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Induction of lymphokine-activated killer cytotoxicity with interleukin-2 and tumor necrosis factor-α against primary lung cancer targets

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Summary. Human peripheral blood mononuclear cells (PBM) activated with recombinant interleukin-2 (IL-2) generate potent lytic activity (LAK) against a variety of malignant cells. IL-2 alone is sufficient for LAK generation, but high concentrations are needed to generate optimal cytotoxicity. Our recent studies based on combinations of biological agents indicated that alternative activation pathways may exist. Synergy for LAK induction was investigated using IL-2 and tumor necrosis factor-α (TNF). Single-cell suspensions of primary human lung carcinomas were prepared from seven established cell lines and 32 fresh tumor specimens. Not only were all cell lines sensitive to allogeneic LAK, but also all fresh tumors were sensitive to some degree to both autologous and allogeneic LAK lysis measured by a 4-h ^{51}Cr-release assay. LAK-mediated cytotoxicity, induced with a combination of human recombinant IL-2 (Cetus, 100 U/ml) and TNF (Genentech, 500 U/ml), showed a mean fourfold increase (range 0.7–16.3) over IL-2 alone. No lytic activity was generated from PBM incubated with media or TNF alone. The sequence dependence of adding IL-2 and TNF in enhancing cytolytic activity was also studied. In vitro kinetics data revealed that the addition of TNF 2–6 h before the addition of IL-2 greatly increased LAK activity over that obtained from the simultaneous addition of the two cytokines. These results demonstrated (a) the synergy of IL-2 and TNF for generating LAK; (b) the lysis of fresh primary lung cancer cells by LAK; and (c) the sequence dependence of IL-2 and TNF for the induction of optimal LAK activity.

Introduction

Lung cancer remains one of the most difficult malignant conditions to treat because of its aggressive nature and because many patients are at an advanced stage of disease at the time of their initial diagnosis. Patient survival remains poor – owing to systemic metastases – despite surgical resection and present adjuvant therapy protocols. Therefore, new systemic therapies are needed. Peripheral blood mononuclear cells (PBM) generate oncolytic activity after incubation with interleukin-2 (IL-2) [9]. This activity can be expressed against the patient's autochthonous tumor as well as against neoplasms unrelated to the major histocompatibility complex.

Tumor regression has been observed in animal models employing adoptive transfer of IL-2 and cells with lymphokine-activated killer (LAK) activity [5, 16, 18, 24] against a number of tumor types including melanomas, sarcomas, and renal cell carcinomas. In recent clinical trials [4, 6, 23, 25, 28–30], this method evoked some responses in patients with melanoma, renal cell carcinoma, colon carcinoma, and non-Hodgkin's lymphoma. The therapeutic efficacy of this therapy in human lung carcinoma has been studied in only a few patients. Those patients with advanced lung cancer responded poorly in reports of clinical trials [13, 23, 25, 30].

LAK activity against non-small cell lung cancer (NSCLC) targets has not been studied extensively either in vivo or in vitro. We undertook this study to determine the in vitro effects of IL-2 and tumor necrosis factor-α (TNF) in the induction of lymphocyte cytotoxicity against both cultured and fresh tumor cells derived from human primary NSCLC.

We previously observed [22] that the combination of IL-2 and TNF was shown to be synergistic in the generation of LAK. Under serum-free conditions, the simultaneous addition of TNF (500 U/ml) to low-dose IL-2 (10 U/ml) resulted in effector cytotoxicity equivalent to that observed with the relatively higher dose of IL-2 alone (100 U/ml). We, therefore, sought in this study to determine whether fresh NSCLC cells are susceptible to LAK lysis, and to evaluate cytotoxic effector activation using sequential combinations of IL-2 and TNF. The concentrations of the cytokines used for this investigation were not intended to duplicate conditions of current clinical trials, but rather to determine alternatives to the use of high-dose IL-2 alone for LAK induction for adoptive transfer, and may be more relevant for in vivo activation of LAK.

