STUDIES ON THE MECHANISMS
OF MACROPHAGE ACTIVATION

II. Parasite Destruction in Macrophages Activated by
Supernates from Concanavalin A-Stimulated Lymphocytes*

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In a previous report (1), we have shown that murine peritoneal macrophages, when cocultivated with syngeneic spleen lymphocytes stimulated by allogeneic cells or concanavalin A (Con A), acquired the capacity to destroy the intracellular parasite Leishmania enriettii. This observation appeared to provide a good model with which to study the mechanisms of macrophage activation.

Several authors have shown that induction in vitro of microbicidal properties of macrophages does not require contact with lymphocytes, but is mediated by soluble products released by the latter cells (2–5). For the sake of convenience, the substance(s) responsible for activation will be hereafter referred to as macrophage-activating factor (MAF). The present report describes the parameters of in vitro MAF production by Con A-stimulated spleen cells, and of its measurement, based on the newly acquired capacity of MAF-treated peritoneal macrophages to destroy intracellular Leishmaniae.

Materials and Methods

Chemicals and Media. Sources of chemicals and media were as indicated in (1). In addition, fetal calf serum was purchased from Seromed, Munich, Germany; starch soluble from Merck, Darmstadt, Germany; Lipopolysaccharide W (Escherichia coli 0111:B4) from Difco Laboratories, Detroit Mich.; [3H]Thymidine from the Radiochemical Centre, Amersham, England; and Limulus Amebocyte Lysate from Microbiological Associates, Walkersville, Md.

Determination of Endotoxin Contamination. The presence of endotoxin in media and reagents was determined by the Limulus Amebocyte Lysate assay, using the microtest introduced by Nguyen and Greppin (6). Sensitivity of the test was found to be of 0.5 ng/ml of endotoxin.

The following items were found to be negative (endotoxin contamination below 0.5 ng/ml): bidistilled water, 2% starch in saline, Seromed FCS (lot 610157), Con A at 5 μg/ml. Borderline positivity was observed for enriched medium (Seromed Dulbecco’s powder medium lot 80254, reconstituted with pyrogen-free water and supplemented with amino acids and vitamins, cf. reference 1). Positivity was lost at 1:2 dilution, and the medium used in all experiments was thus considered to contain endotoxin at a concentration of 0.5 ng/ml or lower.

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1 Abbreviations used in this paper: Con A, concanavalin A; DS, Dulbecco’s medium supplemented with 10% fetal calf serum; FCS, fetal calf serum; MAF, macrophage-activating factor; α-MM, α-methyl-mannoside; SDS, sodium dodecyl sulfate; SNa(s), supernatant fluid from Con A-stimulated lymphocytes; SN, supernate.
Culture Conditions

Macrophages and parasites. Culture and infection of macrophages were performed as described previously (1). Briefly, 2 × 10⁶ starch-induced peritoneal exudate cells were distributed in 35 mm Falcon plastic dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) containing four coverslips, or 0.2 million cells in 16 × 60-mm flat-bottom glass tubes, and incubated for 2 h at 37°C to allow for the attachment of macrophages.

*L. enriettii* amastigotes were prepared as described elsewhere (7) and added to the cultures at a ratio of 3.5–7 parasites per peritoneal cell. After a 24-h incubation at 37°C to allow for phagocytosis, the infected cultures were carefully washed to remove noningested parasites and nonattached cells before the addition of activating or control media.

Preparation of active supernates (SNAs). C57BL/6 spleen cells were prepared as described previously (1), suspended in enriched Dulbecco's medium supplemented with 10% fetal calf serum (DS) (1), and distributed to 60-mm Falcon plastic dishes in 9-ml volumes containing a total of 15 × 10⁶ cells. Stimulation was obtained by the addition of Con A to a 5-μg/ml final concentration. Unless specified otherwise, supernates were harvested after 72 h of incubation at 37°C, centrifuged at 450 g for 5 min to remove contaminating cells, and stored frozen (−20°C) until use.

For the preparation of serum-free SNAs, cells were incubated at a concentration of 30 × 10⁶ for 9 ml of fluids, in the presence of 0.5 μg/ml of Con A.

