STUDIES ON THE MECHANISMS OF MACROPHAGE ACTIVATION

I. Destruction of Intracellular *Leishmania enriettii* in Macrophages Activated by Cocultivation with Stimulated Lymphocytes*

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When interacting with sensitized lymphocytes stimulated by antigen, macrophages undergo a number of metabolic and functional changes, generally referred to as "activation". One distinct feature of activated macrophages is their enhanced capacity to restrict the growth of, or destroy ingested microorganisms (2, 3). In particular, it has recently been shown that under certain conditions, activated macrophages may display considerable cytotoxic activity in vitro towards certain protozoan parasites (4–7).

*Leishmania* parasites are obligate intracellular protozoa which may infect various species of vertebrate hosts, and display a predilection for cells of the mononuclear phagocyte system (8). Preliminary experiments from this laboratory indicated that *Leishmania enriettii*, a parasite of the guinea pig, was readily endocytosed by mouse peritoneal macrophages in vitro, in which it would reside essentially unharmed unless an activating stimulus induced the cells to destroy the micro-organism (4, 9, 10). Killing of *L. enriettii* in mouse macrophages thus constitutes a convenient model system with which to study the mechanisms of activation. The present report describes the effect of cocultivation with lymphocytes stimulated by allogeneic cells, and by the T-cell mitogen concanavalin A (Con A), on the capacity of mouse macrophages to destroy ingested *L. enriettii*. Results of studies on the effects of soluble factors released by such stimulated lymphocytes will be presented in separate articles.

Materials and Methods

Chemicals, Reagents, and Media. Chemicals were purchased as follows: α-methyl-mannoside (α-MM) from Sigma Chemical Co., St Louis, Mo.; Con A from Pharmacia, Uppsala, Sweden.

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1 As pointed out by Allison and Davies (1), the indiscriminate use of this term has led to semantic confusion. In this report, the meaning of the term "activation" will be restricted to that of an enhanced capacity to destroy microorganisms.

2 *Abbreviations used in this paper: α-MM, α-methyl-mannoside; α-MTLA, anti-mouse thymus lymphocyte antigen; Con A, concanavalin A; DS, Dulbecco's medium supplemented with 10% fetal calf serum; MLC, mixed lymphocyte culture; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate.*
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Anti-mouse thymus lymphocyte antigen (α-MTLA) antiserum was a gift from Professor C. Bron, Institute of Biochemistry, Switzerland. This heterologous antiserum is specific for mouse thymus-derived lymphocytes and has been fully characterized (11). Media and sera came from Gibco Bio-Cult Ltd., Paisley, Scotland, and supplementary amino acids and vitamins from Fluka A. G., Buchs, Switzerland. Triple distilled, pyrogen-free water was used for all solutions.

**Culture Conditions.** Dulbecco's medium supplemented with 10% fetal calf serum (DS) was used for the culture of peritoneal macrophages; media for lymphocyte cultures were supplemented (enriched DS) with L-arginine (final concentration: 200 mg/liter), L-asparagine (36 mg/liter), folic acid (10 mg/liter), and L-glutamine (800 mg/liter). Cultures were performed in 35-mm Falcon plastic dishes containing 12 mm diameter round glass cover slips, or in 16 × 60 mm flat-bottom glass tubes, and maintained at 37°C in an atmosphere of 5% CO2 in air.

**Macrophage Cultures.** 2- to 5-mo-old male C57BL/6 mice were injected intraperitoneally with 3 ml of a sterile suspension of 2% starch in phosphate-buffered saline (PBS) prepared with pyrogen-free water. The exudate cells were collected 3 days later in Dulbecco's medium containing 0.5 U/ml of heparin, washed once by centrifugation, and resuspended in DS to a concentration of 1 × 10⁶ nucleated cells/ml. 2-ml vol of the suspension were distributed in 35-mm dishes containing four round glass cover slips, and/or 0.2 ml in flat-bottom tubes. The vessels were incubated at 37°C for 2-4 h before adding parasites.

