Similarities among Factors That Render Macrophages Tumoricidal in Lymphokine and Interferon Preparations

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ABSTRACT

Lymphokine preparations, including supernatants derived from antigen-stimulated Bacillus Calmette-Guerin-immune spleen cell cultures and normal spleen cells incubated with insoluble concanavalin A, were compared with partially purified L-cell interferon for the ability to render resting macrophages nonspecifically tumoricidal in vitro. Significant activation of macrophages by lymphokine preparations occurred at concentrations as low as 0.5 and 0.25% of the assay mixture for antigen-stimulated and concanavalin A-induced lymphokine, respectively. These end point concentrations were each determined to contain 0.3 unit of interferon per ml. Supernatants obtained from unstimulated normal spleen cells, concanavalin A-treated nu/nu spleen cells, or Bacillus Calmette-Guerin-immune spleen cells in the absence of sensitizing antigen did not enhance macrophage tumoricidal function and lacked interferon. Activation by L-cell interferon required at least 1 unit/ml. The macrophage-activating factors contained in lymphokine and interferon preparations were stable at pH 2 and at 56°C, but they were destroyed when heated at 80°C for 30 min, and were inactivated by trypsin. The data demonstrate common properties for the induction of tumoricidal macrophages by these diverse preparations.

INTRODUCTION

A large variety of stimuli can activate macrophages to kill neoplastic cells selectively by a contact-mediated event. Since maintaining this cytotoxic behavior of macrophages appears to be correlated with enhanced host resistance to neoplasia (2, 10, 18, 19), further knowledge of the induction and maintenance of the activation state may allow for improved antitumor therapy. There appear to be 3 separate pathways whereby macrophages are stimulated to kill tumor target cells in vitro (Chart 1). Naive resting macrophages can be rendered nonspecifically tumoricidal after treatment with: (a) lymphocyte-derived MAF (3, 5, 6), (b) fibroblast-derived interferon (17), and (c) a number of polyanions (1, 16, 17). Interferon appears to be intimately involved in each of these schemes that affect macrophage tumoricidal activity, and it may be a unifying mechanism.

Moreover, Youngner and Salvin (20) have reported that the LK's, migration-inhibitory factor and type 2 interferon, elicited in the circulation of BCG-infected mice inoculated with specific antigen (old tuberculin), may not be separate entities. Striking similarities were observed in both their coordinate production and physicochemical properties. Since migration-inhibitory factor appears to be the same as MAF (11), this study was undertaken to determine the relative potencies of MAF's produced by lymphocytes stimulated in vitro by specific antigen or mitogen and to determine their relationship to interferon. Some physicochemical properties of the agents that activate macrophages in MAF and interferon preparations are also described.

MATERIALS AND METHODS

Animals

Adult (6 to 8 weeks old) BALB/c x DBA/2 F1 (hereafter called CD2F1) mice and athymic nude (nu/nu) mice were obtained from the Mammalian Genetics and Animal Production Section of NIH, Bethesda, Md. Animals were given Purina laboratory chow and tap water ad libitum.

Drugs

Sepharose 4B and Con A bound to Sepharose beads (10 mg of Con A per ml of packed beads) were obtained from Pharmacia, Uppsala, Sweden. Partially purified mouse fibroblast interferon (specific activity, 2 × 107 units/mg of protein) was provided by Dr. Kurt Paucker, Department of Microbiology, Medical College of Pennsylvania, Philadelphia, Pa. Interferon was induced in L-cells by Newcastle disease virus and was purified by affinity chromatography on anti-interferon globulin-Sepharose (12).

Target Cells

An established line of MBL-2 [H-2b (C57Bl/6). Moloney murine leukemia virus-induced] lymphoblastic leukemia cells was maintained as suspension cultures in RPMI-FBS. All cultures were kept in a humidified 37°C incubator containing 5% CO2 atmosphere.