Activation of macrophages. After washing, medium in cultures of *L. enriettii*-infected peritoneal macrophages were replaced with suitable dilutions of active or control supernates, to reach 3 ml/Petri dish, respectively, 0.5 ml/flat-bottom tube. Reagents required by the experimental protocol were added immediately in 0.1-ml volumes in dishes only. Parasite destruction was then followed after various incubation periods at 37°C.

Measurement of Parasite and Macrophage Destruction. Killing of intracellular *L. enriettii* in activated cells was followed in two ways, as described earlier (1). (a) By microscopic examination of glutaraldehyde-fixed, May-Grünwald-Giemsa-stained coverslip cultures. (b) By a modification of the detergent-lysis technique (1). 0.5 ml of Hanks' buffer were added to the tube cultures. After a thorough agitation, the fluids were removed by suction and replaced by 0.5-ml of serum-free Dulbecco's medium containing 0.01% sodium dodecyl sulfate (SDS). Tubes were then incubated for 20 min at 37°C to allow for slow, gradual macrophage lysis leaving intracellular parasites unharmed. 0.5 ml of parasite growth medium (7) was then added to the tubes, followed by incubation for 3 d at room temperature, a period required for the transformation of amastigotes to flagellate promastigotes which could be counted in the hemocytometer after immobilization with formalin.

Measurement of DNA Synthesis. Measurement of DNA synthesis was performed as described previously (1). Concentrations of spleen cells used for the test were, respectively, of 0.5 × 10⁶/tube for Con A stimulation in presence of serum, and 10 × 10⁶/tube in absence of serum.

Results

Parasite Killing in Macrophages Exposed to Supernates from Stimulated Lymphocytes. Preliminary experiments showed that incubation of starch-induced, *L. enriettii*-infected peritoneal macrophages with SNAs from Con A-stimulated spleen cells, led to rapid intracellular parasite destruction (Fig. 1 a–d). Controls included macrophages incubated with (a) fresh medium containing 10% fetal calf serum (FCS), (b) supernates from nonstimulated lymphocytes, (c) freshly prepared Con A-containing medium. Intracellular parasite numbers remained stable for 72 h in all of the three control cultures (Table I). Addition of α-methyl-mannoside (α-MM) at a concentration (30 mM) effectively preventing interaction of the lectin with cell surfaces (1, 14) did not inhibit the capacity of SNAs to induce intracellular parasite destruction (Table I), demonstrating that activation was independent of the Con A still present in the supernates.

Similar results were obtained when resident, noninduced macrophages were stim-
Fig. 1. Light microscope observations of parasite destruction in mouse macrophages activated by MAF. Giemsa stain. Linear magnification: × 840. A, L. enriettii-infected C57BL mouse peritoneal macrophages after 24 h of exposure to supernate from spleen cells incubated with 5 μg/ml of Con A for 3 d. Notice rounding of parasites (arrows), an early sign of macrophage activation in this system. B, Same culture as in Fig. 1 A, after 48 h of exposure to SNa. All intracellular parasites have been killed and digested. C, Control culture of L. enriettii-infected macrophages, exposed to control DS medium for 48 h. Parasites have remained elongated; no destruction can be observed. D, Control culture of L. enriettii-infected macrophages, exposed to DS medium supplemented with 5 μg/ml of Con A for 48 h. No parasite destruction can be observed.

### Table I

**Absence of Effect of Control Media on Intracellular Parasite Survival**

| Macrophage incubation medium                                      | Parasites/100 macrophages at 48 h ± SD |
|------------------------------------------------------------------|----------------------------------------|
| Fresh medium (10% FCS)                                           | 326.0 ± 37.63                          |
| Fresh medium (10% FCS) + α-MM 30 mM                              | 258.8 ± 68.43                          |
| Fresh medium + Con A (5 μg/ml)                                   | 312.4 ± 58.09                          |
| SN from normal lymphocytes                                       | —                                      |
| SN from Con A-stimulated lymphocytes                             | 304.2 ± 59.95                          |
| SN from Con A-stimulated lymphocytes + α-MM 30 mM                | 0.00                                   |
|                                                                  | 0.40 ± 0.89                           |
|                                                                  | 0.00                                   |

Macrophage cultures were infected with L. enriettii, then incubated with the various media mentioned. Parasite destruction was determined microscopically after 48 h of incubation. Mean of five counts ± SD.
Macrophage Activation in Vitro

Fig. 2. Effect of endotoxin on macrophage activation induced by MAF. Macrophage cultures were infected with *L. enriettii*, then activated by incubation with various concentrations of serum-containing MAF in presence or absence of 10 ng/ml of endotoxin. Parasite destruction was determined microscopically after 24, 48, and 72 h of incubation. Mean of five counts ± SD.