**Infection of Macrophage Cultures.** *L. enrietti* parasites were prepared as described elsewhere (12), and suspended in DS to a final concentration of 7-14 × 10⁶ amastigotes/ml. 1-ml vol were added to macrophages in Petri dishes, and/or 0.1 in tubes, thus providing a ratio of three and one-half to seven parasites per peritoneal cell in the incubation mixture. The infected cultures were then incubated for 24 h at 37°C to allow phagocytosis of the added parasites.

**Activation of Macrophages.** Lymphocyte suspensions were prepared by homogenization of spleen fragments from appropriate mouse strains in loose-fitting Ten-Broeck glass grinders, followed by centrifugation at 300 g for 5 min. The pellet was suspended in DS and cell clumps and debris were allowed to settle at unit gravity for 15 min in the cold. The supernatant cells were then centrifuged as above and resuspended in enriched DS at appropriate concentrations. 24 h after infection with *L. enrietti*, macrophage cultures were carefully washed free from peritoneal lymphocytes and nonphagocytosed parasites. Spleen cells (either syngeneic with the macrophages, or in the form of mixed cultures), as well as reagents, were then added to the infected macrophages as follows. For mixed cultures, increasing numbers (usually 1.25-20 × 10⁶ cells/35 mm dish) of C57BL/6 lymphocytes, and constant numbers (usually 10 × 10⁶/dish) of irradiated (1,000 rads) allogeneic (DBA/2) or control (C57BL/6) cells were added to the cultures in a final vol of 4 ml of enriched medium per dish. When experiments were done in tubes, 1/16th vol and cell numbers were used. Activation induced by mitogens was obtained in 3-ml (dishes) or 0.3-ml (tubes) vol, using the number of syngeneic lymphocytes and the concentrations of mitogens specified in the text. Controls consisted of infected macrophages incubated with identical numbers of lymphocytes in absence of mitogens, or with mitogens in the absence of spleen cells.

**Measurement of Parasite and Macrophage Destruction.** Killing of intracellular *L. enrietti* in activated cells was followed in two ways: (a) by microscopic examination: cover slip cultures were fixed in 2% glutaraldehyde in PBS and stained with May-Grünwald-Giemsa. The number of parasites in 100 macrophages was then determined under × 400 magnification by counting microorganisms in 300-400 cells in five fields chosen at random on each cover slip. Counts of macrophages were performed in parallel to assess the toxicity of stimulated lymphocytes for these cells. Dead macrophages could easily be distinguished after staining, on the basis of their pyknotic nucleus and their pale, acidophil, disintegrating cytoplasm. (b) To measure more precisely parasite killing in activated macrophages, infected cells were lysed with sodium dodecyl sulphate (SDS), following a technique described in detail elsewhere (13). Briefly, tube cultures were washed to remove nonadherent cells; 0.5 ml of a 0.05% solution of SDS in Dulbecco's medium supplemented with 7.5% fetal calf serum was added to each tube followed by stirring for 60 s on Vortex mixer. This procedure has been shown to provoke the rupture of macrophages and to allow the release of unharmed intracellular leishmaniae. 2 ml of DS medium was then added and the tubes centrifuged at 1,500 g for 10 min. 2 ml of supernate was removed with an automatic pipette fitted with a sterile tip, and replaced by 2 ml of parasite growth medium (12). After 3 days of growth at room temperature, live motile forms of the parasite (promastigotes) were immobilized with formalin and counted in a hemocytometer. Control
experiments (13) indicated that the number of promastigotes counted after 3 days of growth was directly proportional to the number of living intracellular amastigotes originally present inside macrophages in the infected culture.

Standard deviations were calculated from the results of three to five determinations; significance was computed by using Student's t test.

Measurement of DNA Synthesis. To measure the stimulation of DNA synthesis induced by mitogens, 0.5 × 10⁶ spleen cells were cultured in flat-bottom tubes in a total vol of 0.3 ml of enriched DS medium containing appropriate concentrations of Con A. After 48 h of incubation at 37°C, 0.5 μCi of methyl-[3H]thymidine (0.1 ml) was distributed to each tube, and incubation was resumed. 24 h later, the cells were collected by suction on glass-fiber filters, washed twice with cold 5% trichloroacetic acid, and processed for scintillation counting.

