymphocytes) was determined by counting in a hemacytometer. The proportion of mononuclear cells that were monocytes was determined as previously described (19) and ranged from 15–30%. The remainder of the cells were lymphocytes with rare (<0.5%) contamination by granulocytes. More than 98% of these cells were viable as determined by the exclusion of 1.5% trypan blue dye.

Standard sized Leighton tubes (16 × 125 mm) containing glass cover slips were seeded with mononuclear cells in 0.5 ml M199 + 40% AS. Each 0.5 ml contained 1×10^6 monocytes. After the monocytes were allowed to adhere to the cover slips for 2–4 h at 37°C in an atmosphere containing 5% CO_2, the supernate was aspirated and the cell monolayer washed twice with Hanks' balanced salt solution (HBSS) to remove nonadherent cells. Vigorous washing was not carried out, so as to maintain integrity of the monolayer. The monolayers were reincubated with 2 ml of fresh M199 + 40% AS and the medium was changed every 3 days thereafter until the cells were utilized in experiments. After incubation for 5–8 days, the monocytes developed the morphologic criteria for macrophages (20). In all monolayers, a few lymphocytes remained adherent to the cover slips, but accounted for less than 1% of the mononuclear cells. Viability of monolayers was considered satisfactory if >90% of the macrophages excluded trypan blue dye and/or actively incorporated tritium-labeled uridine ([^{3}H]UdR, 28 Ci/mmol sp act, New England Nuclear Corp., Boston, Mass.) as determined by autoradiography. When tested on days 5–10 of incubation, 50–90% of the macrophages in any given monolayer phagocytized heat-killed *Candida albicans*.

Preparation of Toxoplasma.—Purified preparations of Toxoplasma trophozoites of the RH strain were obtained from the peritoneal fluid of 2-day infected mice as previously described (21, 22). The organisms were suspended in cold (4°C) M199 containing 40% heat-inactivated homologous human plasma (M199 + 40% HP) and counted in a hemacytometer. (The human plasma was obtained from a single individual whose plasma was known to be negative in the DT when tested undiluted and not heat inactivated, indicating total lack of measurable Toxoplasma neutralizing antibody. The plasma was heat inactivated at 56°C for 45 min to remove the "accessory factor" necessary for neutralization of Toxoplasma by antibody, [23, 24]). The suspension of organisms was brought to room temperature at the time of challenge of the monolayers. 1 million organisms prepared in this manner killed 100% of mice within 6 days following intraperitoneal injection.

The ability of the extracellular organisms in the challenge inoculum to incorporate [^{3}H]UdR into parasite RNA was assessed by the use of autoradiography (21). At the same time the parasites were being placed on macrophage monolayers, 20×10^6 organisms from the same challenge inoculum were suspended in 2 ml of M199 containing 40% DT negative, heat inactivated, dialyzed AB serum (M199 + 40% DS) and 20 μCi[^{3}H]UdR. This suspension was then continuously agitated at 37°C and after 30–60 min, 0.5-ml aliquots were removed for cytocentrifuge preparations (Shandon-Elliot cytocentrifuge, Shandon Southern Instruments, Inc., Sewickley, Pa.). Autoradiography was performed after fixation of the preparations in 0.4% amino-acridine hydrochloride in 50% ethanol (17). Grain counts were performed on a minimum of 500 Toxoplasma.

Infection of Macrophages.—Before infection, medium from each monolayer was replaced with fresh M199 + 40% AS containing 0.01 M uridine (UdR, Calbiochem, San Diego, Calif.). After reincubation for 4–6 h, cultures were washed with HBSS, decanted, and then infected with 5×10^6 Toxoplasma trophozoites in 0.5 ml of M199 + 40% HP. 30 mins later (zero time) the cultures were washed with HBSS to remove extracellular organisms, fresh M199 + 40% AS was added, and the monolayers reincubated. At zero time and at various intervals thereafter, the incubation medium was decanted and 0.5 ml of M199 + 40% DS containing

5 μCi of [^{3}H]UdR was added to the cultures. After a 30–60 min pulse cover slips were removed, rinsed in isotonic saline, and processed for autoradiography. Duplicate cover slips were prepared for all time intervals. Grain counts were performed on the intracellular organisms in phagocytic vacuoles in a minimum of 200 macrophages per slide.

In evaluation of autoradiographs, close agreement was noted between the results of grain counts over intracellular Toxoplasma obtained on duplicate slides. The vast majority of Toxoplasma were either labeled with a grain count ≥ 5 or <2 (mostly unlabeled); few were labeled with 2–4 grains. Using a grain count of ≥ 5 clearly distinguished morphologically intact organisms from those which had ill-defined borders and the unstained cytoplasm characteristic of dead organisms (23) (Fig. 1 *a*, *b*). However, some Toxoplasma which were not labeled with [^{3}H]UdR had the clear staining qualities of those which had ≥ 5 grains (Fig. 1 *c*). For this reason, staining qualities alone were not considered a satisfactory criterion of viability of Toxoplasma (17). Also, organisms with ≥ 5 grains were easily distinguished from background. Greater than 95% of organisms which had multiplied intracellularly and formed pairs, tetrads, or rosettes had grain counts of ≥ 5 per organism. By using the criterion of ≥ 5 grains per organism to define viable Toxoplasma the population of metabolically active organisms capable of intracellular replication was readily identified.

"Activation" of Macrophages.—Monolayers prepared as described above, with the exception that the nonadherent cells (mainly lymphocytes) were not washed off the cover slips, were incubated with or without antigen for 6–8 days without changing the medium. The antigens were 10 μg/ml Streptokinase-Streptodornase (SK-SD, kindly supplied by Dr. W. M. Sweeney, Lederle Laboratories, Pearl River, N. Y.) prepared as previously described (25) or 10–15 μg/ml Toxoplasma lysate antigen (TLA) (26). Separate experiments had established that these concentrations of antigen and the time period of macrophage exposure to antigen used (6–8 days) resulted in satisfactory macrophage monolayers with evidence of antigen stimulation as determined by morphologic criteria (20, 27) and lymphocyte transformation.

Lymphocyte Transformation.—In all activation experiments, nonadherent cells were removed just before the addition of the UdR to the monolayer. The nonadherent cells from each group of Leighton tubes which had been cultured with or without antigen were pooled. From each group, four plastic tubes (16 × 125 mm, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) each containing 1×10^6 mononuclear cells suspended in 2 ml of M199 + 40% AS with 2 μCi tritium-labeled thymidine ([^{3}H]TdR, 6.0 Ci/mmol sp act, New England Nuclear Corp.) were prepared. After incubation for 24 h, three tubes were used for measuring lymphocyte transformation as previously described (26). The degree of lymphocyte transformation was determined by calculation of the ratio A:C of uptake of [^{3}H]TdR (cpm) obtained in mononuclear cell cultures incubated in the presence (*a*) and the absence (*c*) of antigen. Cytocentrifuge preparations for autoradiography were made from the fourth tube. The mononuclear cells from tubes with or without antigen had comparable viability (>90%) as determined by exclusion of trypan blue dye.

RESULTS

Effect of Macrophages from DT-Negative and DT-Positive Subjects on Toxoplasma.—After challenge with Toxoplasma for 30 min (zero time), a mean of 55% (range 20–92%) of the macrophages were infected by at least one organism (radiolabeled or unlabeled). Many were multiply infected; the mean number of Toxoplasma per infected cell was 3.1 (range 1.7–7.8).

