Initiation of Murine Lymphokine Activated (lak) Cell Cytotoxicity by Adherent Cells and Prostaglandin E2

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INHIBITION OF MURINE LYMPHOKINE ACTIVATED KILLER (LAK) CELL CYTOTOXICITY BY ADHERENT CELLS AND PROSTAGLANDIN E2

BY

KIMBERLY G. ROE
B. S., University of Central Florida, 1984

THESIS

Submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology in the Graduate Studies Program of the College of Arts and Sciences University of Central Florida Orlando, Florida

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ABSTRACT

The effect of adherent cells, prostaglandin E₂ (PGE₂), and indomethacin on LAK cell activity of C57BL/6J (B6) mice was investigated. Depletion of adherent cells from splenocyte cultures prior to LAK cell generation increased LAK cell cytotoxicity by 60% compared to non-depleted splenocyte cultures. Depletion of adherent cells from splenocyte cultures following five days of incubation and prior to the LAK cell assay resulted in a similar enhancement. Indomethacin, an inhibitor of prostaglandin synthesis, enhanced LAK cell cytotoxicity in splenocyte cultures. PGE₂ inhibited LAK cell cytotoxicity of adherent cell depleted splenocytes 28% and 88% at $10^{-6}$ M and $10^{-5}$ M. In vivo treatment of mice with indomethacin increased LAK cell cytotoxicity two fold compared to ethanol treated controls. LAK cell cytotoxicity was greatest in splenocytes from indomethacin treated mice cultured with additional indomethacin at 10.0 ug/ml. This study describes methods which increase murine LAK cell cytotoxicity.
ACKNOWLEDGEMENTS

I would like to thank everyone who assisted in this research and in the preparation of this thesis. To the members of my committee for reviewing the manuscript and for their guidance during research and thesis preparation. To the editorial assistant, Candie Stewart, for assistance far beyond her job description. To my fellow graduate students, Sharareh Sazesh and Bonny Bass for their friendship and technical assistance. And especially, to my parents, for their support and encouragement and for always believing this could be done.
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INTRODUCTION

Immunological approaches to cancer therapy have been the focus of study for the past two decades (1, 2). Early clinical treatment protocols were directed toward immunization against specific tumor cells and/or tumor associated antigens. These attempts to immunize patients were unsuccessful and have been abandoned due to the inability to stimulate sufficiently strong responses to putative tumor antigens. Efforts to establish general immune stimulation that would concomitantly increase an antitumor response were also unsuccessful due to the general immunoincompetence of the tumor-bearing host (1).

Recent approaches in cancer therapy involve adoptive transfer of tumor specific cytotoxic cells (cytotoxic T lymphocytes) in an effort to mediate antitumor effects. This method has the advantage of not being restricted to immunocompetent recipients. Adoptive transfer of tumor specific cytotoxic cells is limited, however, because it is difficult to generate large numbers of these cells. Additionally, most tumors are weakly antigenic or nonantigenic, which prevents generation of cytotoxic T lymphocytes (2, 3).
Yron et al. (4) and Lotze et al. (5) were first to describe lymphocytes which, when cultured with the lymphokine interleukin-2 (IL-2), were capable of lysing fresh autologous tumor cells. These lymphokine-activated killer (LAK) cells can be generated in large numbers and are cytotoxic for many immunogenic and nonimmunogenic tumor cells. LAK cells thus have potential for the successful immunotherapy of cancer.

**Characteristics of LAK cells.** LAK cells are distinct from two other cytotoxic effectors, natural killer (NK) cells and cytotoxic T lymphocytes (CTL) (3, 6-9). LAK cells can be generated in vitro by addition of IL-2 to cultures of fresh human peripheral blood lymphocytes (PBL) from healthy individuals or to PBL cultures from tumor-bearing patients. LAK cells can also be generated by incubation of IL-2 with splenocytes of both normal and tumor-bearing mice.

The in vitro cytotoxicity of LAK cells directed against a variety of tumors has been extensively documented in both rodents and humans (5, 6, 9-14). LAK cells have been characterized as to the stimulus responsible for their activation, their target cell specificity, their mechanism of lysis, and the phenotypes of their precursor and effector cells.
Stimulus for activation. The only stimulus thus far shown to be required for generation of LAK cells is IL-2 (5, 6, 9, 11, 15). Treatment of human PBL with monoclonal antibody against the IL-2 receptor prior to culture with the lymphokine blocked LAK cell activation (9, 16).

Generation of LAK cells in the presence of IL-2 appears to be universal. Lymphocytes from all strains of mice thus far tested (6) and from both normal and tumor-bearing humans exhibited similar patterns of lysis after IL-2 exposure (7-9).

