An Inhibitor of Cell Proliferation Released by Cultures of Macrophages

(thymidine incorporation/leukemia cells/concanavalin A/spleen cells)

JESUS CALDERON, RICHARD T. WILLIAMS, AND EMIL R. UNANUE

Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT Culture fluids from mouse peritoneal exudate cells inhibited [3H]thymidine incorporation by, and proliferation of, EL-4 leukemia cells, 3T3 cells, and mitogen-stimulated spleen lymphocytes. Inhibited EL-4 leukemia cells recovered their normal proliferative capacity when washed and incubated in normal medium. The inhibitory activity resided in a low-molecular-weight substance that could be absorbed by incubation with the tumor cells. This substance was dialyzable and resistant to trypsin digestion and phosphodiesterase treatment. The mononuclear phagocytes in the peritoneal exudate seemed to be the source of the inhibitor. The inhibitory material was found in the same amounts in exudates of normal mice or mice injected with peptone or infected with Listeria monocytogenes; spleen cells adherent to plastic released the inhibitor but in lesser amount. We suggest that this inhibitor may contribute to the deleterious effects found when various cells, including neoplastic ones, are cultured in the presence of macrophages.

The mononuclear phagocyte system comprises circulating and tissue-fixed cells, monocytes and macrophages, respectively, originating from bone marrow precursors. They are endowed with the property of phagocytosis and degradation of extraneous materials (1). A wide variety of functions has been ascribed to the mononuclear phagocyte—phagocytosis and elimination of microorganisms, indeed an essential element in the host defense against infection, killing of tumor cells, interaction with immunocompetent lymphocytes, elimination of detritus and dead materials in inflammation, etc. Phagocytes may accomplish these diverse functions by two possible pathways, not mutually exclusive: by direct contact with the target object (as it necessarily happens in phagocytosis of bacteria) and by the release of pharmacologically active materials. That mononuclear phagocytes can release biologically important materials seems unquestionable at present, although in most cases the chemistry of the putative substances, their function, and the mechanisms of synthesis and release have not been explored. There are reports of activities in macrophage cultures that promote lymphocyte functions (2-4), stimulate growth and differentiation of stem cells (5), stop cell multiplication (6), or kill cells or bacteria (7, 8). The secretion process in macrophages has now been well characterized in the studies of Unkeless et al. (9), and Gordon et al. (10).

We call attention in this report to an activity in cultures of macrophages that stops the growth of various cells without actually killing them. This inhibitory activity is absorbed by the target cell, thus removing it from solution, and it is associated with a dialyzable low molecular weight substance. The effect on cells is readily reversible upon removal of the inhibitor.

MATERIALS AND METHODS

Culture of Peritoneal Exudate Cells (PEC). Cells were obtained aseptically from the peritoneal cavity of normal mice, of mice injected three days before with 1.5 ml of 10% proteose-peptone (Difco Laboratories, Detroit, Mich.) intraperitoneally (i.p.), or of mice injected with Listeria monocytogenes organisms i.p. [2 X 10⁹ organisms 7 days before; the 50% lethal dose (LD₅₀) was 6 X 10¹]. In the latter two instances, the macrophages were presumed to be "activated" on the basis of their cytological characteristics. The mice were of the inbred C57Bl/6J strain (Jackson Laboratories, Bar Harbor, Me.), males, 10-15 weeks old. 10⁶ freshly harvested PEC in 1.0 ml of culture medium were planted in 35 X 10-mm plastic dishes (Falcon Plastics, catalogue no. 3001) and incubated at 37° in 5% CO₂ in air incubator. The medium was RPMI-1640 (Associated Biomedics, Inc.) supplemented with 5% fetal calf serum, 2 mM glutamine, 50 units and 50 µg of penicillin and streptomycin, respectively, and 1% v/v of sodium bicarbonate solution (7.5% w/v, Microbiological Associates, catalogue no. 17-613). After 20 hr of incubation the cell monolayers were washed three times with medium, and, in most experiments, reincubated in 1 ml of culture medium for 48 more hours.