Eight experiments were performed with macrophages from DT-negative individuals and four with macrophages from DT-positive individuals. A

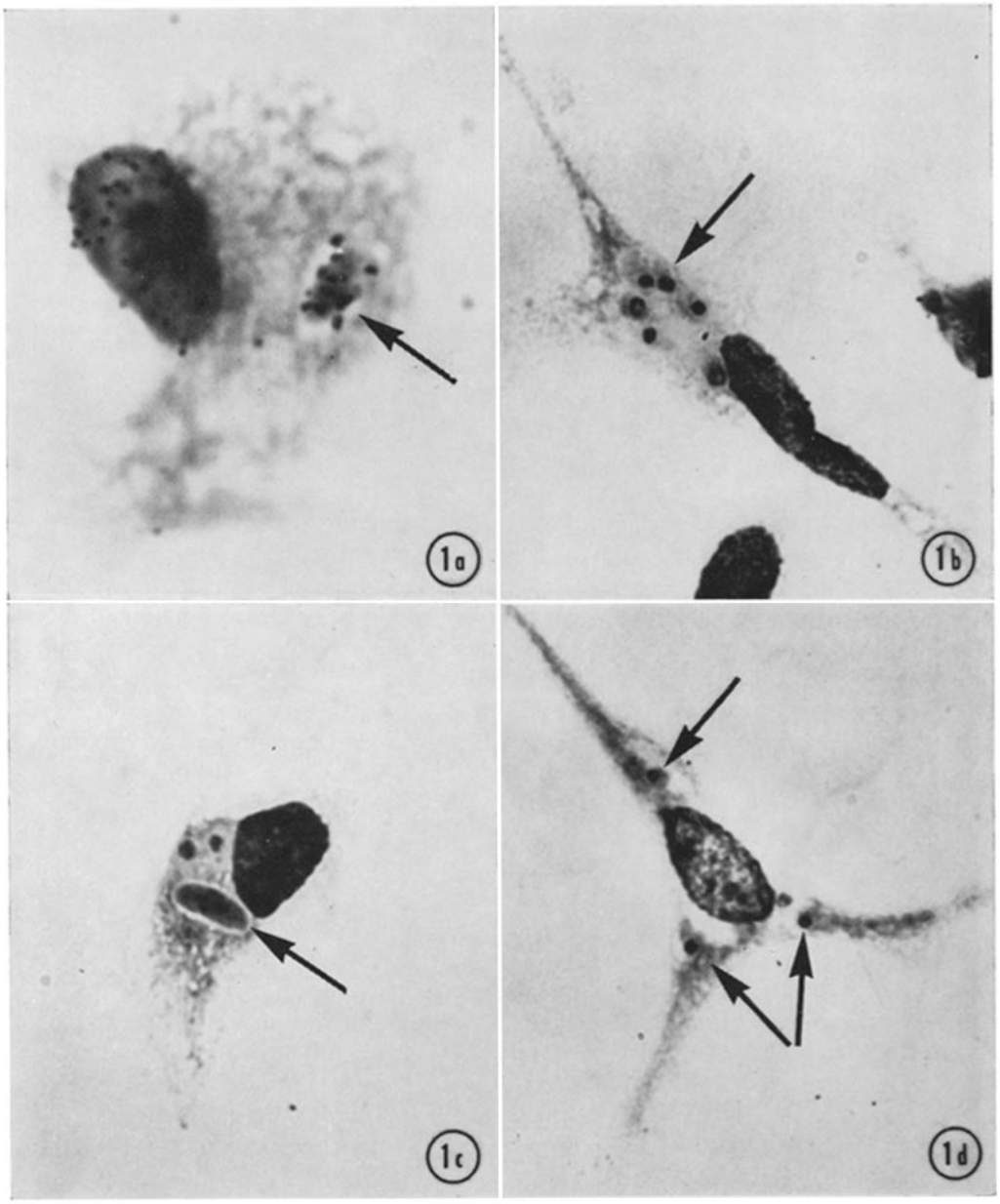


FIG. 1. Variability in appearance of Toxoplasma in autoradiographs of Toxoplasma-infected monolayers of normal human monocyte-derived macrophages. (*a*) 1 h after zero time. Toxoplasma with >5 grains (arrow). Morphology and staining characteristics of such organisms were those associated with viable Toxoplasma. × 1,500. (*b*) 1 h after zero time. Nonlabeled Toxoplasma (arrow). Morphologically intact organisms but with ill-defined borders and lightly stained cytoplasm characteristic of dead organisms (23). (*c*) 1 h after zero time. Toxoplasma pretreated with antibody in the absence of accessory factor (arrow). In some instances, as shown here, the organisms appeared to be viable by morphologic and staining characteristics but failed to incorporate [^{3}H]UdR. Such organisms subsequently underwent dissolution. For this reason, staining qualities alone were not considered a satisfactory criterion of viability of Toxoplasma (17). (*d*) 4 h after zero time. Fragments of Toxoplasma (arrows) demonstrating dissolution of nonlabeled organisms. × 1,200.

representative example of the results is shown in Fig. 2. Over a 20-h period, the number of viable intracellular Toxoplasma in macrophages obtained from DT-negative and DT-positive individuals increased (Fig. 2 *a*, *d*), although the percent of cells infected with viable Toxoplasma remained constant (Fig. 2 *c*). As shown in Fig. 2 *b*, the majority (>85%) of Toxoplasma at 30 min and 90 min after zero time were not labeled with ≥5 grains per organism and almost all such organisms appeared to be dead as judged by staining characteristics (Fig. 1 *b*). With increasing time the percent of organisms with ≥5 grains increased to >90%. After 4–6 h, the total number of Toxoplasma labeled with <5 grains had greatly diminished (from >300 per 100 macrophages to <10 per 100 macrophages), indicating rapid dissolution of those parasites and such organisms were recognizable only as amorphous material or nuclear remnants within cytoplasmic vacuoles (Fig. 1 *d*). These results suggest that the vast majority of organisms in the challenge inoculum were either dead before entry into the macrophages or were "attenuated" to such a degree that they were unable to multiply intracellularly and died within a few hours of causes other than macrophage killing. Further evidence for this was obtained in experiments performed to determine the percent of organisms in the challenge inoculum which were metabolically active such that they were incorporating [^{3}H]UdR. Autoradiographs of the challenge inoculum of each experiment revealed that from 8–20% of the organisms were labeled with ≥5 grains, closely approximating the percent of intracellular organisms that had ≥5 grains at 30–90 min after zero time. In contrast, greater than 85% of the organisms in the challenge inoculum excluded trypan blue dye indicating that dye exclusion is not as sensitive as [^{3}H]UdR uptake as an indicator of those organisms capable of intracellular replication. That greater than 80% of the Toxoplasma in the challenge inoculum were unable to incorporate [^{3}H]UdR can be attributed to the time consuming method of preparing a purified suspension of trophozoites, as trophozoites rapidly lose viability in an extracellular environment (28).

Macrophages obtained from either DT-negative or DT-positive individuals demonstrated no difference in their effect on Toxoplasma; this was true if the incubation medium added to the monolayers after parasite challenge contained DT-negative or DT-positive serum. The photomicrographs in Fig. 3 are representative of the results of these experiments.

Effect of Macrophages on Toxoplasma Exposed to Antibody before Infection.— These experiments were performed to evaluate whether Toxoplasma exposed to antibody in the absence of accessory factor are more readily killed by normal macrophages. Just before macrophage challenge, the organisms were incubated at 37°C for 30 min in medium containing 40% heat-inactivated DT-positive human serum (DT titers ranged from 1:32–1:4,096). Control macrophage monolayers prepared from the same subject were challenged simultaneously with a Toxoplasma inoculum which had been similarly preincubated in medium containing 40% heat-inactivated DT-negative human serum. Follow-