LAK cells are distinguished from other cytotoxic effectors by a requirement of only a single signal for activation. Classical CTLs require at least two, and possibly three, signals for activation: 1) antigen or mitogen, 2) IL-2, and 3) cytoxic cell differentiation factor, CCDF (13, 17, 18). Recently Yang et al. (18) have described the generation of a new class of cytotoxic effector cells, Lymphokine-Induced Cytotoxic Cells (LICC). These cells do not require stimulation with antigen or mitogen but differ from LAK cells in that both IL-2 and CCDF are required for stimulation. Classical NK effectors do not require activation (19). However, NK activity can be augmented by either interferon (20, 21) or IL-2 (22), resulting in a broadened target cell specificity against
fresh tumor cell lines, chemically modified cells, and virus infected cells (23).

**Target cell specificity.** LAK cells are specifically cytotoxic to a variety of fresh, noncultured, autologous, syngeneic or allogeneic primary and metastatic tumor cells. LAK cells lyse freshly isolated tumor cells that are resistant to lysis by NK cells. However, LAK cell activity directed against fresh normal cells has not been reported (6-8). Additionally, all cultured cell lines thus far tested are susceptible to LAK cell mediated lysis (24). Thus, LAK cell lysis is not limited by histocompatibility restrictions or by any currently conceivable antigenic molecular epitope (13). This broad spectrum of tumor lysis distinguishes LAK cells from conventional CTLs, which demonstrate antigen specificity (24), and classical NK cells, which lyse a limited number of tumors (23).

Recent studies have supported the hypothesis that individual LAK cells are "polyspecific" and capable of lysing a variety of target cells (12, 13). A common determinant on tumor cells which is not present on normal PBL may be the recognition structure necessary for LAK cell cytotoxicity. LAK cell mediated lysis of syngeneic concanavalin A (Con A)-induced lymphocyte blasts in the mouse (6), and of trinitrophenyl (TNP)-modified autologous
PBL in the human (13) indicate that LAK cells may operate via recognition of a form of "altered self" (13).

**Mechanism of lysis.** LAK cell cytotoxicity occurs via a granule exocytosis mechanism similar to that of other cytotoxic effectors. Cytoplasmic granules of LAK cells contain a cytolysin that has identical properties to that of NK cells and CTLs (14).

**Phenotypes of effector and target cells.** Distinctions between LAK cell precursors and effectors and those of CTLs or NK cells have been functionally demonstrated by a number of studies. These include: analysis of congenitally immunodeficient mice (24); pattern of precursor regeneration following gamma irradiation (19), bone marrow transplantation (25, 26), or sublethal dose of cyclophosphamide (27); bone marrow fractionation on Percoll discontinuous gradients (28); differential susceptibility to lysis of human oncogene-transfected tumor cells (29); tissue distribution (7); and requirement for proliferation (7).

The phenotype of LAK cell precursors and effectors and their relation to surface markers commonly expressed by NK cells and CTLs remains controversial. Early attempts to characterize the murine LAK cell precursor for the presence of the T cell marker, Thy-1, resulted in mixed findings. Rosenstein et al. (6), and Merluzzi et al. (25),
demonstrated the presence of Thy-1 on LAK cell precursors whereas Mule et al. did not (30, 31). Neither of these groups reported the existence of the NK marker, asialo GM-1, on the surface of LAK cell precursors. After activation with IL-2, however, Thy-1 and other T lymphocyte markers are consistently demonstrated on the surface of LAK effector cells (1, 6, 10, 25, 32-34). In humans, LAK cell precursors have T-cell and NK-cell markers, whereas the mature LAK effector cells have only T-cell markers (1, 30, 34). However, the most recent reports indicate that LAK cell precursors and effectors of mice and humans express NK associated markers on their surfaces (23, 35).

It has been proposed that there is not a single unique progenitor of LAK cell activity, but rather that multiple subsets of lymphocytes (predominantly NK cells) become cytotoxic in response to IL-2 (23). Alternatively, LAK cells may represent the expansion of a common point in the differentiation of CTLs and NK cells (16).

Adoptive transfer of in vitro generated LAK cells in mice. Infusion of in vitro generated LAK cells and systemic administration of low doses of IL-2 mediates significant tumor reduction in mice. Tumors susceptible to LAK cell killing include established pulmonary (3, 31, 33, 36, 37), and hepatic (38, 39) metastasis of varied histological types. No significant reduction of tumor
burden was accomplished using LAK cells alone. This reflects the absolute dependence of LAK cells on the continued presence of IL-2 (3, 5-9). In vivo proliferation of infused LAK cells is directly related to the dose of IL-2 administered (40, 41). Continuously administered low doses of IL-2 are most effective in maintaining the viability of LAK cells. The cytotoxic activity of these LAK cell effectors is enhanced compared to LAK cell effectors maintained with the same amount of IL-2 administered in a single dose (42, 43).