Endotoxin was supplemented with 10 ng/ml of commercial endotoxin before assay on macrophages. This resulted in considerable potentiation of the parasiticidal effect (Fig. 2). That activation could nevertheless be obtained in absence of added endotoxin was evident from the considerable level of parasite destruction in macrophages exposed to undiluted supernates for 72 h. No exogenous endotoxin was added to active media used for other experiments described in this paper.

Supernates from Con A-stimulated spleen cells prepared in serum-free medium had...
Effect of endotoxin on macrophage activation induced by MAF in absence of serum. *L. enriettii*-infected macrophages were preincubated for 1 h with various concentrations of endotoxin, then washed three times, and reincubated with the same concentrations of endotoxin in presence of serum-free MAF. Parasite destruction was determined microscopically after 48 h of incubation. Mean of five counts ± SD. ——, active SN; ---, control medium.

Effect of serum concentration on the induction of activation by SNa. Tube cultures of *L. enriettii*-infected macrophages were activated by serum-free SNa to which increasing concentrations of FCS had been added. Parasite destruction was determined by the SDS method after 48 h of incubation. Mean of three counts ± SD. m = parasite level in control cultures.

little activating properties, when tested as such, indicating that endotoxin contamination of the media was below the threshold of potentiation of MAF. Full activity could be restored by addition of 10 ng/ml or above of endotoxin (Fig. 3), or of increasing amounts of endotoxin-free serum (Fig. 4).

**Kinetics of Parasite Destruction in Macrophages Activated by SNa**. In a previous report (1), it was shown that parasitized macrophages cocultivated with syngeneic lympho-
cytes and Con A killed all intracellular microorganisms within 35–40 h. Using SNa as an activating stimulus, however, the time-course of parasite destruction was considerably shortened (Fig. 5). After a lag period of ≈5 h, the number of intracellular microorganisms decreased rapidly, to reach 50% of the initial population by 10 h. Macrophages killed all parasites within 20 h of the beginning of incubation with SNa.

Fig. 5. Kinetics of parasite destruction in macrophages activated by MAF. Tube cultures of *L. enriettii*-infected macrophages were incubated with 1/3 diluted, serum-containing SNa. Parasite destruction was determined by the SDS method after various times. Mean of two determinations, with range.

Fig. 6. Time-course of MAF-production by Con A-stimulated lymphocytes. Supernates from spleen cell cultures were harvested after stimulation by Con A (5 μg/ml) for various periods (abscissa), then tested at different dilutions for their capacity to activate *L. enriettii*-infected macrophages. Parasite destruction determined by the SDS method after 48 h.
Fig. 7. MAF production as function of Con A concentration and presence of serum. A, *L. enriettii*-infected macrophages were activated by incubation with supernates of lymphocytes stimulated for 72 h with increasing concentrations of Con A, in presence or absence of 10% FCS. FCS was added to serum-free SNa before testing. Dilutions tested were 1/9 for SNa produced in presence of serum, respectively, 1/3 for SNa produced in absence of serum. Parasite destruction was determined microscopically after 48 h. Mean of five counts ± SD. B, Viability at 48 h of spleen cell suspensions incubated with the same concentrations of Con A as in Fig. 7A. Mean of three determinations ± SD. C, Thymidine uptake at 48 h by spleen cell suspensions incubated with the same concentrations of Con A as in Fig. 7A. Mean of three determinations ± SD.

The kinetics of parasite destruction was found to vary slightly between experiments. However, no intracellular parasite survival could be found after 48 h of activation in five experiments, as measured by the SDS method. On the other hand, when results were determined by morphological examination of stained preparations, a 72-h activation period was sometimes used, to allow for killed parasites to be digested and disappear from macrophages.