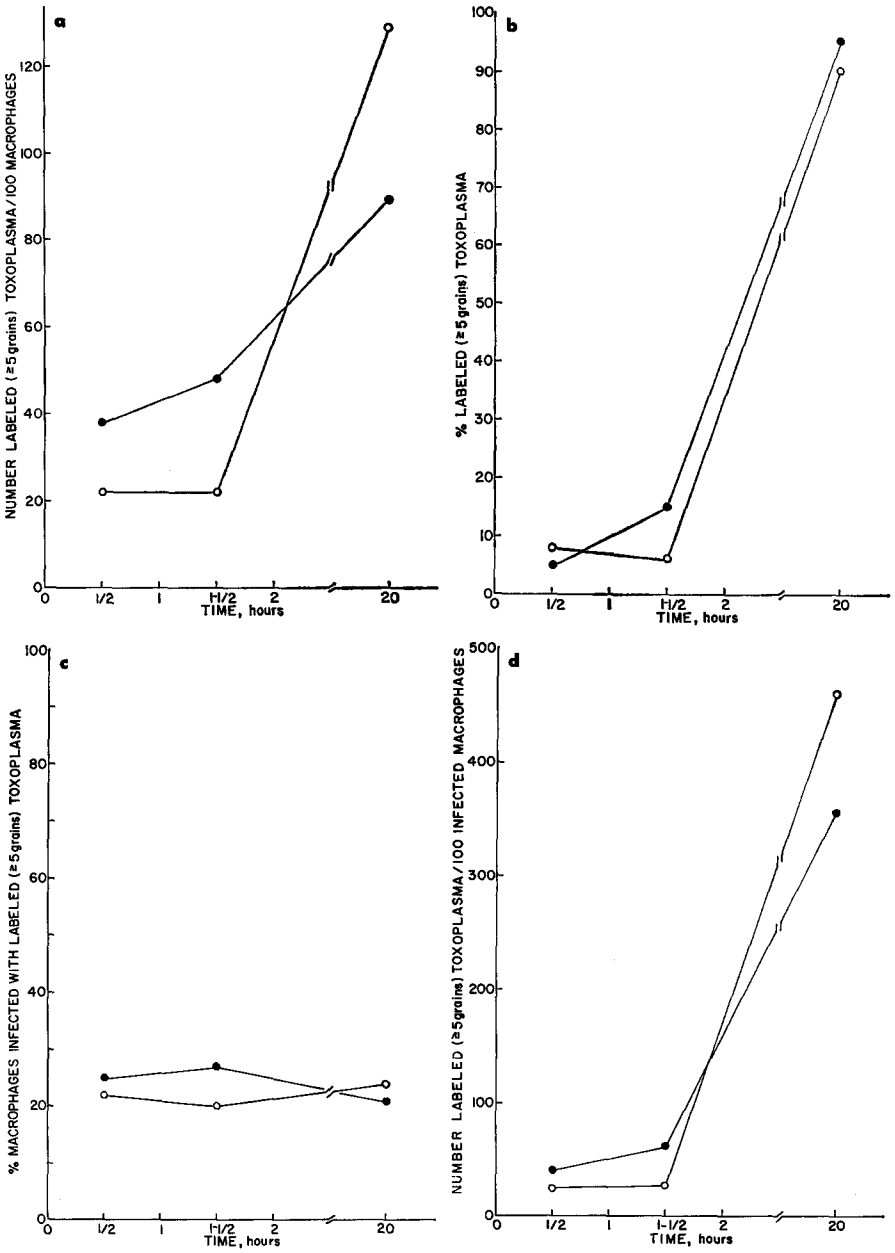
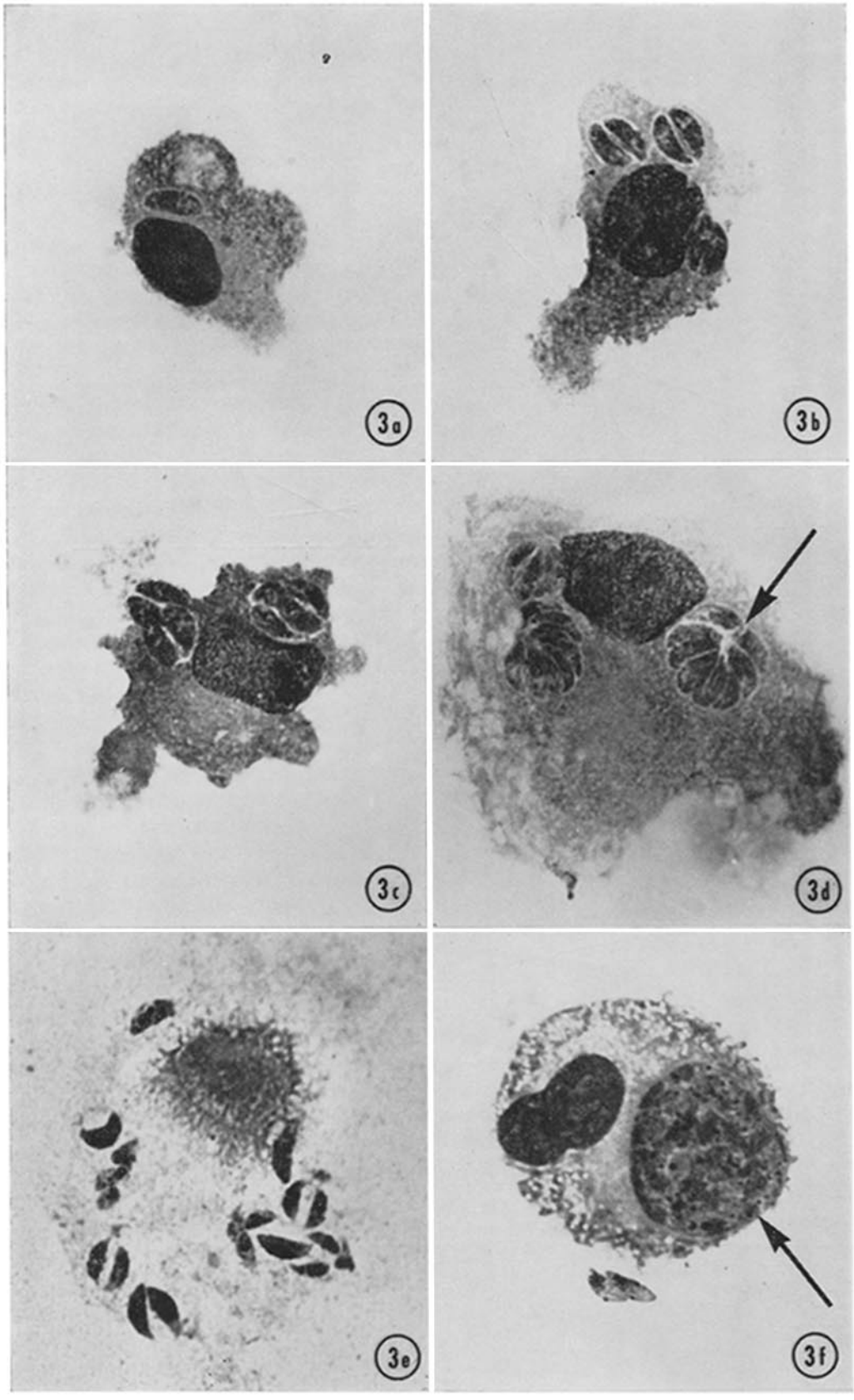


FIG. 2. Effect on Toxoplasma of human monocyte-derived macrophages from DT-negative (●) and DT-positive (○) subjects. (*a*) Total number of viable intracellular Toxoplasma (defined as organisms labeled with ≥ 5 grains, see text) per 100 macrophages. (*b*) Percent of intracellular Toxoplasma which were viable. (*c*) Percent of macrophages infected with viable Toxoplasma. (*d*) Number of viable intracellular Toxoplasma per 100 infected macrophages. It should be emphasized that the denominator is the number of macrophages infected with any Toxoplasma (labeled or unlabeled). This demonstrates the intracellular multiplication of Toxoplasma that has occurred.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ing parasite challenge, the macrophage monolayers were handled as usual except that the fresh medium added was the same as used for preincubation of the challenge inoculum. Results of autoradiography of organisms in the challenge inoculum and of mouse inoculation experiments did not reveal a difference in viability between the antibody-treated and control organisms.

A representative result of six separate experiments is shown in Fig. 4. By 20 h, a marked increase was noted in the number of viable, intracellular Toxoplasma in the control monolayers, whereas no such increase was noted in macrophages infected with organisms which had been treated with antibody. By 40 h, the control monolayers had been destroyed by Toxoplasma. In contrast, monolayers infected with antibody-treated Toxoplasma were relatively intact at this time period. That pretreatment of trophozoites with antibody did not result in the intracellular killing of all Toxoplasma was evidenced by the fact that small numbers of macrophages with radiolabeled replicating Toxoplasma were demonstrable at 20–40 h.

Effect of Activated Macrophages on Toxoplasma.—Macrophages incubated with autologous lymphocytes in the presence of SK-SD or TLA (in the case of DT-positive individuals) are referred to below as stimulated macrophages (SM). Macrophages incubated with autologous lymphocytes in the absence of antigen are referred to as control macrophages (CM). In the SM monolayers there was an increased number of giant multinucleated cells and glass adherent lymphocytes, markedly increased cytoplasmic bridging from macrophages to other macrophages and lymphocytes, increased numbers of mitotic figures, and particularly prominent clumping of cells into "immunological islands" (20), consisting of a central core of one or more macrophages around which were clustered both unlabeled small lymphocytes and larger transformed lymphocytes with heavy uptake of [^{3}H]TdR (Fig. 5 *a–c*). The adherence of lymphocytes to glass and to macrophages in the presence of antigen accounted for our inability to prepare monolayers totally devoid of lymphocytes. Repeated washing failed to remove all the lymphocytes and the presence of even small numbers of lymphocytes resulted in some degree of clustering when antigen was added to the cultures. We were therefore unable to accurately assess the

FIG. 3. Giemsa-stained preparations of normal human monocyte-derived macrophages infected with Toxoplasma in vitro. (*a*) 1 h after zero time. A single Toxoplasma is seen within a vacuole in the cytoplasm. (*b*) 8 h after zero time. The Toxoplasma have undergone a single division here depicted as three separate pairs. The third pair is to the right of the nucleus. (*c*) 12 h after zero time. The Toxoplasma have undergone a second division and four organisms can be seen within each of the two vacuoles. (*d*) 20 h after zero time. Multiple divisions have occurred resulting in rosette formation (arrow). (*e*) 40 h after zero time. Macrophages infected with Toxoplasma appear to be undergoing cell lysis with release of the organisms. (*f*) 40 h after zero time. Cytocentrifuge preparation of supernate of infected macrophage monolayer. Cystlike structures (arrow) can be seen within cytoplasmic vacuoles in some macrophages. × 1,200.