Treatment of tumor-bearing mice with high dose IL-2 alone. High doses of IL-2 alone (25000 - 100000 U/mouse injected intraperitoneally 3 times daily) have been effective in the treatment of intradermal tumors, lung and liver metastasis, and disseminated leukemia in mice (34, 38, 44). This treatment is thought to be mediated by the in vivo induction of LAK cells. This hypothesis is supported by the finding that the antitumor effects of IL-2 are eliminated if mice receive sublethal irradiation. This implies that IL-2 is not directly mediating tumor regression but is acting through a radiosensitive host component (34, 38). It has also been suggested that high dose IL-2 may mediate tumor eradication through the induction of lymphokine secretion (34, 44), or by augmentation of specific T cell immunity (44). These doses of IL-2 cause
significant toxicity in mice, and limit the amount of recombinant IL-2 that can be administered (38).

Adoptive immunotherapy of human cancers with LAK cells and recombinant IL-2. Animal models have been used to define parameters for adoptive immunotherapy of human cancers with LAK cells and recombinant IL-2 (RIL-2) (45-47). Objective regression of cancer has been noted in a number of patients maintained on these treatment protocols (46). However, the high toxicity of RIL-2 has limited the potential uses of such therapy in humans (46, 47).

The role of prostaglandins in regulation of the immune response. One class of compounds which has been shown to mediate killing by both CTLs and NK cells are the prostaglandins (48-56). Studies in animal models have demonstrated that prostaglandins of the E series (PGEs) modulate many immune responses (57-60). These include T lymphocyte proliferation (57, 58) and the production and secretion of IL-2 (59, 60). PGEs also inhibit human blood mononuclear cell proliferation by interfering with the production of IL-2 and with IL-2 independent proliferation (61-63). IL-2 receptor expression is not affected by treatment with PGEs (64). Walker et al. (63) reported that PGE$_2$ inhibited the IL-2 dependent cell cycle event which is required for T cell proliferation. Prostaglandin E$_2$
primarily exerts its inhibitory effect on lymphocyte proliferation through an inhibition of IL-2 production (63).

A population of glass-adherent mononuclear cells suppresses T-cell mitogenic activity through production of PGEs (57). These findings suggest that PGEs may serve as endogenous modulators of human immune reactions. Removal of glass-adherent cells from mononuclear populations results in increased IL-2 production by nonadherent cells.

Most of the particulate and soluble agents known to stimulate macrophages result in increased production of PGEs (58). This is consistent with the earlier finding that macrophages synthesize and release prostaglandins in response to inflammatory stimuli (65). Thus, PGE$_2$ secreted by macrophages may serve to regulate the production of IL-2 under physiological conditions (61).

Prostaglandins suppress NK activity. Addition of PGEs to murine NK cell assays results in marked and dose-dependent suppression of cytotoxicity (48, 53). Maximal suppression of cytotoxicity occurred when PGEs were present throughout the entire assay period. This indicates that PGEs act at the effector level in mediating inhibition of NK cell function. After treatment with indomethacin or aspirin, compounds which block PGE production, NK cell activity is restored to normal or near normal levels in tumor-bearing animals. Thus, PGEs are potent inhibitors of
NK cell activity in vitro and, under certain conditions, PGEs may inhibit NK cell activity in vivo (48).

In humans, NK cellular cytotoxicity can be activated or inhibited by PGE2 (50). T cells may not be involved in the control of PGE2 production. Their presence is necessary, however, for PGE2 mediated inhibition of NK cell activity (56).

Prostaglandins suppress CTL activity. PGE2 suppresses CTL activity (49, 51, 52, 54, 55). Wolf and Droege (55) have shown that endogenously produced PGE inhibits the production of IL-2. However, the addition of exogenous PGE2 to CTL cultures also inhibits cytotoxicity in a dose-dependent manner. These results support the hypothesis that PGE2 directly inhibits CTL responses.

The mechanism of inhibition by PGEs seems to be via an increase in intracellular levels of 3', 5' cyclic adenosine monophosphate (cAMP) (51, 52, 54). The modulating effects of the intracellular levels of cAMP upon the attacking cell population are dependent upon the concentration of this cyclic nucleotide at the moment of initial interaction with the target cells (54). Agents that increase cAMP levels, including PGEs, inhibit the activity of in vivo-generated CTLs. These agents have little inhibitory effect on the activity of in vitro-generated CTLs. It has been suggested
that culture induces nonspecific desensitization to cyclic AMP-active agents (52).