More than 90% of the cells attached to the dishes were well-spread macrophages forming an interrupted monolayer. Fluids were obtained at various times after initiation of cultures, centrifuged at 500 X g for 10 min in order to remove detached cells, and filtered through sterile filters of 0.45 µm pore diameter (Swinnex-25, catalogue no. SXHA 02505, Millipore Corp.). We tested the capacity of the PEC culture fluids to influence the growth of various kinds of cells: EL-4 leukemia, spleen lymphocytes stimulated by mitogens, 3T3 fibroblasts, and polyoma-transformed 3T3 fibroblasts. These various cells were cultured in the presence of graded dilutions of the PEC culture fluids and examined for their incorporation of [3H]thymidine after several hours. (The medium was the same one described above, i.e., RPMI-1640 with 5% fetal calf serum and supplements.)

EL-4 leukemia cells of C57Bl/6J mice were maintained by in vivo i.p. passages. They were harvested from the mice and cultured at a density of 5 X 10⁶ cells per ml of culture medium in 12 X 75-mm plastic tubes (Falcon Plastics, catalogue no. 2054). Four hours before the end of the incubation, 1 µCi of [3H]thymidine in a 50 µl volume was added to the culture (2 Ci/mmol, catalogue no. NET-027A, New England Nuclear Corp.).
Fig. 1. This graph shows the inhibition of \[^{3}H\]thymidine incorporation in EL-4 cells. EL-4 cells (5 \times 10^5) were incubated 8 hr in culture medium with graded concentrations of supernatants obtained from 48-hr culture of PEC from mice injected with proteose-peptone. Here and in the following figures, error bars indicate one standard error of the mean.

Co., Boston, Mass.). Trichloroacetic-insoluble fractions from the cultured cells were collected on glass fiber filters in a sampling manifold (Millipore Co.) and radioactivity was measured in a liquid scintillation spectrometer. Each experimental group was done in triplicate.

Spleen cells from C57Bl/6J mice were cultured with concanavalin A or Escherichia coli lipopolysaccharide by standard procedures.

3T3 fibroblasts and polyoma virus-transformed fibroblasts were obtained from Dr. Thomas Benjamin of our department.

Reagents. The important reagents were 3'5'-cyclic phosphodiesterase (P-0134, Sigma, St. Louis, Mo.), N6,O2'-dibutyryl adenosine-3':5'-cyclic monosphosphoric acid (D-0627, Sigma), trypsin-TPCK (Worthington Biochemical Corp., New Jersey), and trypsin inhibitor (T-9003, Sigma), concanavalin A twice crystallized (79-001, Miles Laboratories, Ill.), and LPS (lipopolysaccharide from E. coli obtained from Difco Labs., Michigan).

RESULTS

Inhibition of Cell Growth. The PEC culture fluid consistently inhibited the proliferation and growth of the various cells. A great many of the experiments were done with EL-4 leukemic cells, in which the inhibition of proliferation was striking. In the experiment shown in Fig. 1, the EL-4 cells were cultured for 8 hr in different amounts of PEC culture fluids. If the culture medium was made entirely of the PEC fluid, there was about 90% inhibition of \[^{3}H\]thymidine incorporation; as little as 20% PEC culture fluid, the smallest amount used in the experiment, inhibited about 20%. Similar results were obtained by culturing EL-4 cells 24 or 48 hr in the PEC fluids. Fig. 2 depicts the growth of EL-4 cells in PEC culture fluids and in normal medium. The number of tumor cells cultured in PEC fluids was reduced to 52% and 31% of the normal values at 24 and 120 hr of incubation, respectively. The mitotic indexes at different times were 2.3%, 2.3%, and 1.5% at 24, 48, and 72 hr after culture in normal medium and 0.6%, 0.7%, and 0.9% after culture in PEC fluids.