Effector Cells

Noninduced peritoneal cells were harvested from CD2F1 mice as previously described (16). Cells from 20 mice were pooled, washed twice in 30 ml of Hanks' balanced salt solution, and resuspended in RPMI-FBS. The peritoneal cells were kept in an ice bath prior to use to prevent adherence.
BCG-PPD MAF. LK prepared from PPD-stimulated BCG-immune spleen cells was generously provided by Dr. M. S. Meltzer, Immunopathology Section, National Cancer Institute, NIH, Bethesda, Md. Briefly, mice were immunized intradermally with viable Mycobacterium bovis strain BCG of the cultures, and the cell suspensions were incubated for 72 hr at 37°. Control cultures received PPD following the incubation period. Supernatant fluids were stored at 4° until use. Con A MAF. Lymphocytes were obtained from CD2F, mice by aseptic removal of spleen and thymus. The organs were minced finely with scissors in RPMI-1640 and triturated to obtain single-cell suspensions. The suspension was filtered through sterile gauze to remove tissue debris. The cells were then centrifuged at 250 x g for 10 min at 4°, treated with Tris-buffered NH4C1 solution to lyse RBC, and resuspended at 2 x 10^7 cells/ml in RPMI-1640 supplemented with 5% fetal bovine serum. Spleens from homozygous nude mice, processed in a similar fashion, were used as controls. Sepharose-bound Con A was added to the cell suspensions at a final concentration of 100 μg of bound Con A per ml. The Con A Sepharose-stimulated spleen cell cultures, spleen cells in medium with uncoated Sepharose beads, or Con A Sepharose in medium (without lymphocytes) were put into 75 sq-cm tissue culture flasks (Falcon Plastics, Oxnard, Calif.). The flasks were placed on a gently rocking platform at 37° and allowed to incubate for 72 hr. The various spleen cell or control cultures were then centrifuged at 3000 rpm for 20 min to remove cells and Sepharose beads. The cell-free supernatants were filtered through a sterile 0.45-μm Millipore membrane, brought up to 20% fetal bovine serum, and stored at -80°.

Macrognage Activation Assay

A modification of our previously described technique (16, 17) was used to measure the ability of biological and synthetic agents to produce growth-inhibitory macrophages. This assay is advantageous for measuring macrophage-mediated cytotoxicity due to its sensitivity and allows for direct quantitation of viable target cell number. Approximately 4 x 10^6 peritoneal macrophages contained in 1 ml of RPMI-FBS were seeded in 16-mm wells on tissue culture cluster plates (Costar, Cambridge, Mass.). After 2 hr of incubation at 37° in a 5% CO_2-in-air atmosphere, nonadherent cells were removed by 3 cycles of aspiration with RPMI-1640. The adherent cells (macrophages) were then overlaid with 4 x 10^6 MBL-2 cells contained in 1.0 ml of RPMI-FBS. LK preparations or control materials were added in the desired concentrations in an additional 1.0 ml of RPMI-FBS. Toxicity controls consisting of MBL-2 cells alone in the presence of LK were also included in each experiment. The LK's were kept in the culture medium for the duration of the incubation. All cultures were maintained in a humidified, 5% CO_2-in-air incubator at 37°, and viable leukemia cells were counted daily with a hemocytometer. Triplicate cultures were quantitated at each time interval, and each experiment was repeated at least 3 times. The percentage of growth inhibition of MBL-2 cells due to macrophage-LK interaction was calculated by comparison to MBL-2 cells grown in the presence of normal macrophages alone.

RESULTS

Macrophage Activation in Vitro by LK Preparations. Both BCG-PPD MAF and Con A MAF were tested at a 10% final concentration for the ability to render macrophages tumoricidal (Charts 2 and 3). Allogeneic macrophages alone did not influence MBL-2 proliferation. Similarly, LK preparations were generally not directly cytotoxic for the leukemia cells, with the exception of a >48 hr exposure to BCG-PPD MAF (Chart 2). However, both LK preparations transformed normal resting macrophages into cytotoxic effector cells in vitro (Charts 2 and 3). Macrophage tumoricidal capacity was accompanied by increased spreading of macrophages on glass and increased adherence of tumor cells on macrophage membranes. Control materials consisting of supernatants from spleen cells alone, spleen cells incubated with Sepharose beads, or Con A Sepharose in medium (without lymphocytes) did not influence MBL-2 proliferation in the presence of macrophages.