In vivo treatment with indomethacin inhibits metastasis in tumor-bearing mice. In vivo treatment of tumor-bearing mice with indomethacin causes a reduction in tumor metastasis (66-68). The mechanism of indomethacin mediated inhibition of tumor growth has been proposed to be via reversal of immunosuppression caused by endogenously produced prostaglandins (66). Alternatively, indomethacin may block PGE mediated inhibition of NK cell activity (68). The effects of indomethacin on LAK cell activity in mice have not been reported.

The purpose of the research presented here is to characterize the regulatory effects of adherent cells, PGE$_2$, and indomethacin on the murine lymphokine activated killer cell. Additionally, this report describes methods which significantly enhance the cytotoxic response of murine LAK cells.
MATERIALS AND METHODS

Animals. C57BL/6J (B6) mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Experiments were performed with male mice, 6 to 10 weeks of age.

Cell lines. P815 mastocytoma cells (69, 70) were maintained in culture by in vitro passage in Dulbecco's Modified Eagle medium (GIBCO, Grand Island, NY) supplemented with 10% v/v heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 ug/ml streptomycin, 60 ug/ml L-glutamine, and 5 x 10^{-3} M 2-mercaptoethanol.

Preparation of non-depleted splenocyte suspensions. Pooled whole splenocyte suspensions (non-depleted splenocytes) were prepared in a manner similar to the method of Merluzzi et al. (10). The spleens of 2 to 6 animals were aseptically removed and disassociated in supplemented RPMI-1640 medium (GIBCO) using a sterile syringe plunger. The resulting single-cell suspension was incubated at 0°C for 5 min to allow large debris to settle. The cells remaining in the supernatant were removed and washed once with RPMI-1640 medium. Viable cell counts were determined by trypan blue exclusion and cells were adjusted to the appropriate concentration in RPMI-1640 medium.
Adherent cell depletion prior to LAK cell generation.

Adherent cell depleted splenocyte cultures were prepared by adjusting non-depleted splenocyte suspensions to a concentration of $2 \times 10^6$ cells/ml. Aliquots of 15-20 ml were added to 100 x 20 mm plastic tissue culture dishes (Falcon, Becton Dickinson, Oxnard, CA) and incubated for 1 hr at 37°C in a 5% CO$_2$-95% air atmosphere. Following incubation the plates were washed vigorously with warm RPMI-1640 medium to remove non-adherent cells.

**RIL-2.** A recombinant DNA preparation of human IL-2 cloned in *Escherichia coli* was provided by the Cetus Corporation (Emeryville, CA) (15). Units of IL-2 were calculated from the total specific activity reported by the manufacturer. Activity was verified using the IL-2 dependent cell line HT-2 (71) and the standard microassay (72). One unit of activity is defined as the reciprocal of the dilution which results in one-half of the maximal proliferation of HT-2 cells (71, 72).

**Generation of LAK cells.** LAK Cells were generated by culturing non-depleted or adherent cell depleted splenocytes in 25-cm$^2$ tissue culture flasks (Corning Glassware, Corning, NY) containing 5-10 ml of RPMI-1640 medium. The final concentration of responders was adjusted to $5 \times 10^6$/ml. RIL-2 was added to these cells at a final
concentration of 1000 U/ml, the optimal concentration determined for generation of LAK cell cytotoxicity.

Culture flasks were incubated upright for 5-8 days at 37°C in 5% CO2-95% air atmosphere. After incubation, LAK cells were removed from the flasks and were used for in vitro cytotoxicity studies. Adherent cell-depleted LAK cell suspensions were obtained by removal of the flask contents only. Whole spleen (non-depleted) LAK cell suspensions were obtained as follows. Flask contents were removed as described above. Five ml of RPMI-1640 medium at 4°C was added to the flask and the flask was incubated at 0°C for 2 hr. The contents were then combined with that of the original flask. This low-temperature incubation allowed recovery of cells which had adhered to the plastic surface of the tissue culture flask during LAK cell generation.

**Determination of cytotoxicity.** A standard chromium-release assay was performed to determine LAK cell activity (10). Briefly, 100 uCi of $^{51}$Cr (New England Nuclear, Boston, MA) was added to a pellet of 8-10 x $10^6$ P815 tumor cells and incubated for 2 hr in a 37 C water bath. Cells were washed three times in Dulbecco's medium, and 100 ul containing $5 \times 10^4$ cells was added to 12 x 75 mm sterile test tubes (Fisher Scientific Products). LAK cells were harvested, washed once in RPMI-1640 medium, and added to each of three tubes to produce 100:1, 50:1, and 25:1
effector (LAK):target (P815) cell ratios. The cultures were mixed with a MLA pipette, centrifuged for 3 min at 30 x g, and incubated at 37°C for 4 hr in a humidified atmosphere with 5% CO₂ - 95% air. Following incubation, the tubes were recentrifuged at 400 x g for 5 min to pellet cells. The amount of radioactivity released into 0.150 ml of the supernatant of each culture was determined in a gamma counter. Spontaneous release controls were obtained from supernatants of target cells incubated in medium alone. Maximum release controls were determined from cultures in which target cells were incubated with a 1% v/v solution of NONIDET P40 detergent (Bethesda Research Laboratories, Rockville, MD). The percentage $^{51}$Cr release was determined from the following formula:

$$\frac{\text{Experimental Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release}} \times 100\%$$