The inhibitory activity of the PEC fluids was also noted in cultures of normal spleen lymphocytes stimulated by addition of concanavalin A or E. coli lipopolysaccharide (Table 1). In one experiment normal and polyoma-transformed 3T3 cells 14 hr after being planted in a dish were inhibited 64% and 40%, respectively, in their incorporation of \[^{3}H\]thymidine. In unpublished experiments the PEC fluids markedly inhibited the production of antibody-forming cells in cultures of spleen cells with antigen (sheep erythrocytes) following the procedures of Mishell and Dutton (11).

Cytostatic Effects of PEC Culture Fluids. We investigated the behavior of EL-4 cells incubated with PEC fluid if later washed and incubated in normal medium (Figs. 3 and 4). In the two experiments in Fig. 3, EL-4 cells were cultured with various dilutions of PEC fluid for 7 or 8 hr, washed, and then incubated in normal medium for 19–48 hr, the last four in the presence of \[^{3}H\]thymidine. (The studies detailed previously indicated a marked inhibition of \[^{3}H\]thymidine incorporation by culturing from 8 to 24 hr; the period of 7–8 hr was selected.

Table 1. \[^{3}H\]Thymidine uptake by spleen cells in the presence of PEC fluids

<table>
<thead>
<tr>
<th>Addition to culture</th>
<th>cpm of [(^{3})H]thymidine at 72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7,580 ± 1,939</td>
</tr>
<tr>
<td>Concanavalin A, 1.5 μg</td>
<td>126,405 ± 10,350</td>
</tr>
<tr>
<td>Concanavalin A, 1.5 μg + PEC fluid, 100%</td>
<td>18,102 ± 957</td>
</tr>
<tr>
<td>Lipopolysaccharide, 5 μg</td>
<td>43,910 ± 3,573</td>
</tr>
<tr>
<td>Lipopolysaccharide, 5 μg + PEC fluid, 100%</td>
<td>6,359 ± 205</td>
</tr>
</tbody>
</table>

Spleen cells (1.5 \times 10^6) were cultured for 72 hr; \[^{3}H\]thymidine was present for the last 12 hr of culture. Figures represent cpm in trichloroacetic acid-insoluble fraction (± one standard error of the mean). All results are means of triplicate cultures.
INCUBATION (hr)

**Fig. 3.** Two experiments demonstrating the restoration of [3H]thymidine incorporation by EL-4 cells after incubation in PEC fluids. Cultures of 5 X 10⁶ cells were preincubated in normal medium or with different concentrations of supernatants from 48-hr culture of PEC; then the cells were washed once and incubated in normal medium for the periods of time indicated.

for practical considerations.) There was a marked inhibition of [3H]thymidine incorporation during the first 8 hr in which the EL-4 cells were placed in PEC fluid, but the proliferative capacity was regained after culture in normal medium but not in PEC fluids (data not shown). The kinetics of reconstitution of the proliferative activity were studied in cultures that were incubated at 2-hr periods with [3H]thymidine for up to 36 hr. In Fig. 4 one can note a brief early rise in [3H]-thymidine incorporation followed by a lag period of about 8 hr before a steady increase in the incorporation of [3H]thymidine. The kinetics of incorporation of [3H]thymidine into EL-4 cells shown in Fig. 4 are complicated, and it would be premature to try to interpret them. Suffice to say that removal of the inhibitor does allow the cells to regain their capacity to incorporate thymidine.

**Fig. 4.** The figure shows the kinetics of [3H]thymidine incorporation of EL-4 cells after incubation in PEC or with different concentrations of supernatants from 48-hr culture of PEC for the periods of time indicated.

**Cells Responsible for Inhibitory Activity.** A most likely candidate for the inhibitory activity of the PEC fluid is the macrophage, since it is the most abundant cell in such fluids. Inhibitory activities, however, have been ascribed to activated thymic-derived (T) lymphocytes, also present to a small extent among the PEC (12). We tested for the possible contribution of the T cells by comparing the activity of fluids of normal PEC to PEC deprived of T lymphocytes by treatment with anti-0 serum and complement. Treatment with anti-0 and complement kills thymic-derived lymphocytes. The inhibitory activity of PEC treated with anti-0 was the same as that of untreated PEC, ruling out the role of T cells in the phenomenon. In this experiment fluid from untreated PEC inhibited the incorporation of [3H]thymidine by 92% (±0.7). Fluids from anti-0-treated PEC inhibited 89.7% (±0.2%).