Materials and methods

Cell lines. All cell lines were derived from resected NSCLC lesions representing the three major histological types of

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NSCLC. The 5949 cell line was a gift from Dr. Eva Singletary, M.D. Anderson Cancer Center. NCI lines H596, H226, H322, H157, H460 (A), and H522 [2, 14] were generously supplied by Dr. A. Gazdar of the National Cancer Institute's Naval Medical Center, Bethesda, Md, USA.

Culture media. The culture media used to maintain all cell lines consisted of RPMI 1640, supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml glutamine, and either 1%, 5%, or 10% heat-inactivated fetal calf serum (Irvine Scientific, Santa Ana, Calif, USA) depending on the cell line. Serum-free medium (AIM-V) was generously given by Gibco Laboratories (Grand Island, NY, USA). Complete medium, consisting of RPMI 1640 and 5% pooled human AB serum (Gibco), was used to wash thawed PBM and target cells.

Cytokines. Human recombinant IL-2, produced from recombinant *E. coli*, was generously provided by the Cetus Corporation (Emeryville, Calif, USA), and human recombinant TNF by Genentech Inc. (San Francisco, Calif, USA). The specific activities of IL-2 and TNF were 3×10^6 U/mg and 5×10^7 U/mg protein, respectively. IL-2 was stored at −70° C in aliquots of 10^4 Cetus U/ml and TNF was stored at 4° C in stock concentrations until used to generate LAK activity.

PBM Preparation. PBM were obtained from healthy volunteers and from patients with primary lung carcinoma. Heparinized blood was diluted with Hanks balanced salt solution (HBSS, Hazelton Research Products Inc., Lenexor, Kan, USA) and fractionated on Histopaque (Sigma Chemical Co., St. Louis, Miss, USA) at 450 g for 30 min. PBM were isolated at the serum:gradient interface, removed carefully, washed three times in HBSS, and then either used fresh or cryopreserved in a −70° C freezer at a concentration of 10^8 cells/ml in 90% human AB serum and 10% dimethyl sulfoxide (Sigma). There was no significant difference in the level of LAK activation between fresh and frozen PBM (data not shown), therefore the latter was used for the present investigation.

Preparation of fresh lung cancer cells. Thirty-two fresh sterile surgical specimens were collected consecutively from 31 patients directly from the operating suite and transported under sterile conditions to the laboratory in RPMI 1640 with 100 U/ml penicillin and 100 μg/ml streptomycin on ice. The patients with NSCLC included 16 men and 15 women with a median age of 61 years (range, 42–88 years). Of the 31 patients, 2 had received chemotherapy and 4 had received radiotherapy before their operations.

Thirty-one specimens were derived from lesions in the lung parenchyma and one from a hilar lymph node. Pathological analysis confirmed the diagnosis of NSCLC in all patients. Histological diagnoses of these patients included adenocarcinoma (20), squamous cell (10) and large-cell carcinoma (1), and mesothelioma (1).

Single-cell suspensions of the tumor sample were prepared as described previously [10], with slight modifications. Visible clots of blood on the specimen were gently removed with forceps, followed by sharp dissection of normal tissue, necrotic areas, and blood clots away from viable tumor tissue. The tumor sample was then minced into 1–2-mm^2 pieces in 2 ml transport medium. The fragments, along with 10 ml transport medium, were then placed in a trypsinizing flask with constant stirring into a 37° C water bath for 15 min to dislodge attached red blood cells and necrotic cells. The supernatant was removed and replaced with 15 ml digestion medium consisting of 0.1% collagenase (Sigma type IV), 0.01% hyaluronidase (Sigma type V), and 0.002% DNase (Sigma type I) in RPMI 1640 with constant stirring at 37° C. After 15 min, the supernatant was removed and replaced with 15 ml digestion medium; this was repeated twice. The supernatant from the three fractions was collected and centrifuged at 200 *g* for 10 min. The pellet obtained was resuspended in 40 ml HBSS and fractionated on 10 cm^3 Histopaque (Sigma) to remove red blood cells and debris. The fraction at the gradient interface was carefully removed and washed three times in HBSS. A viable cell count was performed with trypan blue; the tumor cells were then cryopreserved at −70° C in 90% human AB serum and 10% dimethyl sulfoxide until the cytolytic studies were performed.