Prostaglandin and indomethacin. Prostaglandin E₂ (PGE₂) and indomethacin were purchased from Sigma Chemical Co., St. Louis, MO. PGE₂ was dissolved in absolute ethanol to a concentration of $2 \times 10^{-3}$M, and stored at -20°C. This stock solution was later diluted in RPMI-1640 medium and added to cultures at concentrations of $10^{-7}$ to $10^{-5}$M. Controls containing equivalent concentrations of ethanol were included for each dilution of PGE₂ tested.
In the in vitro studies, indomethacin was dissolved in absolute ethanol at a concentration of 10 mg/ml and diluted to the appropriate concentrations with RPMI-1640 (see Results section for concentrations used). In the in vivo studies, groups of mice were provided with 14 ug/ml indomethacin in 0.16% ethanol ad libitum in drinking water for 21 days. The drinking water was changed twice weekly. Control animals received ethanol in their drinking water at a concentration equivalent to that present in the indomethacin preparations.

Statistics. Statistical differences in responses to various treatment protocols were determined by chi-square analysis.
RESULTS

Effect of RIL-2 concentration and culture period on LAK cell generation and cytotoxicity. Whole splenocytes (1.5 x 10^7) obtained from a single animal were incubated in the presence of RIL-2 for 3 or 5 days. Total viable cell counts were determined and cytotoxic activity was measured for duplicate cultures at 100:1 effector:target (E:T) cell ratios. At all RIL-2 concentrations, cell recovery was consistently less than 100%. Chi square analysis determined that no significant difference (p = 0.80) exists between the number of cells recovered at varying RIL-2 concentrations and culture periods (Fig. 1).

Cytotoxicity increased with increasing incubation time at all concentrations of RIL-2 tested (Fig. 2). Cell numbers were similar after both time periods (Fig. 1). Greatest LAK cell cytotoxicity was generated following 5 days incubation (Fig. 2).

Enhancement of cytotoxicity by centrifugation. Effectors and radiolabeled target (P815) cells were combined and centrifuged at 30 x g for 3 min prior to LAK

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Figure 1. Effect of RIL-2 concentration and culture period on growth of murine splenocytes.
Figure 2. Effect of RIL-2 concentration and culture period on LAK cell cytotoxicity.
cell assay. Centrifugation enhanced LAK cell cytotoxicity at higher concentrations of RIL-2 (>125 U/ml) (Fig. 3).

Effect of adherent cell depletion immediately prior to LAK cell assay. Splenocytes were cultured with RIL-2 for 5 days. Following incubation, adherent cell depleted and non-depleted splenocyte cultures were assayed for LAK cell cytotoxicity. Higher concentrations of RIL-2 (>125 U/ml) enhanced LAK cell cytotoxicity of both cell types assayed in a dose-dependent manner. The median enhancement of cytotoxicity of adherent cell depleted cultures was 60% compared to non-depleted cultures. Highest LAK cell cytotoxicity occurred with 1000 U/ml RIL-2 at 50:1 and 100:1 E:T ratios (Fig. 4).

Effect of adherent cell depletion prior to culture or prior to assay on LAK cell cytotoxicity. Adherent cell depleted (-ADH/CULTURE) and non-depleted splenocytes were cultured with 1,000 U/ml RIL-2 for 5 and 8 days. Some LAK cells generated from the non-depleted splenocyte cultures were depleted of adherent cells prior to the assay (-ADH/ASSAY). Cytotoxicity increased in all adherent cell depleted cultures compared to non-depleted cultures at each E:T ratio tested. The mean percent enhancement was 29.7% for suspensions depleted of adherent cells prior to assay and 40.5% for those depleted prior to culture (5 day incubation). Following 8 day culture, the mean percent
Figure 3. Enhancement of LAK cell cytotoxicity by centrifugation.
Figure 4. Effect of adherent cell depletion immediately prior to assay on LAK cell cytotoxicity. Non-depleted preparations contain both adherent and nonadherent cells. "ADH DEPLETED" indicates depletion of adherent cells immediately prior to assay (after culture with RIL-2).
RIL-2 CONCENTRATIONS:

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<th>Concentration</th>
<th>0 U/ml</th>
<th>125 U/ml</th>
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EFFECTOR:TARGET CELL RATIOS (X:1)

PERCENT CYTOTOXICITY

CELLS ASSAYED:
- NON-DEPLETED
- ADH DEPLETED
enhancement was 124% in cultures depleted of adherent cells prior to assay and 119% in those depleted prior to culture (Fig. 5). Adherent cell depletion, either before or after culture with RIL-2 resulted in a significant enhancement in cytotoxicity compared to non-depleted splenocytes (p < 0.005 for depletion prior to or following LAK cell generation).