Destruction of intracellular parasites did not result from death of the phagocytes.
Fig. 8. Induction of macrophage activation by SNa pulsing. Tube cultures of macrophages were infected with
$L. enriettii$, pulsed for various periods of time with MAF-containing supernates, then washed and reincubated in control medium. Parasite destruction was determined by the SDS method, 48 h after the start of the SNa pulses. Mean of three counts ± SD.

themselves; these were fully viable at the time of parasite elimination (Fig. 1 b). In four experiments, the number of macrophages in activated cultures was found to reach 95.5–121.1% of the control population after 48 h, at a time when 98.1–100.0%
of the parasites had been destroyed.

Time-Course of MAF Production by Con A-Stimulated Lymphocytes. The capacity of SNAs to induce intracellular parasite killing was found to depend both on the concentration of the supernates and on the duration of lymphocyte stimulation by Con A (Fig. 6). At the highest dilution tested (1/27), complete parasite destruction was observed only when using supernates from lymphocytes stimulated for 48 h or longer. On the other hand, 24-h supernates led to complete parasite killing when tested undiluted.

MAF Production as a Function of Con A Concentration and Presence of Serum. To determine the concentration of Con A producing supernates of highest activity, spleen cells were incubated for 72 h with increasing amounts of lectin, in presence or absence of 10% FCS. Before assay on parasitized macrophages, SNAs without serum were supplemented with 10% FCS. MAF production was maximal at 5 μg/ml of Con A in presence of serum, respectively, 1.0 μg/ml in its absence (Fig. 7 A). Decreased MAF production at Con A concentrations above optimum was probably a result of reduced lymphocyte survival (Fig. 7 B).

Little correlation was found between DNA synthesis and MAF production in Con A-stimulated spleen cell cultures. In absence of serum, DNA synthesis was highest at a 0.5-μg/ml Con A concentration, below that inducing maximum MAF activity (1.0 μg/ml, three experiments; Fig. 7 C compared to 7 A). In the presence of serum, DNA synthesis was detectable over a wider range of lectin concentrations, but was always reduced at 5 μg/ml (five experiments), the concentration inducing supernates with highest activity. Interestingly, macrophages could be fully activated by undiluted SNAs from spleen cell cultures incubated with 10 μg/ml of Con A, at which concentration little or no DNA synthesis was induced (five experiments). This activity was essentially lost at a 1/9 dilution, however, suggesting that only limited amounts of MAF were produced under these conditions.

Induction of Macrophage Activation by SNa Pulsing. To determine whether the continuous presence of MAF was required for activation, infected macrophages were pulsed
with SNa for various periods of time, then washed and reincubated in MAF-free medium. Parasite destruction was determined using the SDS technique, 48 h after the start of the SNa pulses.

As described previously, very little reduction in intracellular parasite numbers could be observed in macrophages exposed to SNa for 6 h (Fig. 5). However, when such macrophages were incubated further in MAF-free medium, >80% of the parasites were found to be destroyed by 48 h (Fig. 8). A 10-h pulse of SNa was sufficient to induce macrophages to kill all microorganisms within the next 38-h period. In five experiments, the duration of the SNa pulse required for complete subsequent parasite killing was comprised between 6 and 15 h.

Discussion

Interaction of immune lymphocytes with antigen (8, 9), or of normal lymphocytes with mitogens (9, 10), induces the release by the latter cells of soluble mediators (lymphokines [11]) which alter macrophage functions in several ways (for reference, cf. 12). Among these is an increased capacity to destroy or restrict the growth of ingested microorganisms (2), a phenomenon referred to in the present report as activation.

The experiments described here are based on the observation that activated mouse peritoneal macrophages acquire the capacity to destroy parasites of the genus Leishmania (1, 13). Supernates with macrophage-activating properties (SNas) were obtained by cultivation of mouse spleen cells with the T-cell mitogen Con A. The activating properties of lymphocyte supernates were clearly independent of residual lectin: a-MM, which interferes with binding of Con A to cell surfaces (14) did not prevent macrophage activation; moreover, Con A alone did not activate macrophages in the absence of lymphocytes.