Treatment with Anti-MTLA Antiserum. 30 million spleen cells were incubated in 1 ml of a 1:10 dilution of rabbit anti-mouse MTLA antiserum for 10 min at 37°C; rat complement was then added, and the suspensions were further incubated for 20 min. The number of viable cells was determined by dye exclusion after anti-MTLA antiserum treatment; it was found not to differ considerably from control preparations incubated without complement or with complement in the presence of normal rabbit serum. Cell concentrations in treated and control preparations were adjusted to the same value before addition to macrophage cultures.

Results

Phagocytosis of L. enriettii by Mouse Macrophages. Addition of 2.0 × 10⁶ starch-induced nucleated peritoneal cells to 35-mm Petri dishes resulted in the attachment of 1.12-1.46 × 10⁶ cells (five experiments), of which over 98% were phagocytic, as determined by their capacity to ingest L. enriettii (a parasite which does not actively penetrate cells). These cells will be hereafter referred to as macrophages.

24 h after infection of such cultures by addition of 7.0-14.0 × 10⁶ leishmania amastigotes, the number of intracellular micro-organisms determined on stained preparations was comprised of between 19 and 44% of the input (five experiments), resulting in an infection ratio of between one and four parasites per macrophage. The rest of the amastigotes could be recovered from the supernatant fluids of the infected cultures, suggesting that neither destruction nor multiplication occurred intra- or extracellularly during this period.

After washing to remove nonphagocytosed amastigotes, and in the absence of activating stimulus, intracellular parasite numbers remained essentially unchanged for 72-96 h, at which time the experiments were terminated (controls, Fig. 3). In experiments (not reported) where cultures of parasitized macrophages were maintained for longer periods, the number of intracellular micro-organisms was seen to decrease slowly with time; live intracellular amastigotes could nevertheless be observed for as long as 10 days after phagocytosis.

Macrophage Activation by Mixed Lymphocyte Cultures (MLCs)

Effect of increasing concentrations of responding spleen cells. Incubation of leishmania-infected macrophages in presence of MLCs resulted in rapid morphological disappearance of the intracellular micro-organisms (Fig. 1A-D). To determine the best conditions for this effect, tube cultures of infected C57BL/6 macrophages were exposed to mixtures consisting of increasing numbers of syngeneic spleen cells and constant numbers of irradiated allogeneic or syngeneic cells. Parasite destruction was assessed after 96 h of incubation by lysis of macrophages with SDS followed by culture of the released amastigotes.
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Fig. 1. Light microscope observations of parasite destruction in mouse macrophages activated by MLC. Giemsa stain. Linear magnification: × 750. 1A: L. enriettii-infected C57BL mouse peritoneal macrophages, before activation. Notice elongated parasites (P) in a clear vacuole. Inset: higher magnification of a parasitized macrophage, showing parasite nucleus (N) and kinetoplast (K). 1B: Same culture as in Fig. 1A, after 48 h of exposure to a MLC consisting of a mixture 5 × 10^6 C57BL spleen cells and 10 × 10^6 stimulator DBA/2 spleen cells per 35 mm Petri dish. Notice rounding of parasites (arrows), an early sign of macrophage activation in this system. 1C: Same culture as in Fig. 1B, after 96 h of exposure to MLC. All intracellular parasites have been killed and digested. 1D: Control culture of L. enriettii-infected macrophages, exposed to 5 × 10^6 C57BL spleen cells and 10 × 10^6 irradiated syngeneic (C57BL) spleen cells for 96 h. Essentially no damage has been done to the intracellular parasites.

As shown in Fig. 2, parasite destruction was seen to increase with increasing numbers of responding lymphocytes in the MLC, until complete disappearance of the micro-organisms from infected macrophages was observed in cultures exposed to the highest number of responding lymphocytes (Figs. 1C and 2). A slight decrease in parasite numbers could also be observed in control macrophages incubated with high ratios of syngeneic spleen cells (Fig. 2). However, this reduction was not significant.