A further extension of this kind of experiment is shown in Fig. 5, which compares the inhibitory activity on EL-4 cells of culture fluids of PEC to those of spleen cells that adhere to dishes, of normal spleen cells, and of spleen cells deprived of macrophages, on growth of EL-4 cells. (All fluids came from 24-hr cultures of cells. Spleen cells were cultured, in suspension, on dishes at a density of 10⁷ per ml. After culture, the suspension was centrifuged, and the fluids were obtained and tested. Nonadherent spleen cells were obtained after 1 hr of culture of 10⁷ spleen cells in a 35 X 10-mm dish. This procedure was repeated three times in order to obtain a confluent monolayer of cells to be used as adherent cells.) The PEC cultures were strongly inhibitory, followed by the spleen adherent cells and normal spleen cells. The amounts of culture supernatants that produced 50% inhibition were 8%, 28%, 62%, and 72% for PEC, spleen adherent cells, normal spleen, and spleen cells depleted of adherent cells, respectively. We interpret this to indicate that the inhibitory activity is a property of cells adhering to dishes but more actively represented in peritoneal exudate than in normal spleen sus-
spleen cells (○), or spleen cells after removal of adherent cells (●). The fluids were obtained after 24-hr incubation with the cells. The experiment was done with EL-4 cells incubated 8 hr in the fluids.

Since macrophages in the peritoneal cavity tend to be more mature and differentiated than those found in spleen, we have ascribed the inhibitory activity to mature macrophages.

Supernatant fluids from PEC of normal, peptone-injected, or Listeria-infected mice inhibited [3H]thymidine incorporation into EL-4 cells to the same extent, indicating that the state of "activation" of macrophages did not influence the phenomenon being described. The production of inhibitory activity from PEC of normal or peptone-injected mice was highest during the first 24 hr of culture, decaying slightly during 4 days of culture.

Attempts to Establish the Nature of the Inhibitory Activity. The inhibitory activity of PEC fluids on EL-4 cells was lost by dialysis, suggesting that it was caused by a low-molecular-weight chemical. If the PEC fluid was dialyzed against an equal volume of medium, the inhibitory activity was distributed equally on both sides of the membrane. (The dialysand inhibited the incorporation of [3H]thymidine by 78% and the dialysate inhibited 69.5%.) After extensive filtration on Amicon PM-10 and UM-2 filters, the inhibitory activity was found in the filtered fraction, indicating a material of less than 1400 daltons.

The inhibitory activity was resistant to trypsic digestion (100 µg/ml of trypsin for 1 hr) and was stable to freezing and thawing (five times); we have routinely maintained the culture fluids at -20° without loss of activity. The possibility that the inhibitory activity was cyclic AMP secreted by the cells was considered. The PEC culture fluids, treated with 3'5'-phosphodiesterase (100 µg/ml with 0.014 M MgSO₄, 30 min) were as inhibitory as normal fluids.

Absorption of Inhibitory Activity to Tumor Cells. Can the inhibitory activity of PEC fluids be absorbed by the tumor cells? One milliliter aliquots of PEC fluids were incubated with 10⁶ EL-4 cells for 1 hr at 4°, the cells were removed by centrifugation, and the fluid was diluted and tested for its capacity to inhibit EL-4 growth. Fig. 6 shows that a significant percent of activity was indeed removed by incubation with EL-4 cells. Not shown in Fig. 6 is the observation that incubation of PEC fluid with EL-4 at 37° resulted in a very minor degree of absorption. We interpret this to mean that the PEC fluid activity can indeed be absorbed to cells but that the binding is weak at 37°.