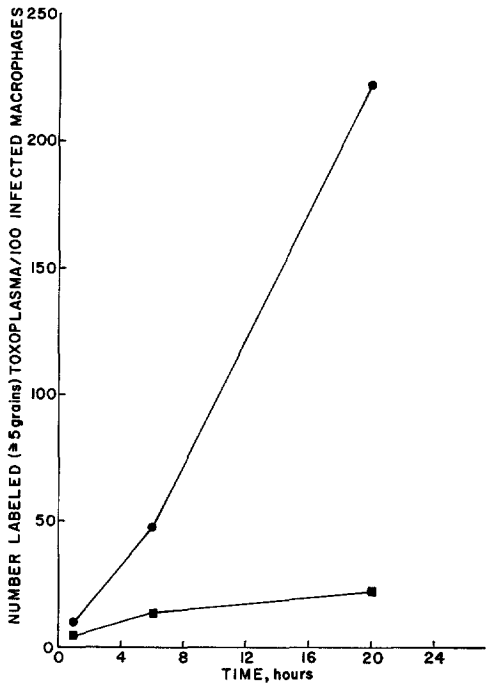

FIG. 4. Effect of normal human monocyte-derived macrophages on Toxoplasma which had been exposed to anti-Toxoplasma antibody before infection. (●), macrophages infected with Toxoplasma which had not been exposed to antibody; (■), macrophages infected with Toxoplasma which had been exposed to antibody.

role of the lymphocyte in this in vitro model, since we could not determine the effect of antigen stimulation alone on monolayers of macrophages totally free of lymphocytes.

Evidence for antigen stimulation of the lymphocytes was the increased uptake of [^{3}H]TdR (determined both by scintillation counting and autoradiography) by nonadherent cells from SM cultures as compared to CM cultures. The mean ratio A:C of [^{3}H]TdR uptake in cell cultures stimulated with SK-SD was 4.0 (range 2.3–7.4) and in cell cultures prepared from DT-positive individuals and stimulated with TLA was 5.7 (range 3.7–9.2). However, when macrophages from DT-negative individuals were incubated with autologous lymphocytes and TLA, there was no evidence of antigen stimulation, either morphologically or by lymphocyte transformation. Autoradiographs revealed blastogenic transformation (Fig. 5 *d*) of 8–23% (mean 14.5%) of lymphocytes from antigen-stimulated cultures (either SK-SD or TLA) compared to $<2\%$ (mean 1.6%) in control cultures.

Six experiments with SK-SD SM were performed and the results were similar in each. As shown in Fig. 6, the SM monolayer from a DT-negative individual

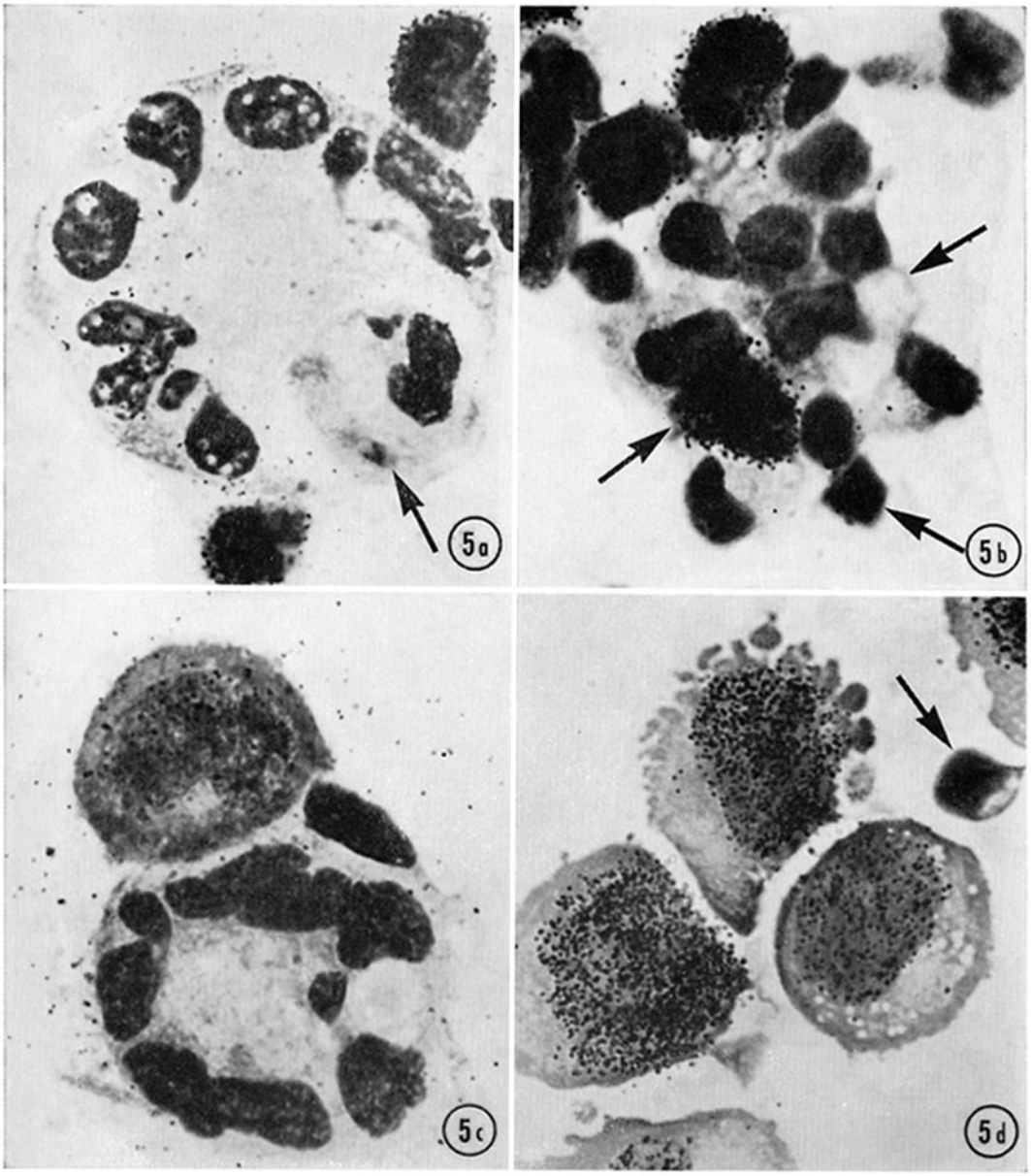

FIG. 5. Morphologic changes observed in macrophage monolayers cultured in the presence of sensitized lymphocytes and antigen. (*a*) 1 h after zero time. Multinucleated giant cell which has phagocytized a macrophage containing a single Toxoplasma (arrow). The Toxoplasma is not labeled by [^{3}H]UdR and appears to be undergoing dissolution. (*b*) Monolayer labeled with [^{3}H]TdR. Note the clustering of macrophages (arrow, upper right), transformed lymphocytes incorporating [^{3}H]TdR (arrow, lower left), and nontransformed unlabeled lymphocytes (arrow, lower right) into an "immunologic island" (20). (*c*) Multinucleated giant cell and lymphoblast which has incorporated [^{3}H]TdR, demonstrating characteristic juxtaposition of lymphocytes and macrophages in antigen stimulated monolayers. (*d*) Autoradiography of a cytocentrifuge preparation of supernatant cells from a macrophage monolayer which had been incubated for 6 days in the presence of sensitized lymphocytes and antigen. Just before infection of the monolayer, the supernatant cells were removed and incubated for 20 h with [^{3}H]TdR. Note lymphoblasts which have incorporated [^{3}H]TdR in comparison to the nontransformed lymphocyte (arrow). × 1,200.