Time-course of parasite killing in macrophages cocultivated with MLCs. When parasite destruction was followed microscopically, no detectable effects were observed for up to 24 h after addition of MLCs to infected macrophage cultures. Changes in parasite morphology became evident after 48–72 h (Fig. 1B); parasite destruction then progressed rapidly, until complete
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Fro. 2. Activation of macrophages by MLCs. Macrophage cultures originating from 2 x 10^6 C57BL peritoneal cells per tube were infected with L. enriettii, then activated by incubation with increasing numbers of syngeneic spleen cells exposed to a constant number (10^6) of irradiated DBA/2 cells (C57BL/14 DBA/2) or to 10^6 irradiated syngeneic cells in controls (C57BL/14 C57BL). The number of live intracellular parasites was determined in each preparation after 96 h of incubation, by using the SDS technique.

disappearance of the micro-organisms was observed after 96 h of incubation (Figs. 1 C and 3 A).

To determine more precisely the kinetics of parasite killing in macrophages activated by MLCs, the fate of intracellular leishmaniae was monitored by SDS lysis of infected cells followed by culture of the released amastigotes. As shown in Fig. 3 B, intracellular parasite killing occurred abruptly between 48 and 72 h after exposure of macrophages to MLCs, at a time when no reduction in parasite numbers could be observed in stained preparations (Fig. 3 A). This suggested that death of the micro-organisms was not followed by their immediate morphological disappearance from the macrophages.

In six experiments, the number of parasites in macrophages incubated for 96 h with MLCs consisting of 5 x 10^6 responding and 10 x 10^6 stimulator cells per 35 mm dish, was consistently found to be reduced to zero. After the same period, intracellular parasites in control preparations (macrophages incubated with identical numbers of syngeneic spleen cells) averaged 87-127% of the starting population. No evidence of toxicity of test or control lymphocyte preparations for macrophages could be observed microscopically at any time within this period.

Macrophage Activation by Con A-Stimulated Lymphocytes

EFFECT OF LECTIN CONCENTRATION ON MACROPHAGE ACTIVATION. Incubation of infected macrophages with syngeneic lymphocytes and Con A resulted in intracellular parasite destruction (Fig. 4). Maximum activation was observed by using 1-5 µg/ml of Con A in the incubation mixtures (Fig. 5 A). A marked decrease in parasite killing was observed at concentrations in excess of 10 µg/
FIG. 3. Time-course of parasite killing in macrophages activated by MLCs. Macrophage cultures originating from 2 × 10⁶ C57BL peritoneal cells per tube (respectively, 2 × 10⁶ per 35 mm dish) were infected with *L. enriettii*, then exposed to MLCs containing 5 × 10⁶ (respectively, 5 × 10⁶) syngeneic responding and 10⁶ (respectively, 10⁶) irradiated allogeneic DBA/2 stimulator spleen cells (●—●) or 10⁶ irradiated syngeneic cells (○—○) in controls. 3 A: Intracellular parasite numbers recorded microscopically on stained preparations (dishes). 3 B: Survival of intracellular parasites determined by SDS release from tube cultures.

ml. That this effect might be attributed to the toxicity of Con A for lymphocytes was suggested by the fact that both stimulation of DNA synthesis and cell survival were considerably depressed at the highest concentrations tested (Fig. 5 B).

In the absence of lymphocytes, incubation of infected macrophages with 5 μg/ml of Con A had no effect on the survival of intracellular parasites (Fig. 4 D and Fig. 8); nor were lymphocytes capable of inducing macrophages to destroy leishmaniae in the absence of Con A (Fig. 5 A).

To determine the lymphocyte concentrations best able to induce intracellular killing in the presence of Con A, parasitized macrophages were incubated with various numbers of syngeneic spleen cells and 5 μg/ml of lectin. As judged from SDS release of live parasites, optimum activation after 48 h was obtained in
FIG. 4. Light microscope observations of parasite destruction in mouse macrophages activated by Con A-stimulated spleen cells. Giemsa stain. Linear magnification: × 750. 4 A: *L. enriettii*-infected C57BL macrophages, before activation. 4 B: Same culture as in Fig. 4 A, after 72 h of exposure to 1.25 × 10⁶ syngeneic spleen cells per 35 mm Petri dish, in presence of 5 μg/ml of Con A. All parasites have been digested. 4 C: Same culture as in Fig. 4 A, after 72 h of exposure to 20 × 10⁶ spleen cells per 35 mm Petri dish, in presence of 5 μg/ml of Con A. Notice the presence of elongated parasites, indicating lack of activation, and the poor condition of some of the macrophages (arrows). 4 D: Same culture as in Fig. 4 A, after 72 h of incubation with 5 μg/ml of Con A, in the absence of added spleen cells. Con A alone does not activate macrophages in this system.