**Effect of RIL-2 on cell number.** RIL-2 added to splenocyte cultures increased cell numbers compared to splenocytes cultured without RIL-2. The highest number of cells was recovered from adherent cell depleted splenocytes cultured with RIL-2 for 8 days (Fig. 6).

**Enhancement of LAK cell cytotoxicity by indomethacin.** Splenocytes were cultured with RIL-2 and indomethacin, an inhibitor of prostaglandin synthesis. Controls consisted of splenocyte cultures with RIL-2 plus medium (MEDIUM) or ethanol (EtOH). Splenocytes were collected after 5 days incubation and LAK cell cytotoxicity was determined. Indomethacin at concentrations greater than 2.5 ug/ml enhanced LAK cell cytotoxicity compared to ethanol and medium controls (Fig. 7).

**Effect of indomethacin addition immediately prior to assay on LAK cell cytotoxicity.** Adherent cell depleted and non-depleted splenocytes were cultured with RIL-2 for 5 days. Some non-depleted splenocyte cultures were depleted
Figure 5. Effect of adherent cell depletion prior to culture or prior to assay on LAK cell cytotoxicity. Non-depleted preparations contain both adherent and nonadherent cells. "-ADH/ASSAY" indicates adherent cell depletion on the surface of plastic tissue culture flasks prior to assay and following culture with RIL-2. "-ADH/CULTURE" indicates adherent cell depletion on plastic petri plates prior to culture with RIL-2.
5 DAY INCUBATION

8 DAY INCUBATION

RIF-2 CONCENTRATIONS:

- ADH/ASSAY
- ADH/CULTURE

EFFECTOR:TARGET CELL RATIOS (X:1)

PERCENT CYTOXICITY

[Graph showing concentration levels and cytotoxicity percentages]
Figure 6. Effect of RIL-2 and adherent cell depletion on growth of murine splenocytes. Legend is in Fig. 5.
Figure 7. Enhancement of LAK cell cytotoxicity following culture with indomethacin. Medium control (MEDIUM) consisted of splenocytes cultured with RIL-2 and RPMI-1640 medium only. Ethanol control (EtOH) consisted of splenocytes cultured with RIL-2 and RPMI-1640 medium plus 100% ethanol at concentrations equivalent to those present in indomethacin supplemented cultures.
CONCENTRATION INDOMETHACIN (ug/ml)

PERCENT CYTOTOXICITY

0 2.5 5.0 10.0

MEDIUM
EtOH
INDOMETHACIN

1000 U/ml RIL-2
5 DAYS CULTURE
of adherent cells before assay. Indomethacin was added to effector/target combinations immediately prior to LAK cell assay. Indomethacin did not significantly affect cytotoxic activity of any cell population at the concentrations tested (p-values = 0.50 for non-depleted splenocytes, 0.70 for adherent cell depletion prior to assay, and 0.30 for adherent cell depletion prior to culture) (Fig. 8).

Effect of PGE₂ on LAK cell cytotoxicity. Higher concentrations of PGE₂ inhibited LAK cell cytotoxicity of adherent cell depleted splenocyte cultures. This inhibition was 28% and 88% at $10^{-6}$M and $10^{-5}$M, respectively, compared to the appropriate alcohol controls (mean of 2 separate experiments) (Fig. 9). Prostaglandin E₂ suppressed LAK cell cytotoxicity of non-depleted splenocytes from the same animals at the highest concentration ($10^{-5}$M) tested (Fig. 10).

Effect of in vivo treatment of mice with indomethacin followed by in vitro culture of non-depleted splenocytes with indomethacin. Mice were treated with indomethacin or an equivalent concentration of ethanol in drinking water for 21 days. Prior to culture with RIL-2, indomethacin treated mice had elevated splenocyte counts compared to ethanol treated controls. Cell numbers were not significantly different for the treated or untreated groups following 5 days of culture (p=.90) (Table I).
Figure 8. Effect of indomethacin addition immediately prior to assay on LAK cell cytotoxicity. Legend is in Fig. 5.
PERCENT CYTOTOXICITY

1000 U/ml RIL-2
E:T RATIO = 50:1

CONCENTRATION INDOMETHACIN (ug/ml)