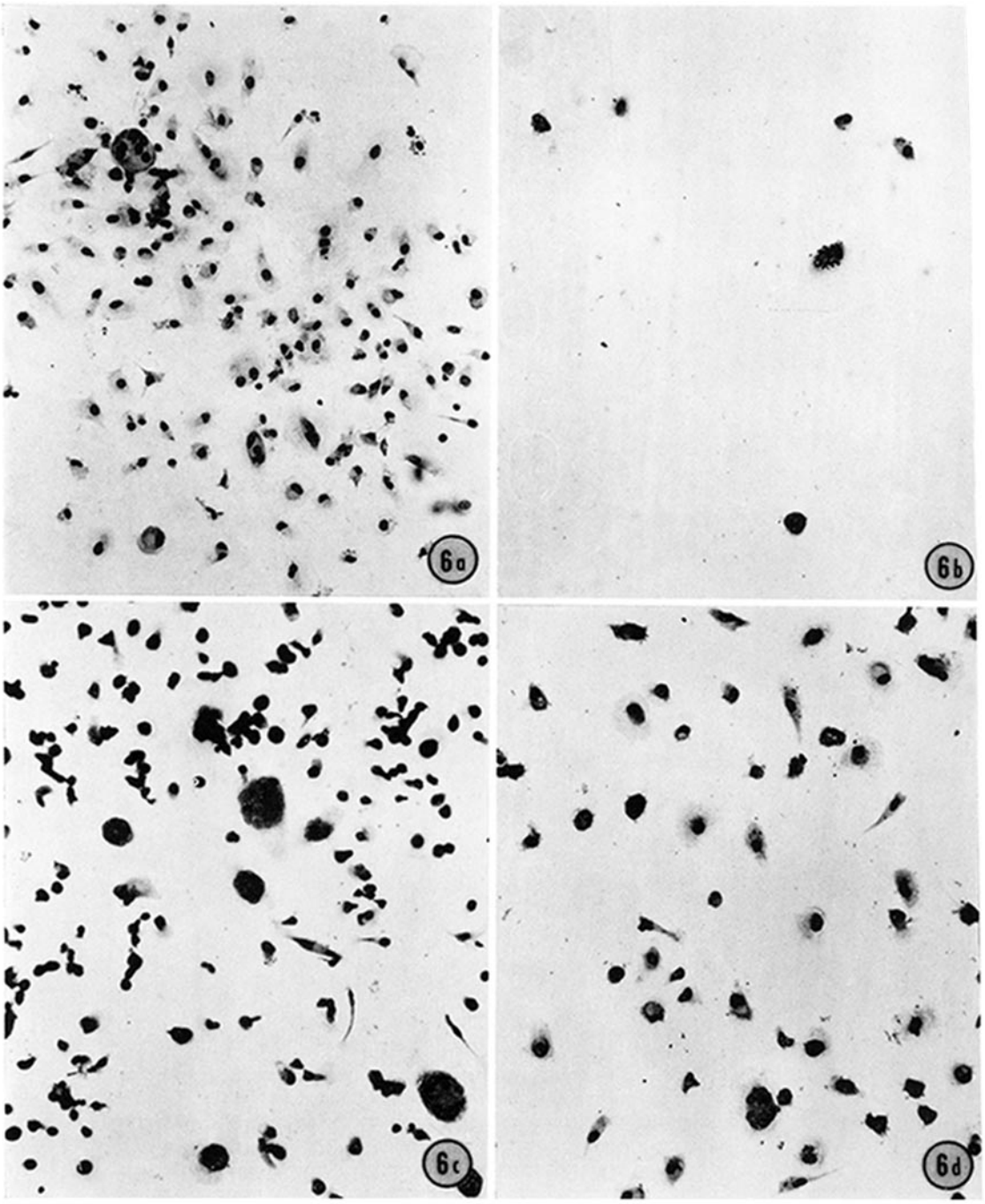

FIG. 6. Resistance of monolayers of SK-SD stimulated and control human monocyte-derived macrophages to infection with Toxoplasma. (*a*) Control macrophages 1 h after zero time. (*b*) Control macrophages 70 h after zero time. There is almost complete destruction of the monolayer. (*c*) Stimulated macrophages 1 h after zero time. (*d*) Stimulated macrophages 70 h after zero time. There is remarkable sparing of the monolayer. × 120.

showed marked resistance to necrotization by Toxoplasma whereas the CM monolayer was almost completely destroyed by 70 h after parasite challenge. This difference in resistance was demonstrable as early as 20 h after zero time.

A representative example of the quantitative results of these experiments is shown in Fig. 7. By 20 h, the number of viable intracellular Toxoplasma in CM had increased rapidly; in marked contrast, the number of viable organisms in SM had declined (Fig. 7 *a*, *d*). After 20 h, there was evidence of multiplication of some intracellular Toxoplasma in SM. In SM, at 8 and 20 h (Fig. 7 *b*), the great majority (>85%) of organisms had <5 grains (most were unlabeled) and were only identifiable as poorly stained fragments inside a cytoplasmic vacuole (Fig. 8 *a*). During the same time interval, >95% of organisms in CM incorporated ≥5 grains (Fig. 7 *b*), had normal morphology and staining characteristics, and most had multiplied (Fig. 8 *b*). The percent of CM infected with viable Toxoplasma remained constant until 40 h and then increased rapidly, suggesting that there was disruption of parasitized cells followed by invasion of uninfected macrophages and this was confirmed by microscopic observation. The percent of SM infected with viable Toxoplasma decreased over the first 20 h and then increased gradually (Fig. 7 *c*).

The mean percent of cells infected with Toxoplasma and mean number of Toxoplasma per infected cell were generally greater in CM than SM monolayers. At 1 h, a mean of 56% (range 24–85%) of CM were infected with Toxoplasma (labeled or unlabeled) with a mean of 3.0 (range 1.7–5.3) organisms per infected cell; the corresponding figures for SM monolayers were 44% (range 32–78%) and 2.3 (range 1.7–4.0). (These differences between CM and SM were not statistically significant [$P > 0.2$, Student's t test]). The differences observed could not be attributed to any difference in the number of viable organisms in the challenge inoculum for CM and SM since both were infected simultaneously with the same Toxoplasma preparation. The differences were most likely the result of the inhibition of [^{3}H]UdR uptake by Toxoplasma and the more rapid dissolution of the organisms within SM during the first hour after initial infection, as was also observed in the mouse model (17). The clustering of cells noted in SM monolayers may also have been a contributing factor.

Three experiments were performed in which macrophages from DT-positive individuals were incubated with autologous lymphocytes and TLA. Results were similar to those observed in monolayers of SK-SD SM. However, when macrophages from DT-negative individuals were incubated with autologous lymphocytes and TLA these macrophages were similar to CM in their effect on Toxoplasma (i.e., there was no evidence of monolayer resistance to nor macrophage killing of Toxoplasma).

DISCUSSION

The results described above demonstrate that normal human monocyte-derived macrophages cultured in vitro are incapable of significant killing or