preparations containing 1.25–10.0 × 10⁶ lymphocytes per tube culture (Fig. 6). Interestingly, increasing spleen cell concentration up to 2 × 10⁶ per tube (respectively, 2 × 10⁷ per dish) resulted in a significant reduction of macrophage activation compared to maximum activation obtained with lower cell numbers (Table I, Figs. 6 and 4 C).

Macrophage activation by lymphocytes incubated with 5 μg/ml of Con A could be abolished almost completely by addition of 0.05 M α-MM to the culture media (Fig. 7). At this concentration, the glycoside displayed no toxicity towards either lymphocytes or macrophages.

Cytotoxicity of Con A-stimulated spleen cells for macrophages. As shown in Fig. 6, incubation with high numbers of Con A-stimulated spleen cells also resulted in a toxic effect for macrophages, demonstrable already after 48 h by the decreased number of living phagocytes attached to the coverslips. Interestingly, maximum toxicity for macrophages occurred at lymphocyte con-
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Fig. 5. Activation of macrophages by spleen cells stimulated by Con A. 5A: Macrophage cultures originating from $2 \times 10^5$ C57BL peritoneal cells per dish were infected with *L. enriettii*, then activated by incubation with $5 \times 10^6$ syngeneic spleen cells in presence of increasing concentrations of Con A. Parasite destruction was determined microscopically after 48 h of activation. Mean of five determinations, ± SD. 5B: Thymidine uptake and viability of spleen cell suspensions incubated with the same concentrations of Con A as in Fig. 5A. Mean of three determinations, ± SD.

centrations ($2 \times 10^6$/tube) for which parasite destruction was incomplete. Conversely, parasite killing was highest in cultures containing $1.25-5.0 \times 10^6$ stimulated lymphocytes, where no evidence of toxicity for macrophages could be seen for up to 72 h (Fig. 8). In these preparations, however, a reduction of the number of phagocytes was nevertheless observed as incubation was prolonged further, compared to macrophages in the starting population and to control cultures incubated with identical numbers of nonstimulated spleen cells (Table II).

**TIME-COURSE OF PARASITE DESTRUCTION IN MACROPHAGES EXPOSED TO CON A-STIMULATED LYMPHOCYTES.** Parasite killing in macrophages incubated with Con A-stimulated lymphocytes was monitored by SDS lysis of infected cells
TABLE I

Inhibitory Effect of High Ratios of Con A-Stimulated Lymphocytes on Macrophage Activation

| Con A-stimulated lymphocytes per culture (× 10^5) | Live intracellular parasites per culture at 48 h (10^-3 ± SD) | Exp. 1 | Exp. 2 | Exp. 3 |
|-------------------------------------------------|-------------------------------------------------------------|-------|-------|-------|
| 0                                               | 296.5 ± 79.0                                               | 1,060.0 ± 145.0 | 1,066.0 ± 82.0 |
| 1.25                                            | 226.5 ± 17.5                                               | 126.0 ± 40.0 | 36.6 ± 16.0 |
| 5.0                                             | 0                                                           | 13.3 ± 4.5 | 8.2 ± 6.2 |
| 10.0                                            | 16.5 ± 3.0                                                 | 70.0 ± 6.0 | 14.0 ± 22.0 |
| 20.0                                            | 75.0 ± 40.0                                                | 353.0 ± 76.0 | 412.0 ± 114.0 |

P 0.01 > P > 0.001 0.01 > P > 0.001 0.01 > P > 0.001

Macrophage cultures originating from 2 x 10^4 peritoneal cells were infected with *L. enrietti*, then activated by incubation with various numbers of syngeneic spleen cells in presence of 5 μg per ml of Con A. Parasite destruction was determined by using the SDS technique after 48 h of activation. Mean and SD calculated from the results of triplicate cultures. Values of P computed for differences between cultures exposed to 5 x 10^5 and to 20 x 10^4 spleen cells.