NON-DEPLETED
-ADH/ASSAY
-ADH/CULTURE
Figure 9. Effect of PGE$_2$ on LAK cell cytotoxicity of adherent cell depleted splenocyte cultures. "MEDIUM" consisted of splenocytes cultured with RIL-2 and RPMI-1640 medium only. "EtOH" consisted of splenocytes cultured with RIL-2 and RPMI-1640 medium plus 100% ethanol at concentrations equivalent to those present in PGE$_2$ supplemented cultures.
CONCENTRATION PGE₂

PERCENT CYTOTOXICITY

MEDIUM
EtOH
PGE₂

1000 U/ml RIL-2
5 DAY CULTURE
Figure 10. Effect of PGE$_2$ on LAK cell cytotoxicity of non-depleted splenocyte cultures. No IL-2 control (NO IL-2) consisted of splenocytes cultured with RPMI-1640 medium only. "MEDIUM" consisted of splenocytes cultured with RIL-2 and RPMI-1640 medium. "EtOH" consisted of splenocytes cultured with RIL-2 and RPMI-1640 medium plus 100% ethanol at concentrations equivalent to those present in PGE$_2$ supplemented cultures.
CONCENTRATION PGE$_2$

PERCENT CYTOTOXICITY

- NO IL-2
- MEDIUM
- EtOH
- PGE$_2$

1000 U/ml RIL-2
5 DAYS CULTURE
# TABLE I

**EFFECT OF IN VIVO TREATMENT WITH INDOMETHACIN ON SPLEEN CELL NUMBER AND LAK CELL GENERATION**

<table>
<thead>
<tr>
<th></th>
<th>Prior to Culture with RIL-2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Following Culture with RIL-2&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin Treated&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.2</td>
<td>8.9</td>
</tr>
<tr>
<td>Ethanol Controls&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.5</td>
<td>9.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Pooled spleen cell suspensions from 5 mice in each treatment group.

<sup>b</sup>Identical cell numbers (2.5 x 10<sup>7</sup>) cultured in the presence of 1000 U/ml RIL-2 for 5 days, washed once with supplemented RPMI-1640 medium, and enumerated.

<sup>c</sup>Animals maintained on 14 ug/ml indomethacin in drinking water for 21 days.

<sup>d</sup>Animals maintained on equivalent amounts of ethanol in drinking water for 21 days.
LAK cells were generated from indomethacin treated or alcohol treated animals. Indomethacin was added to some splenocyte cultures from both treatment groups. Indomethacin treatment of mice increased LAK cell cytotoxicity compared to that of alcohol treated controls. The highest cytotoxicity was expressed by splenocytes from indomethacin treated mice cultured with additional indomethacin at 10.0 ug/ml (Fig. 11).
Figure 11. Enhancement of LAK cell cytotoxicity by in vivo treatment with indomethacin followed by culture with additional indomethacin. Ethanol treatment indicates animals treated with ethanol in drinking water at a concentration equivalent to that used in indomethacin treatment.
IN VIVO TREATMENT:
- ETHANOL
- INDOMETHACIN
  14 ug/ml, 21 days

CONCENTRATION INDOMETHACIN (ug/ml)
ADDED AT BEGINNING OF CULTURE PERIOD
DISCUSSION

Various culture methods have been used by different investigators for generation of LAK cells and assay of their cytotoxic activity (4, 7, 10, 13, 16). At present, no standard protocol for LAK cell generation and/or assay exists. This study identifies parameters which directly affect LAK cell analysis. The most important of these include centrifugation of effector/target cell combinations and removal of adherent cells.

Low speed centrifugation of effector/target combinations greatly enhanced cytotoxicity in this study. Cytotoxicity is probably enhanced due to an increase in effector and target cell contact necessary for lysis. Some investigators have also incorporated a centrifugation step into their LAK cell assay (7-10, 35).

LAK cell precursors and effectors are nonadherent (6, 8, 35), however, many investigators do not report the routine depletion of adherent cells prior to murine LAK cell assay or adoptive immunotherapy (12, 19, 44-46). This study demonstrates a significant enhancement in murine LAK cell cytotoxicity if adherent cells are depleted either
before or after culture with RIL-2. This emphasizes the importance of adherent cell depletion in the preparation of LAK cells for in vitro studies or immunotherapy. This finding is consistent with recent reports of a similar suppressive action of adherent cells on human LAK cell generation (11, 73).

Adherent cell depleted cultures, which show maximum LAK cell cytotoxicity, probably contain a higher proportion of LAK cells compared to non-depleted cultures. However, this higher proportion of LAK cells cannot fully account for the enhancement of cytotoxicity shown here. At the lowest E:T ratio tested, adherent cell depleted splenocytes demonstrated increased cytotoxicity compared to the highest E:T ratios of non-depleted splenocytes. Even when the number of non-depleted effector cells is increased 4-fold (from 25:1 to 100:1 E:T ratios), adherent cell depleted cultures continue to express higher levels of cytotoxicity.