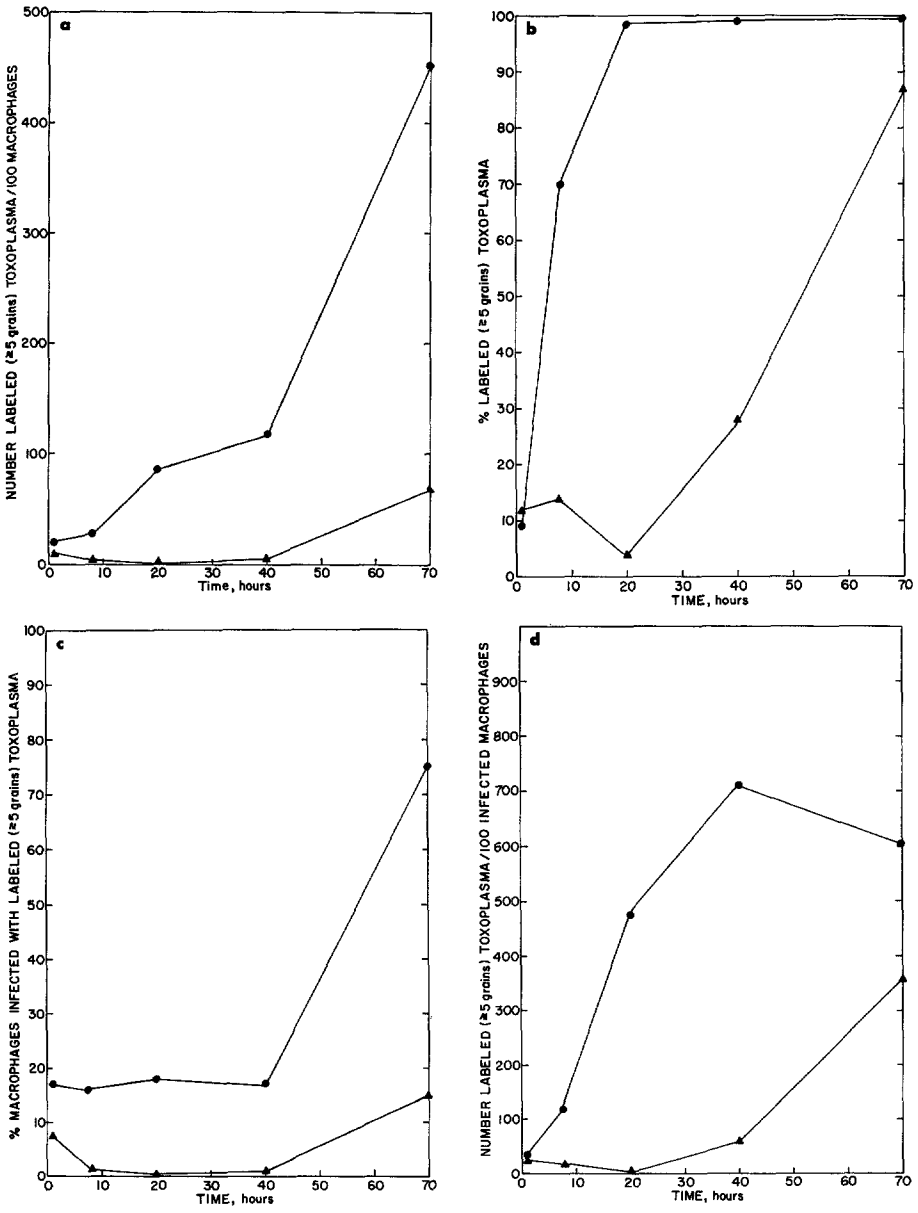

FIG. 7. Effect of SK-SD stimulated (▲) and control (●) human monocyte-derived macrophages on Toxoplasma as determined by autoradiography. (*a*) Total number of viable intracellular Toxoplasma per 100 macrophages. (*b*) Percent of intracellular Toxoplasma which were viable. (*c*) Percent of macrophages infected with viable Toxoplasma. (*d*) Number of viable intracellular Toxoplasma per 100 infected macrophages. The decrease in number of viable intracellular Toxoplasma in CM between 40 h and 70 h represents the lysis of macrophages by the parasites and subsequent invasion of uninfected macrophages.

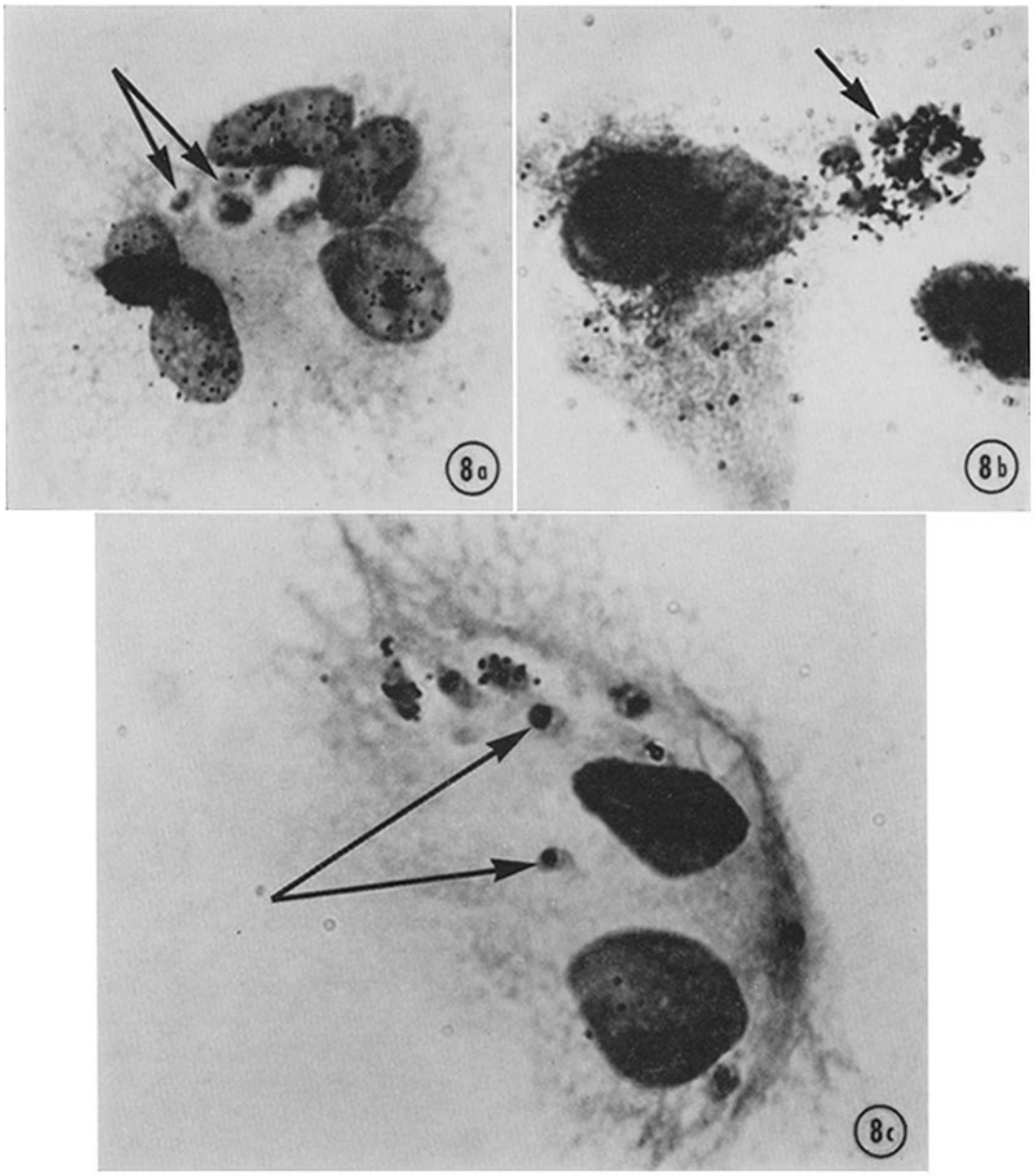

FIG. 8. Observations in monolayers of SK-SD stimulated and control human macrophages from a single experiment. (*a*) 8 h after zero time. Macrophage in stimulated monolayer. Toxoplasma in vacuoles (arrows) appear fragmented and are undergoing dissolution. × 1,200. (*b*) 20 h after zero time. Macrophage in control monolayer. Toxoplasma have formed a rosette (arrow) and have incorporated [^{3}H]UdR. (*c*) 1 h after zero time. Radiolabeled and nonradiolabeled Toxoplasma in a single nonactivated macrophage. The nonlabeled organisms (arrows) are altered in morphology and staining characteristics, indicative of early dissolution, compared to labeled organisms. × 1,500.

inhibition of replication of Toxoplasma. While normal human monocyte-derived macrophages were apparently incapable of killing Toxoplasma, they were capable of the intracellular digestion of attenuated or dead organisms. These results in human macrophages are similar to our previous observation of the survival and multiplication of Toxoplasma in peritoneal macrophages of normal mice (17). Jones and Hirsch (29), utilizing data derived from electron microscopy studies, concluded that the inability of the peritoneal macrophages of normal mice to kill Toxoplasma was linked to the lack of fusion of lysosomal granules with phagosomes containing viable Toxoplasma. They considered that this was due to some property of the parasite rather than of the macrophage, since single macrophages were noted in which dead Toxoplasma undergoing digestion and viable organisms existed side by side in separate vacuoles. Similarly, we frequently observed human macrophages in which Toxoplasma that had incorporated [^{3}H]UdR and nonlabeled fragmented Toxoplasma apparently undergoing digestion were present in the same cell (Fig. 8 *c*).

In the present study, macrophages from Toxoplasma-infected individuals did not differ from macrophages from noninfected individuals in their effect on Toxoplasma. In contrast, peritoneal macrophages from Toxoplasma-infected mice inhibit or kill Toxoplasma whereas macrophages from normal mice support the growth of the organism (17). While our in vitro results did not reveal a difference between macrophages from normal individuals and those from humans with toxoplasmosis, this may not necessarily reflect the situation as it exists in vivo. The macrophages employed in the present study were not obtained directly from the host, as was true for the mouse model, but were derived from monocytes by long-term culture in vitro.