![Graph of Macrophage Activation](image)

**Fig. 6.** Macrophage activation by increasing concentrations of Con A-stimulated spleen cells. Macrophage cultures originating from 2 x 10^4 peritoneal cells per tube were infected with *L. enrietti*, then incubated with increasing numbers of syngeneic spleen cells in the presence of 5 μg/ml of Con A. Parasite destruction was determined by the SDS method after 48 h of incubation. The numbers of live macrophages were determined in each preparation by microscope counts of stained cultures incubated in parallel and under the same conditions.

after different periods of activation; a representative experiment is shown in Fig. 8. In three experiments, with 5 x 10^6 spleen cells per tube and 5 μg/ml of Con A, no decrease in parasite counts could be detected after 15 h; killing thereafter progressed rapidly, to reach completion within 30-40 h of activation. Within this period, no toxicity for macrophages could be detected on morphological examination of parallel cultures using identical macrophage/lymphocyte ratios (Fig. 8 and Table II).
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FIG. 7. Effect of α-MM on macrophage activation induced by Con A. Tube cultures of macrophages originating from 2 × 10⁵ peritoneal cells were infected with *L. enriettii,* then activated by incubation with 1–5 × 10⁵ syngeneic spleen cells in the presence of 5 μg/ml of Con A and of 50 mM α-MM. Parasite destruction was determined by the SDS method after 48 h of incubation.

### TABLE II

Cytotoxic Effect of Con A-Stimulated Lymphocytes for Macrophage Cultures

| Lymphocytes per tube (× 10⁵) | Con A 5 μg/ml | Macrophages (× 10⁴) per tube culture ± SD after |
|-----------------------------|--------------|---------------------------------------------|
|                             |              | 24 h | 48 h | 72 h | 96 h |
| —                          | —            | 1.41 ± 0.10 | 1.48 ± 0.10 | 1.55 ± 0.24 | 1.30 ± 0.15 |
| 10                         | —            | 1.43 ± 0.08 | 1.58 ± 0.18 | 1.54 ± 0.15 | 1.48 ± 0.16 |
| 1.25                       | +            | 1.30 ± 0.11 | 1.44 ± 0.13 | 1.31 ± 0.13* | 0.98 ± 0.23 |
| 5.0                        | +            | 1.28 ± 0.14 | 1.35 ± 0.06* | 1.19 ± 0.12 | 0.62 ± 0.13 |
| 10.0                       | +            | 1.24 ± 0.06 | 1.28 ± 0.13* | 1.24 ± 0.16 | 0.65 ± 0.22 |

Tube cultures originating from 2 × 10⁵ peritoneal cells were infected with *L. enriettii,* then activated by incubation with various numbers of syngeneic spleen lymphocytes in the presence of 5 μg/ml of Con A. The numbers of macrophages attached to the bottom of the vessels were determined by microscope counts of the stained preparations after increasing periods of incubation.

* Time of complete intracellular parasite destruction as determined under the microscope.

**INHIBITION OF ACTIVATION BY ANTI-THYMOCYTE ANTISERUM.** To show that macrophage activation by Con A-stimulated spleen cells was due to an effect of the lectin on thymus-derived lymphocytes, C57BL/6 spleen cells were treated with a rabbit antiserum specific for α-MTLA, in the presence or absence or rat complement, or with normal rabbit serum and complement as control. Lymphocyte concentrations in the various preparations were then adjusted to the same value, and incubated with parasitized macrophages in the presence of Con A. In two experiments, α-MTLA treatment in the presence of complement resulted in
Fig. 8. Time-course of parasite destruction in macrophages activated by Con A-stimulated spleen cells. Tube cultures of macrophages originating from $2 \times 10^5$ peritoneal cells were infected with \textit{L. enriettii}, then activated by incubation with $5 \times 10^5$ syngeneic spleen cells in the presence of 5 $\mu$g/ml of Con A. Surviving parasites were determined by the SDS method. --- numbers of intracellular parasites in macrophages exposed to Con-A stimulated spleen cells. — — —: numbers of live macrophages in parallel cultures incubated under the same conditions, as determined microscopically on stained preparations. Controls: parasite numbers in macrophage cultures incubated without spleen cells nor Con A ( ), with spleen cells only ( ), and with Con A only ( ).