CD2F1 spleen cells, nu/nu spleen cells, and CD2F1 thymus cells were incubated with insoluble Con A for 72 hr in an attempt to compare MAF production, and the resultant supernatant from each culture was added at different dilutions to normal macrophages. Tumoricidal macrophage activity was still present in supernatants from CD2F, spleen cells at the lowest concentration tested (3.75%) (Chart 4). Thymus cells from CD2F, mice proved to be a very poor source for MAF, as exemplified by the necessity of 60% supernatant in the culture medium to attain any degree of macrophage activation. These ranges of activity have been consistently observed for 3 separate preparations. Supernatants derived from nu/nu spleens lacked demonstrable MAF, indicating a T-cell requirement for the production of this mediator. No direct cytotoxicity was demonstrable by any of the supernatant materials in the absence of macrophages.
Macrophage Activation for Tumor Cytotoxicity

Similarly, the dose dependency of macrophage activation by 72-hr supernatants of PPD-stimulated BCG-immune spleen cell cultures was tested. Supernatants from BCG-immune spleen cell cultures with PPD added after collection of culture fluids were used as controls. Tumoricidal macrophages were present at final concentrations of BCG-PPD MAP at 1.9 to 60% in the assay medium (Chart 5). Dilutions of control supernatants did not induce macrophage activation.

Macrophage Activation in Vitro by L-Cell Interferon. The partially purified fibroblast interferon preparation at 10 units/ml transformed normal resting macrophages into cytotoxic effector cells in vitro in a fashion similar to MAP (Chart 6). Interferon preparations or allogeneic macrophages alone did not influence MBL-2 proliferation. Treated macrophages showed accelerated spreading on plastic as compared to control cultures.

For determination of the dose dependency of activation by interferon, the partially purified preparation was tested at log_{10} dilutions ranging from 10^4 to 10^8 units/ml in the culture medium. The percentage of growth inhibition by interferon-treated macrophages was 66 and 37% at 10 and 1 unit/ml, respectively; >10 units/ml produced 100% inhibition of MBL-2 cell growth. Direct inhibition of MBL-2 cell growth by interferon preparations in the absence of macrophages was not observed.

Since exposure of naive lymphocytes to Con A or treatment of BCG-immune lymphocytes with sensitizing antigen(s) produces substantial interferon levels, we attempted to determine whether MAF activity in LK preparations was related to interferon titer. Undiluted BCG-PPD MAP and Con A MAP were each determined to contain 54 and 125 units of interferon antiviral activity per ml, respectively. Since these LK's could be diluted to 0.5 and 0.25% of the assay medium, respectively, and still render macrophages cytotoxic for leukemia cells, their end point interferon titers were each 0.3 units/ml. Macrophage activation by L-cell interferon required exposure of resident peritoneal macrophages to at least 1 unit of interferon activity under our...
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Chart 5. Comparison of BCG-PPD MAP and control supernatant for ability to render macrophages tumoricidal. MAP was derived from BCG-immune spleen cells incubated for 72 hr in the presence of 100 μg of PPD per ml (•). Control supernatants were obtained from 72-hr BCG-immune spleen cultures that received PPD following the incubation period (○). Growth inhibition of MBL-2 cells after 48 hr in the presence of 4 x 10⁵ peritoneal macrophages plus the conditioned supernatants at the concentrations shown.