In 1965, Stadtsbaeder (3) described results of studies of the effect of normal human leukocytes cultured in vitro on Toxoplasma and concluded that human monocytes support the intracellular multiplication of Toxoplasma. Although it is unclear from his publication whether the observations were made on early or late monocyte cultures, it appears that Stadtsbaeder's studies were with monocyte-derived macrophages and that his findings were similar to ours. Abell and Holland (30) described viable Toxoplasma trophozoites in the cytoplasm of mononuclear cells in the bone marrow of a child with acute toxoplasmosis, acquired during a drug-induced remission of acute lymphoblastic leukemia. Although this patient's mononuclear cells may have been defective in microbicidal capacity due to the underlying disorder, the chemotherapeutic agent used, or both, this case may represent an in vivo correlate of the in vitro findings of Stadtsbaeder and the present study.

Although relatively little is known about the microbicidal capacities of human monocyte-derived macrophages against facultative or obligate intracellular organisms, some data are available on the ability of these cells to handle *Listeria monocytogenes*, *Mycobacterium leprae*, *Cryptococcus neoformans*, and *Rickettsia mooseri*. Human monocyte-derived macrophages cultured in vitro

from normal subjects have been reported to be capable of killing *L. monocytogenes* (4) but not *M. leprae* (10), *C. neoformans* (8), and *R. mooseri* (11), which survive and multiply within these cells. Diamond and Bennett (8) suggested that macrophages might contribute to the extension and dissemination of cryptococci by providing an intracellular shelter for the organism with a favorable environment for growth. Such a means of dissemination may also be postulated for Toxoplasma since it has the ability to survive within the normal human macrophage.

Lycke and his colleagues have shown that, even in the absence of accessory factor, antibody will have an effect on Toxoplasma in a tissue culture system in vitro (31). To gain information on the effect of macrophages per se, we therefore considered it necessary to perform studies in the absence of conventional humoral antibody. Once Toxoplasma were intracellular, addition to the medium of antibody alone or antibody plus accessory factor had no demonstrable effect on the interaction of macrophages and Toxoplasma. However, exposure of Toxoplasma to antibody plus accessory factor before entry into macrophages resulted in rapid extracellular killing of the organisms (less than 0.1% of extracellular Toxoplasma which had been preincubated at 37°C for 30 min with antibody and accessory factor were viable) and any additional effect macrophages may have had under these conditions could not be evaluated. Of interest was the observation that although no difference in viability could be distinguished between extracellular Toxoplasma which had been treated with heat-inactivated antiserum or with serum which contained no antibody, the former were inhibited or killed by normal human macrophages. This observation is similar to that observed with *R. mooseri* which, when treated with human antiserum, were rendered susceptible to destruction within nonimmune human monocyte-derived macrophages in vitro (12). The fact that not all intracellular Toxoplasma which had been treated with heat-inactivated antiserum were killed by macrophages might be explained by either a heterogenous population of infected macrophages (e.g., a subpopulation of macrophages incapable of inhibiting or killing Toxoplasma), or the method used for preincubation of Toxoplasma with antiserum may not have resulted in adequate "preparation" for killing of all the organisms. The latter explanation appears most likely since even the addition of accessory factor did not kill 100% of extracellular organisms with the preincubation method employed. In separate studies, we have observed that monolayers of normal human peripheral blood monocytes cultured for 2 h are rapidly destroyed by Toxoplasma. However, pretreatment of Toxoplasma with heat-inactivated antiserum markedly inhibited the cytocidal effect of the organisms, evidently due to inhibition or killing of antibody-coated Toxoplasma by the monocytes (Anderson and Remington, unpublished data). Whether cell-bound antibody may play a role in ingestion or killing, or both, by macrophages from Toxoplasma-infected individuals, was not answered. If cell-bound antibody to Toxoplasma were

present on the monocytes in vivo, it is unlikely we would have demonstrated any functional activity it may confer because of the prolonged time these cells were cultured in vitro before challenge.

Since activated mouse peritoneal macrophages can inhibit or kill Toxoplasma (15–17) and since animal macrophages can be activated in vitro by sensitized lymphocytes cultured in the presence of specific antigen to kill intracellular organisms (32–36), we attempted to activate human macrophages in vitro by culturing them in the presence of sensitized lymphocytes and specific antigen. The activation by lymphocytes was shown to be antigen-specific and conferred upon normal human macrophages the capacity to destroy intracellular Toxoplasma. Killing, in contrast to mere inhibition of the organism, was demonstrated by the fact that viable organisms identified intracellularly at 1 h after zero time either disappeared or could be identified only as fragments within a few hours thereafter. In addition to the autoradiographs, examination of duplicate macrophage monolayers (taken at identical time intervals from the same experiment) by phase microscopy revealed a significant decrease in the number of live organisms during the first 4–20 h after zero time in macrophages which had been cultured in the presence of lymphocytes plus specific antigen but could not be demonstrated in macrophages which had been cultured in the presence of lymphocytes without antigen.

Of interest is the observation that monolayers of SM which showed remarkable resistance to necrotization by Toxoplasma and which inhibited the organism's replication and ultimately killed it, appeared to lose this resistance by 40 h of infection. By that time, an increased percentage of cells could be seen to be infected with viable organisms that were actively multiplying. This loss of resistance was also observed in the Listeria model (15) in which monolayers of activated macrophages which were initially remarkably protected against destruction by Listeria were ultimately destroyed due to overwhelming reinvasion by organisms released from destroyed cells. A possible explanation is that macrophages lose their "activation" during the course of the 70-h time period examined. Despite the increase in the number of SM infected with viable Toxoplasma observed after 40 h, allowing incubation to continue for an additional 30 h did not allow for replication and reinvasion comparable to that observed in CM. Another possible explanation is that not all macrophages in SM monolayers were activated. As is true for other models in which monolayers of activated macrophages have been compared with monolayers of normal macrophages, it is unclear as to what percent of the macrophages were actually activated. In the present study, the percent of macrophages that were activated can be approximated from the percent of SM infected with viable Toxoplasma at 1 h compared with the percent at 8 h; the data from six separate experiments revealed that 70–80% of the SM infected with viable Toxoplasma were activated to kill Toxoplasma (i.e., 7.5% of SM were infected with viable organisms at 1 h and only 1.5% had viable organisms at 8 h, suggesting that 80% of infected SM were activated). Although by our method of evaluation the

number of viable Toxoplasma in SM appears to decline during the initial 20 h of infection, these results actually reflect a summation of destruction of organisms by 70–80% of the infected cells and replication of organisms in approximately 20% of the infected cells. The effect of the latter is not clearly seen in our graphed data since they account for so few of the total.

Interestingly, Cline noted that monocyte-derived macrophages from subjects with active tuberculosis, leprosy, or Hodgkin's disease had an enhanced capacity to kill *L. monocytogenes* when compared with monocyte-derived macrophages from normal subjects (4). Despite the enhanced microbicidal capacity of macrophages from patients with active leprosy for *L. monocytogenes*, it appears that *M. leprae* is able to survive and multiply within monocyte-derived macrophages from patients with lepromatous or tuberculoid leprosy (6, 10). Godal et al. (37) demonstrated that human monocyte-derived macrophages from patients with tuberculoid leprosy, but not from patients with lepromatous leprosy, were stimulated to undergo morphologic changes and proliferation by exposure to specific antigen (killed *M. leprae*) in the presence of sensitized lymphocytes. However, the microbicidal capacity of these cells for *M. leprae* was not tested. Diamond and Bennett (8) found no evidence of intracellular killing of *C. neoformans* by monocyte-derived macrophages of subjects with active cryptococcosis. They also noted that normal human monocyte-derived macrophages which had been activated in vitro by culturing them in the presence of autologous lymphocytes and SK-SD, cryptococcin or viable cryptococci, did not acquire a capacity to kill *C. neoformans* despite the fact that they had an increased capacity to kill *L. monocytogenes*. Magliulo et al. (9) stated that human macrophages obtained from subjects convalescing from tuberculosis could be activated in vitro by exposure to sensitized lymphocytes and PPD. Such macrophages had increased phagocytic and bactericidal activity for Paracolonbacter compared to macrophages obtained from normal subjects who did not react to tuberculin skin testing. The cells used in their study were obtained directly by a modification of Rebuck's skin-window technique. Whether these authors were indeed studying macrophages rather than monocytes is unclear from their report. From the results obtained in each of the studies described above, it appears that both normal and activated human monocyte-derived macrophages vary considerably in their ability to handle different microorganisms.

Although the relationship of the in vitro phenomena we and others have observed to the actual function of the human macrophage in vivo remains to be elucidated, further efforts are warranted to attempt to define means whereby macrophages might be activated in vivo to kill or inhibit opportunistic pathogens.