**Table III**

| Treatment of lymphocytes | Con A (5 $\mu$g/ml) | Live intracellular parasites per culture at 48 h ($\times 10^{-3}$, ± SD) |
|-------------------------|--------------------|------------------------------------------------------------------|
|                         | Exp. 1             | Exp. 2                                                           |
| None                    | 183.7 ± 37.0       | 220.0 ± 29.2                                                     |
| None +                  | 0.00               | 0.00                                                            |
| Anti-MTLA + C           | 124.0 ± 38.2       | 231.3 ± 48.9                                                     |
| Normal serum + C        | 1.66 ± 1.5         | ND                                                              |

Macrophage cultures originating from $2 \times 10^5$ peritoneal cells were infected with \textit{L. enriettii}, then activated by incubation with 5 $\mu$g per ml of Con A in the presence of $5 \times 10^5$ syngeneic spleen cells treated with anti-MTLA or normal rabbit serum with or without complement. Parasite destruction was determined from triplicate cultures by using the SDS technique after 48 h of incubation.

Discussion

Activated macrophages are characterized by an increased capacity to kill and digest intracellular micro-organisms, which can be observed in vitro using a variety of microbes as targets (2, 6, 7, 10, 14–16). In particular, inhibition of the multiplication of \textit{Listeria monocytogenes} has been often utilized as an index of macrophage activation. However, such bacteria present a number of disadva-
tages: they multiply rapidly both intra- and extracellularly, leading to macrophage disruption, and the addition of antibiotics to control extracellular growth renders interpretation of the results difficult (17). The use of intracellular parasites, such as leishmania, to monitor macrophage activation, is an interesting alternative. L. enriettii is insensitive to the antibiotics commonly used in tissue culture. It is readily endocytosed by mouse macrophages in vitro; it is not destroyed by nonactivated cells, nor does it destroy them, within the time period of the experiment. However, activation of host macrophages leads to rapid intracellular killing and digestion of the micro-organism.

Activation was obtained in vitro by exposure of macrophages to mixtures consisting of syngeneic responding and irradiated, allogeneic stimulator spleen cells. No parasite destruction was observed in control preparations containing syngeneic lymphocytes alone, indicating that activation resulted from the stimulatory effect of allogeneic cells. A similar situation appears to exist in vivo, where macrophage activation occurs as a result of graft-versus-host reactions (18).

The choice of the test system proved to be critical for the precise measurement of the kinetics of parasite killing in activated cells. A comparison of the two systems used (Materials and Methods) indicated that SDS lysis of macrophages, followed by counts of the live (motile) parasites in culture, allowed an earlier detection of macrophage activation than simple microscopic examination of stained cultures. Thus, parasite death was not followed by their immediate morphological disappearance from the macrophages. Alternatively, parasite inactivation may be a gradual process, a step of which is the inhibition of the capacity of amastigotes to transform to motile promastigotes in culture.

Con A-stimulated spleen cells induced strong macrophage activation. However, a decrease in intracellular parasite killing was regularly observed at high lymphocyte:macrophage ratios (Table I). Failure of the macrophages to become fully activated when incubated with large numbers of lymphocytes may reflect a suppressor activity developing in cultures of Con A-stimulated spleen cells under crowded conditions (19). Alternatively, activation may occur only within a certain range of concentrations of the activating lymphokine, above which macrophages are adversely affected and fail to become activated.

Concomitantly with decreased activation, macrophage monolayers exposed to high concentrations of Con A-stimulated lymphocytes were partially destroyed within 48 h. This toxic effect was perhaps related to a nonspecific lectin-induced cytotoxic activity of the added lymphocytes (20), or to the release by activated macrophages themselves of cytolytic substances (21).