Effector cell activation. Autologous and allogeneic PBM were cultured in AIM-V medium at a concentration 1×10^6 cells/ml for 5 days at 37° C with 5% CO_2. During initial experiments, concentrations of IL-2 at 10 U/ml and 100 U/ml (Cetus units) with or without 500 U/ml TNF were used to activate PBM against culture cell lines. Maximal synergy was attained using 100 U/ml IL-2 and 500 U/ml TNF, and these concentrations were used as the standard conditions for activating PBM against fresh tumor targets. Cell recovery from cultures after 5 days, using IL-2 alone at both concentrations and with TNF, was found to be equivalent. For the sequence and interval-dependent kinetic experiments, IL-2 (10 U/ml) and TNF (500 U/ml) were used to activate PBM, either IL-2 first and then TNF, or in the reverse order, varying the time interval after the first cytokine was added. Culture conditions were the same as described above.

Cytotoxicity assay. LAK activity was determined from a standard 4-h ^{51}Cr-release assay as previously described [10]. Briefly, cryopreserved cultured or fresh tumor targets were rapidly thawed on the morning of the assay and washed twice in complete medium. The target cells were then labeled with 400 μCi ^{51}Cr (DuPont, Wilmington, Del, USA) for 2 h. After an additional 30 min of incubation with 5 ml complete medium, the cells were washed three times in AIM-V, adjusted to 2.5×10^4 cells/ml, and 0.1 ml target suspension was added to round-bottom microtiter plates (Costar, Cambridge, Mass, USA) in triplicate patterns. Activated effectors were washed three times in HBSS to remove all unbound IL-2 and TNF. Effector:target ratios of 100:1, 25:1, 6.3:1, and 1.5:1 were used, for a total volume of 0.2 ml/well. The plates were first centrifuged at 45 *g* for 5 min, then incubated for 4 h at 37° C in 5% CO_2. The supernatants were then harvested onto filter strips using the Skatron Collecting System (Skatron, Lier, Norway) and recorded on a gamma counter as counts per minute (cpm). The percentage of specific tumor lysis (% SL) was calculated by the formula:

$$\% \text{ SL} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\%$$

Spontaneous and maximum release (cpm) were considered to be ^{51}Cr released with the addition of 0.1 ml medium and

0.1 ml 0.1 M HCl alone, respectively, to 0.1-ml targets. Both spontaneous and maximum release were calculated as the mean of six wells each.

Calculation of lytic activity (LU). Specific lysis for each fresh and cultured tumor target was calculated from the mean of triplicate data obtained. The use of multiple effector:target ratios yields a sigmoidal killing curve, whereby a linear regression line of best fit is calculated from the linear portion of the curve. Lytic activity was then determined as 10^6 × the inverse of the number of effectors required to elicit 30% specific lysis of the targets.

Statistical analysis. Significant differences among lytic activities obtained from the assays were determined from the Wilcoxon rank sum test. Two-sided P values are represented in all experiments.

Results

Synergism of IL-2 and TNF on LAK generation measured against cultured cell lines

Previous investigations have shown that the maximal effector activation with IL-2 alone routinely occurs at IL-2 concentrations of 100 U/ml in serum-free medium and does not increase significantly when higher concentrations are employed [8, 11]. When PBM activated with 100 U/ml IL-2 alone were compared to PBM activated with 10 U/ml IL-2 and 500 U/ml TNF, the level of target lysis achieved was shown to be greater for the former conditions in a majority of the cell lines examined (Fig. 1). PBM incubated with 10 U/ml IL-2 alone had low levels of LAK activity. Further, the addition of 500 U/ml TNF to 100 U/ml IL-2 enhanced lytic activity compared to 100 U/ml IL-2 for the cultured targets examined. LAK activation using 1000 U/ml IL-2 with or without 500 U/ml TNF, however, did not significantly enhance lytic activity over that obtained using 100 U/ml IL-2 alone against these cell lines and fresh tumor targets (data not shown).