Serum-free SNas were unable to activate macrophages, when tested as such, indicating that trace contamination by endotoxin was not sufficient to promote MAF-induced activation. The finding that addition of nanogram amounts of LPS rendered such preparations fully active is in accordance with reports from other laboratories (15-17), showing that minute amounts of endotoxin potentiate macrophage toxicity for tumor target cells.

Addition of LPS-free FCS to otherwise inactive, serum-free SNas also allowed these preparations to express their activating potential, indicating that MAF-induced activation can be obtained in absence of endotoxin. The role of serum in promoting activation is unclear. Albumin and γ-globulin fractions are inducers of pinocytosis (18), a process related to the formation of lysosomes and hydrolytic enzymes (19) probably required for parasite killing and digestion. In addition, serum contains inhibitors of serine-esterases, which are known to potentiate some of the effects of lymphokines on macrophages (20). Still other factors have been shown to modulate macrophage tumoricidal activity (21). Experiments are in progress to test for the possible role of such substances in the present system.

The concentration of mitogen producing supernates with highest activity was found to depend on the presence of serum in the medium used for lymphocyte stimulation. The requirement for higher concentration of Con A in presence of FCS may be a result of binding of the lectin to serum proteins (22), thus decreasing the amount of mitogen available for reaction with cell surfaces. The decreased thymidine uptake
observed at high lectin concentrations appears to correlate with impaired lymphocyte survival (Fig. 7 B), perhaps a result of the inactivation of surface receptors necessary for membrane function (23). Little correlation was observed in Con A-stimulated lymphocyte cultures between DNA synthesis and MAF production; these may constitute two independent phenomena triggered simultaneously by lectins. A similar independence of lymphokine production and DNA synthesis has been reported by others (24, 25).

No parasite killing could be observed during the first 5 h of incubation of macrophages with active supernates. Parasite destruction then progressed rapidly, to reach completion within 20 h. The time-course of parasite destruction in macrophages exposed to lymphokine-rich supernates was thus considerably shorter than that obtained by direct cocultivation with spleen cells and mitogen (1). The longer lag phase observed in the latter system probably reflects the time required for lymphokine synthesis by Con A-stimulated lymphocytes.

Pulse experiments indicated that the presence of MAF was required essentially in the inductive phase of activation. Although no significant parasite destruction was observed in macrophages exposed to SNa for 6 h (Fig. 5), complete killing was eventually obtained by 48 h when such cells were further maintained in control medium (Fig. 8). In another system, Nogueira and Cohn (5) recently demonstrated that removal of activating factor before achieving complete destruction of intracellular Trypanosoma cruzi trypomastigotes led to resumed growth of the surviving parasites. The difference with our findings may be a result of higher pathogenicity of this microorganism for mouse macrophages compared to L. enriettii. The observations reported in this paper suggest that lymphokine-containing supernates trigger biochemical events, the completion of which is independent of further presence of activating substance(s) in the surrounding fluids.

Summary

Activation of mouse peritoneal exudate macrophages, as evidenced by destruction of the intracellular protozoan parasite Leishmania enriettii, was obtained by incubation with supernates from concanavalin A (Con A)-stimulated syngeneic spleen cells. Parasites were not destroyed in macrophages exposed to control media.

Supernate-induced activation was independent of the presence of Con A. The activating principle (macrophage activating factor, or MAF) was produced by Con A-stimulated lymphocytes in presence or absence of serum. In absence of serum, MAF synthesis was highest at Con A concentrations far below those required in serum-containing media. MAF production was reduced at Con A concentrations of 10 μg/ml or above, probably a result of toxicity of the lectin for lymphocytes. MAF was detectable after 24 h of lymphocyte stimulation and increased up to 72 h; production appeared to be independent of DNA synthesis.

Serum-free MAF was inactive when tested as such on macrophages. Full activity could be restored by addition of nanogram amounts of endotoxin or of FCS before assay. Endotoxin also considerably potentiated MAF activity in serum-containing supernates.

Full intracellular parasite destruction was observed after contact of macrophages with MAF for 20 h. The continuous presence of MAF was not necessary for activation;
a 10-h pulse was sufficient to induce macrophages to destroy all intracellular microorganisms within the next 38 h.

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