DISCUSSION
We have consistently found a cytostatic activity in cultures of lymphoid cells rich in mononuclear phagocytes. The culture fluids inhibited both the incorporation of radioactive thymidine into, as well as the proliferation of, various cells. The activity responsible for the inhibitory effect was dialyzable and absorbable by incubation with EL-4 cells, suggesting that it resides in a small molecule capable of interacting with some cell constituent. It is possible that this molecule may block at the cell membrane the uptake and transport of nucleosides or nutrients; alternatively, since it is smaller than 1400 daltons, the possibility exists that it readily enters the cell to block in some way, directly or indirectly, the biosynthesis of DNA. Clearly, these points need investigation.

Two further points need clarification: One, we have not answered conclusively the question of whether different kinds of cells react differently to the inhibitor, since only limited experiments were performed with nonlymphoid cells—the point which is clear is that lymphoid cells are very sensitive to the inhibitory effect. Secondly, all evidence indicates that the mononuclear phagocyte is the main source of the inhibitor, but we cannot exclude the participation of some other lymphoid cells besides macrophages. In one experiment (Fig. 5) cultures of spleen cells presumably depleted of macrophages inhibited, albeit at high concentration. It is difficult to eliminate all macrophages by their adherence to surfaces, so the possibility remains that the effect seen with spleen was caused by some remaining phagocytes.

The biological activities found in cultures of macrophages fall into two groups: one showing stimulation; the other, inhibition. Among the first are those described in the report of Hoffman and Dutton (4) showing that 24-hr culture fluids of murine macrophages allow lymphocytes to respond immunologically to sheep erythrocytes. The activity was absorbable...
by the erythrocytes and hence was directed against the antigen rather than at the lymphocytes. Gery et al. found that cultures of adherent cells stimulated by endotoxin enhanced the response of thymocytes to lectins (2, 3). Chervenick and LoBuglio obtained evidence for a factor in culture of monocytes that stimulated granulocyte colony formation (5). The inhibitory activities from macrophages fall into two categories: those associated with a lytic effect (7) and those associated with inhibition of growth (6, 13). Kramer and Granger found a "toxin" in cultures of "immune macrophages" and active against many kinds of cells. This toxin was mainly generated under conditions where antigen was added to the macrophage cultures (7). Bast et al. have recently found a factor that lyses bacteria in cultures of macrophages activated by immune processes (8). An activity similar, if not identical, to the one reported here was found by Waldman and Gottlieb (6) using rat macrophages. It should be recalled that in the past many investigators have found inhibition of lymphocyte function by addition of excessive numbers of macrophages to the cultures (reviewed in ref. 14). The presence of different activities in cultures of mononuclear phagocytes may not be surprising. It is possible that the phagocytes may release various kinds of biological activities in accordance with their maturational stage and/or response to external stimuli (microorganisms, lymphocyte products, etc.). (Our experiments suggest some quantitative differences in the inhibitory activity of spleen macrophages and peritoneal ones, but no differences among the latter cells regardless of whether they were "activated" or not.) In favor of the presence of different biological activities in macrophage culture fluids are recent observations we have made when testing antibody formation in vitro. In dilutions of 25–100%, the macrophage culture fluids consistently inhibited antibody formation; but when further diluted to 10%, some culture fluids exhibited stimulatory effects.

One fundamental question concerns the relevance of the inhibitory substance secreted by the macrophage in culture to various inhibitory phenomena associated in vivo with macrophage activation or seen in vitro when macrophages are mixed with other cells (15). Although we are impressed by the phenomenon that we have studied because of its reproducibility and its relatively strong activity, we are still skeptical about its biological role, knowing that it is a small molecule that presumably diffuses readily—unless one considers its role in circumstances where macrophages agglomerate around target cells, as in granulomas.

**Note Added in Proof.** Experiments made after the manuscript was submitted have disclosed the presence in the peritoneal cell culture fluid of a nondialyzable material which enhances the proliferation of thymocytes or spleen cells in response to lectins. The nondialyzable material does not enhance proliferation of EL-4 cells. The stimulatory activity for thymocytes or spleen cells is partially or completely masked by the inhibitor described herein and can be revealed following extensive dialysis. The stimulatory activity is probably similar to that described by Gery et al. (2, 3), and discussed above.

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