Similar Properties of MAP and Interferon. A number of physicochemical treatments were used to differentiate macrophage activation factors in LK and interferon preparations. However, all 3 of these diverse preparations behaved essentially the same. Prior exposure of either BCG-PPD MAF, Con A MAF, or L-cell interferon preparations to trypsin (100 μg/ml for 30 min at 37°) or heating at >80° for 30 min destroyed their ability to render macrophages nonspecifically tumoricidal, whereas all agents were stable at 56° for 30 min or at pH 2.0 for 24 hr. Preliminary molecular weight measurements as determined by filtration through Sephadex G-100 show that macrophage-activating and interferon activities in both BCG-PPD MAF and L-cell interferon preparations coincide at a molecular weight of approximately 45,000.

DISCUSSION

The significance of macrophage activation in various physiological processes is now receiving just recognition. The activated macrophage both protects the host against intracellular pathogens (7) and modulates cell proliferation, destroying cells with abnormal growth properties (8, 9). The ability of macrophages to discriminate between normal and tumor cells (9) and the proposed causal relationship between antitumor activity of immunoadjuvants and acquisition of macrophage tumoricidal function are of major importance with regard to the immunotherapy of neoplasia.

Interaction of T-lymphocytes with sensitizing antigen, as well as nonspecific mitogenic stimulation, allows for the release of biologically active substances into the culture medium. Among these mediators are MAF and interferon, and collaboration is required between both macrophage and lymphocyte in their production (4, 13). It is not clear whether the macrophage converts the stimuli to a cell-bound form to stabilize the interaction between T-lymphocyte and stimulatory agent (14) or whether the macrophage produced a "second signal" after the addition of stimulatory agent to the culture medium (15). Con A-treated spleen cells from CD2F1, but not from nu/nu mice, were a potent source of LK. In contrast, thymus cells were ineffective in production of both MAF and interferon. The inability of these cells to produce mediators under our culture conditions may reflect differences in the relative amount or maturity of macrophages in the thymus cell population as compared to the spleen. As noted by Fidler et al. (6), one cannot evoke a direct macrophage response to low doses of eluted Con A, since Con A in itself does not render macrophages cytotoxic, although tumor cell adhesion to macrophages is facilitated (R. M. Schultz, unpublished finding).

Due to the concurrent production of MAF and interferon in LK supernatants and the proposed involvement of fibroblast-derived interferon in modulating macrophage tumoricidal function, we sought to define further the agents that activate macrophages in both specific (antigen-stimulated) and nonspecific (mitogen-stimulated) LK preparations and to determine their possible relationship to interferon. The involvement of leukocyte interferon in macrophage activation appeared particularly appealing, since other known pathways of macrophage activation (i.e., polyanions, viruses, etc.) are conditions for interferon induction.

Several lines of evidence demonstrate the striking similarities between both specific and nonspecific MAF and fibroblast-derived interferon in the ability to render macrophages nonspecifically tumoricidal. (a) The kinetics of direct macrophage activation was similar with <24 hr of...
incubation, allowing for significant inhibition of target cell growth. This observation is in contrast to macrophage activation by polyanions, where cytotoxic activity is weaker and delayed (16). (b) Both LK and partially purified interferon were inactivated by trypsin, stable at pH 2, and stable at 56°, but were inactivated by 80° for 30 min. (c) Preliminary studies show that these agents that render macrophages cytotoxic are eluted in the same peak fractions when filtered through Sephadex G-100 (M.W., ~45,000). (d) The presence of interferon reside on the same or different molecules in LK preparations appeared to correlate with levels of interferon, and control supernatants that lacked MAF were similarly devoid of interferon. (e) End point MAF activity (by dilution) can be equated to similar interferon antiviral titers.

It is possible to envision that, in the scheme of immunological surveillance, antigen-induced LK's may have many of the antitumor and antimicrobial functions that have been ascribed to interferon induced by viruses, nucleic acids, and bacterial endotoxins. The questions that still remain unanswered are: Do the biological activities of MAF and interferon reside on the same or different molecules in LK preparations? Is MAF the same as the macrophage-activating factor elaborated in fibroblast cultures treated with interferon? This report shows similarities in the properties of these 2 agents, but much additional evidence is required to answer these questions definitively.

REFERENCES

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