SUMMARY

Human macrophages derived from in vitro culture of peripheral blood monocytes were studied under a variety of conditions to determine their microbicidal

capacity for the obligate intracellular protozoan, *Toxoplasma gondii*. The effect of macrophages on intracellular Toxoplasma was evaluated morphologically by light and phase microscopy and by autoradiography. When macrophages from dye test (DT)-negative or DT-positive individuals were infected with Toxoplasma in the presence of normal human serum, the organisms were able to multiply intracellularly with resultant destruction of the monolayer. Once organisms were intracellular, the presence of antibody-containing serum in the medium did not alter this inability of the macrophages to kill Toxoplasma. However, when Toxoplasma were incubated in the presence of heat-inactivated DT-positive serum just before infection of the monolayers, the intracellular organisms were inhibited or killed by normal macrophages. Attempts were made to activate macrophages in vitro to kill Toxoplasma. Macrophages incubated in the presence of sensitized lymphocytes and Streptokinase-Streptodornase (SK-SD) or Toxoplasma lysate antigen (TLA) were found to kill Toxoplasma when compared to macrophages incubated in the presence of lymphocytes from DT-negative individuals and TLA or lymphocytes alone. Thus, in vitro induction of resistance (both specifically and nonspecifically) in human macrophages was accomplished by culturing these cells in the presence of specifically sensitized lymphocytes and antigen. These results suggest that, as in the mouse model, activated human macrophages have the ability to inhibit or kill intracellular Toxoplasma and that these cells may be important as effector cells in cell-mediated immunity (CMI) to toxoplasmosis in man.

Simeon C. Bautista provided valuable technical assistance.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

REFERENCES

1. Cell-mediated immunity and resistance to infection. 1973. *WHO Tech. Rep. Ser.*, No. 519.
2. van Furth, R., Z. A. Cohn, J. G. Hirsch, J. H. Humphrey, W. G. Spector, and H. L. Langevoort. 1972. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bull. WHO* **46**:845.
3. Stadtsbaeder, S. 1965. Multiplication des toxoplasmes dans les globules blancs humains mis en culture. *Rev. Belg. Path. Med. Exp.* **31**:280.
4. Cline, M. J. 1970. Bactericidal activity of human macrophages: analysis of factors influencing the killing of *Listeria monocytogenes*. *Infect. Immunol.* **2**:156.
5. Cline, M. J. 1970. Drug potentiation of macrophage function. *Infect. Immunol.* **2**:601.
6. Drutz, D. J., and M. J. Cline. 1972. Incorporation of tritiated thymidine by leprosy bacilli in cultures of human lepromatous macrophages. *J. Infect. Dis.* **125**:416.
7. Drutz, D., and M. J. Cline. 1972. A technique for assessing the bactericidal capability of individual human macrophages. *Clin. Res.* **20**:527 (Abstr.).

8. Diamond, R. D., and J. E. Bennett. 1973. Growth of *Cryptococcus neoformans* within human macrophages *in vitro*. *Infect. Immunol.* **7**:231.
9. Magliulo, E., V. DeFeo, A. Stirpe, C. Riva, and D. Scevola. 1973. Enhanced *in vitro* phagocytic power of macrophages from PPD-stimulated skin sites in human subjects hypersensitive to PPD. *Clin. Exp. Immunol.* **14**:371.
10. Samuel, D. R., T. Godal, B. Myrvang, and Y. K. Song. 1973. Behavior of *Mycobacterium leprae* in human macrophages *in vitro*. *Infect. Immunol.* **8**:446.
11. Gambrill, M. R., and C. L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections. II. Multiplication of typhus rickettsiae in human macrophage cell cultures in the nonimmune system: influence of virulence of rickettsial strains and of chloramphenicol. *Infect. Immunol.* **8**:519.
12. Gambrill, M. R., and C. L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections. III. Influence of immune serum and complement on the fate of *Rickettsia mooseri* within human macrophages. *Infect. Immunol.* **8**:631.
13. Vischer, W. A., and E. Suter. 1954. Intracellular multiplication of *Toxoplasma gondii* in adult mammalian macrophages cultivated *in vitro*. *Proc. Soc. Exp. Biol. Med.* **86**:413.
14. Frenkel, J. K. 1967. Adoptive immunity to intracellular infection. *J. Immunol.* **98**:1309.
15. Ruskin, J., and J. S. Remington. 1969. Role for the macrophage in acquired immunity to phylogenetically unrelated intracellular organisms. *Antimicrob. Agents. Chemother.* 474.
16. Ruskin, J., J. McIntosh, and J. S. Remington. 1969. Studies on the mechanisms of resistance to phylogenetically diverse intracellular organisms. *J. Immunol* **103**:252.
17. Remington, J. S., J. L. Krahenbuhl, and J. W. Mendenhall. 1972. A role for activated macrophages in resistance to infection with Toxoplasma. *Infect. Immunol.* **6**:829.
18. Frenkel, J. K., and L. Jacobs. 1958. Ocular toxoplasmosis. *Arch. Ophthalmol.* **59**:260.
19. Steigbigel, R. T., L. H. Lambert, Jr., and J. S. Remington. 1974. Phagocytic and bactericidal properties of normal human monocytes. *J. Clin. Invest.* **53**:131.
20. Hanifin, J. M., and M. J. Cline. 1970. Human monocytes and macrophages: Interaction with antigen and lymphocytes. *J. Cell Biol.* **46**:97.
21. Remington, J. S., M. M. Bloomfield, E. Russell, Jr., and W. S. Robinson. 1970. The RNA of *Toxoplasma gondii*. *Proc. Soc. Exp. Biol. Med.* **133**:623.
22. Remington, J. S., T. Yagura, and W. S. Robinson. 1970. The effect of rifampin on *Toxoplasma gondii*. *Proc. Soc. Exp. Biol. Med.* **135**:167.
23. Sabin, A. B., and H. A. Feldman. 1948. Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoon parasite (Toxoplasma). *Science* (*Wash. D. C.*). **108**:660.
24. Feldman, H. A. 1956. The relationship of Toxoplasma antibody activator to the serum-properdin system. *Ann. N. Y. Acad. Sci.* **66**:263.
25. Gaines, J. D., M. A. Gilmer, and J. S. Remington. 1973. Deficiency of lymphocyte antigen recognition in Hodgkin's disease. *Natl. Cancer Inst. Monogr.* **36**:117.
26. Krahenbuhl, J. L., J. D. Gaines, and J. S. Remington. 1972. Lymphocyte transformation in human toxoplasmosis. *J. Infect. Dis.* **125**:283.

27. Ferraresi, R. W., A. Mazzolli, and E. L. Suarez. 1968. Effects of antigen on *in vitro* cultures of sensitized peritoneal cells. *Experientia (Basel).* **24:**68.
28. Jacobs, L., F. E. Jones, and M. L. Melton. 1952. The survival of *Toxoplasma gondii* in various suspending media. *J. Parasitol.* **38:**293.
29. Jones, T. C., and J. G. Hirsch. 1972. The interaction between Toxoplasma gondii and mammalian cells. II. The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *J. Exp. Med.* **136:**1173.
30. Abell, C., and P. Holland. 1969. Acute toxoplasmosis complicating leukemia. Diagnosis by bone marrow aspiration. *Am. J. Dis. Child.* **118:**782.
31. Lycke, E., E. Lund, Ö. Strannegård, and E. Falsen. 1965. The effect of immune serum and activator on the infectivity of *Toxoplasma gondii* for cell culture. *Acta Pathol. Microbiol. Scand. Suppl.* **63:**206.
32. Patterson, R. J., and G. P. Youmans. 1970. Demonstration in tissue culture of lymphocyte mediated immunity to tuberculosis. *Infect. Immunol.* **1:**600.
33. Krahenbuhl, J. L., and J. S. Remington. 1971. *In vitro* induction of nonspecific resistance in macrophages by specifically sensitized lymphocytes. *Infect. Immunol.* **4:**337.
34. Simon, H. B., and J. N. Sheagren. 1972. Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. *Cell. Immunol.* **4:** 163.
35. Krahenbuhl, J. L., L. T. Rosenberg, and J. S. Remington. 1973. The role of thymus-derived lymphocytes in the *in vitro* activation of macrophages to kill *Listeria monocytogenes. J. Immunol.* **111:**992.
36. Fowles, R. E., I. M. Fajardo, J. L. Leibowitch, and J. R. David. 1973. The enhancement of macrophage bacteriostasis by products of activated lymphocytes. *J. Exp. Med.* **138:**952.
37. Godal, T., R. J. W. Rees, and J. O. Lamvik. 1971. Lymphocyte-mediated modification of blood-derived macrophage function *in vitro*; inhibition of growth of intracellular mycobacteria with lymphokines. *Clin. Exp. Immunol.* **8:**625.