When activated by lower numbers of Con A-stimulated spleen cells, macrophages were affected at a later time and to a smaller extent, and remained intact for more than 24 h after parasite destruction, indicating that disappearance of the micro-organisms was not due to impaired survival of the host cells. In addition, macrophages activated by MLCs were not observed to lose viability for up to 96 h. Absence of toxicity may be related to the weaker activation obtained under these conditions compared to activation induced by Con A-stimulated lymphocytes, as parasite destruction occurred at a later time and required higher lymphocyte ratios to reach completion.
A considerable decrease in intracellular parasite killing was also observed when macrophages and lymphocytes were incubated with Con A at concentrations above 10 μg/ml. Con A has been shown to inhibit phagosome-lysosome fusion, due to its interaction with membrane receptors (22). However, concentrations of the lectin required to demonstrate this effect are well above those for which decreased activation was noted in our system. Within our test conditions, increasing concentrations of Con A led to increased toxicity for lymphocytes and decreased thymidine uptake, a phenomenon also noted by others (23), presumably resulting in a diminished capacity of the spleen cells to activate macrophages in this system.

α-MM inhibited the capacity of lymphocytes to activate macrophages when incubated with Con A. This glycoside interferes with binding of the lectin to cell surfaces, thus preventing lymphocyte stimulation (24). Although Con A also binds to macrophages (22), it is unlikely that inhibition of activation by α-MM was due to decreased binding of the lectin to macrophages, as Con A alone has no macrophage-activating properties in the present system. Neither did α-MM prevent interaction of macrophages with a soluble activating factor released by spleen cells, as supernates of Con A-stimulated lymphocytes could activate macrophages even in presence of this sugar derivative.

Macrophage activation in vitro can be shown to depend on the release by stimulated spleen cells of soluble factor(s) (lymphokines) inducing rapid intracellular parasite killing when added to macrophage cultures free from lymphocytes. Different cell types may be involved in the production of these substances. Macrophage activation in vivo is a T-cell-dependent phenomenon (25), which occurs as result of cell-mediated responses to living and non-living antigens. In accordance, activation was inhibited in this study by treatment of the responding spleen cells with anti-MTLA antiserum and complement before stimulation by Con A. However, experiments described here do not rule out the possibility that part of the activating effect occurred through the agency of B cells present concomitantly in the incubation mixtures. Spleen cells from thymo-deficient animals, when stimulated by endotoxin, could promote parasite destruction comparable to that induced by normal spleen cells interacting with T-cell stimulants. This suggests that macrophage activating substance(s) can be produced by B lymphocytes, in addition to chemotactic factor (26), colony-stimulating factor (27), and migration-inhibitory factor (28).

Summary

When cultures of normal mouse peritoneal macrophages were infected with the intracellular protozoan parasite Leishmania enriettii, the micro-organism was found to survive intracellularly for several days, apparently without multiplication. However, exposure of infected macrophages to certain stimuli led to rapid parasite killing and digestion, providing a sensitive assay with which the mechanisms of macrophage activation can be studied.

Microbicidal activity was induced by incubation of macrophages with syngeneic spleen lymphocytes, which were stimulated either by allogeneic cells in

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mixed lymphocyte culture (MLC) or by the plant lectin concanavalin A (Con A). Cocultivation with MLCs led to parasite killing within 48-72 h, whereas exposure of infected cells to Con A-stimulated lymphocytes resulted in substantial destruction of the micro-organism within less than 24 h, an effect which was dependent on the presence of thymus-derived lymphocytes and was inhibited by α-methyl-mannoside.

Incubation with Con A-stimulated lymphocytes also led to lysis of part of the macrophage monolayer. However, parasite killing did not result from decreased macrophage survival, as destruction of the micro-organism was highest under culture conditions which were the least detrimental to the phagocytes. Conversely, excess numbers of Con A-stimulated lymphocytes were less efficient at inducing macrophage activation and displayed marked toxicity to the macrophage monolayer.

When spleen cells were stimulated by Con A at concentrations above 10 μg/ml, a decrease was noted in the capacity of macrophages to destroy the parasite, probably reflecting a toxicity of the lectin for lymphocytes resulting in impaired activating capacity.

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