This explanation does not rule out the possibility that adherent cell depletion increases LAK cell cytotoxicity on a per cell basis. Limiting dilution experiments and development of a method to enumerate LAK cells would serve to determine whether this is the case.

The data in the present study showed that optimal LAK cell cytotoxicity was generated following 5 days culture with 1000 U/ml RIL-2. Total viable cell numbers recovered
were similar for all RIL-2 concentrations tested and both 3 and 5 day incubation periods. Viable cell recovery after culture with RIL-2 was consistently less than 100%. This is probably due to loss of non-RIL-2 responsive cells during the culture period. Measurable cytotoxicity was evident only in cultures incubated for 5 days at RIL-2 concentrations routinely used by others (<1000 U/ml) (3, 10, 13, 14, 19, 25, 26, 28-32, 36-39). These results suggest that while cell recovery remains relatively constant, the proportion of LAK cells continues to increase with increasing culture period. Increasing the amount of RIL-2 in splenocyte cultures 10-fold, increased cytotoxicity at both culture periods. However, lower concentrations were routinely used since quantities of RIL-2 available for this study were limited.

The enhancement of LAK cell cytotoxicity following culture with indomethacin, an inhibitor of prostaglandin synthesis, is indirect evidence that products of the cyclooxygenase pathway modulate LAK cell activity. Darrow and Tomar (49) reported that indomethacin enhanced the proliferation and induction of cytotoxicity of CTLs generated from mixed lymphocyte cultures. They further reported that indomethacin had no effect if added just prior to the chromium release assay. These results may be directly related to those of the present study in which
indomethacin had no effect when added immediately prior to the LAK cell assay.

Prostaglandins of the E series suppress cytotoxic activity of NK cells and CTLs in vitro at concentrations greater than $10^{-7}$ M (48, 49, 52, 59). Suppression of LAK cell cytotoxicity by direct addition of prostaglandin has not been reported to date. This study describes inhibition of LAK cell cytotoxicity when PGE$_2$ is added to adherent cell depleted cultures at similar concentrations. The low cytotoxic response observed at the highest concentration of PGE$_2$ tested ($10^{-5}$ M) may be due to direct toxic effects of this compound on the LAK cell effectors themselves. Prostaglandin E$_2$ was less effective on the inhibition of LAK cell cytotoxicity of non-depleted splenocytes. This may indicate that endogenous levels of PGs necessitate the addition of higher levels of exogenous PGE$_2$ to mediate the same inhibition.

Parhar and Lala (67) have recently described regression of metastasis after in vivo treatment of mice with indomethacin and RIL-2. This combination therapy also increased in vitro cytotoxicity directed against various tumor targets. The studies presented here indicate that in vivo treatment with indomethacin alone was sufficient to increase in vitro LAK cell cytotoxicity. Cytotoxicity could be further enhanced by culturing splenocytes from
indomethacin treated mice with additional indomethacin. This represents a new method for maximizing LAK cell cytotoxicity by a combination of in vivo and in vitro treatment with indomethacin.

The standard protocol for human adoptive immunotherapy includes infusion of in vitro generated autologous LAK cells and relatively high doses of RIL-2 (46). This approach is limited due to the toxicity of the RIL-2, and not the LAK cells themselves (46, 47). A pharmacological agent that would reduce RIL-2 toxicity without eliminating its clinical benefit would increase the application of this type of immunotherapy.

Indomethacin increased LAK cell cytotoxicity without observable side effects at the concentration used in this study for in vivo treatment. Although it has not been tested, it is quite possible that indomethacin might also alleviate the adverse side effects of RIL-2 through its reported anti-inflammatory properties (74).

Higher doses of indomethacin have been associated with a variety of adverse reactions in humans including gastrointestinal complications and bone marrow depression resulting in anemia and leukopenia (74, 75). During this study, in vivo indomethacin treated animals were examined daily and found to be healthy with no evidence of gastrointestinal complications, hair or weight loss.
Hematological depression was not observed in this study. These results suggest that treatment of humans with low doses of indomethacin might increase LAK cell cytotoxicity and reduce RIL-2 toxicity. Further studies will have to be undertaken to determine: (1) if in vivo treatment with both RIL-2 and indomethacin would increase LAK cell cytotoxicity and reduce IL-2 toxicity in mice, and (2) if similar levels of indomethacin could be safely achieved in humans before such treatment is attempted.

The studies presented here identify important parameters for LAK cell generation and assay. Additionally, these data indicate that adherent cells inhibit LAK cell cytotoxicity through production of prostaglandins and that these effects can be reversed by treatment with indomethacin.
LITERATURE CITED