For these studies, all targets were resistant to NK (natural killer, non-activated) lymphocyte effectors and to those effectors incubated with TNF alone. Additionally, no enhanced lysis was observed when TNF was added directly to the cytotoxic assay with either non-activated or IL-2-activated lymphocytes (data not shown).

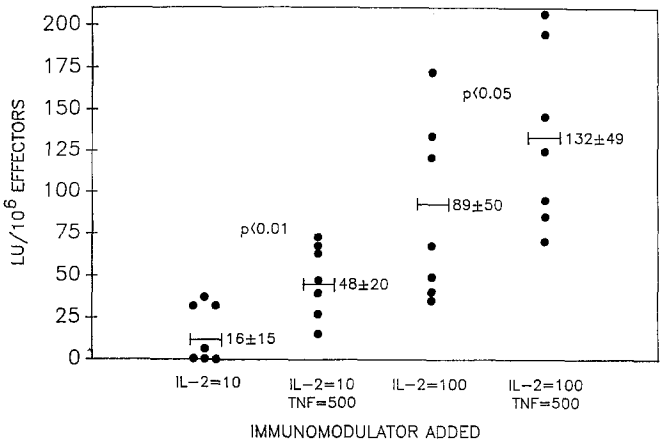

Fig. 1. Lysis of cultured human non-small cell lung cancer (NSCLC) cell lines. Normal donor peripheral blood mononuclear cells (PBM) were activated in AIM-V for 5 days under the four immunomodulator conditions listed. Oncolytic activity of each effector was then tested against seven NSCLC lines in a 4-h ^{51}Cr-release assay. All lines were sensitive to lymphokine-activated killer activity (LAK) induced with interleukin-2 (IL-2) alone. Activation with TNF significantly enhanced lytic activity against all cell lines over levels with IL-2 alone at low dose ($P < 0.01$) and high dose ($P < 0.05$) by the Wilcoxon rank-sum analysis

Lysis of fresh human lung carcinoma targets

All 32 primary NSCLC specimens were lysed to some degree by normal donor lymphocytes activated with 100 U/ml IL-2 alone, most being poorly sensitive. There was a wide range of lytic activity (0.1–22.0 LU), with a mean of 3.5 ± 4.6 LU. Again, all tumors were resistant to NK and TNF, and not lysed by lymphocytes incubated with TNF alone. The relative lytic potency of effectors activated by 100 U/ml IL-2 and 500 U/ml TNF against the fresh tumors was significantly increased ($P < 0.01$) to 8.1 ± 8.0 LU (range 0.3–35.1).

When directly compared within each experiment, the overall mean increases (-fold) seen with the addition of TNF over IL-2 alone were 4.0 ± 3.8 (median 3.8), with a range of 0.7–16.3, as depicted in Fig. 2. Augmentation of cytolysis with the combination of IL-2 and TNF was observed in all specimens (including those poorly sensitive) except the one from patient 8. The magnitude of cytolysis observed and prior therapy, histological cell type, degree of differentiation, or clinical stage of disease did not correlate significantly. However, of the 31 samples demonstrating lytic augmentation with TNF compared with IL-2 alone (24 had a greater than twofold increase), 15 of these were histologically classified as adenocarcinoma (Table 1). Lymphocytes from 9 adenocarcinomas evaluated showed a threefold or greater increase in lysis when the IL-2/TNF combination was used for effector activation.

Cytolysis of autologous compared with allogeneic LAK

The above data were generated using lymphocytes from normal volunteer donors. Therefore, it was of interest to determine whether a similar effect could be produced using PBM obtained from patients bearing NSCLC. For these studies, fresh tumor cell suspensions were tested for lytic susceptibility using normal donor autologous and allogeneic effectors. PBM were activated in medium containing IL-2 with and without TNF. All tumor samples were lysed by LAK from all three PBM sources. Tumor lysis was increased when PBM were cocultured with TNF and IL-2. When all IL-2/TNF-generated LAK effectors were tested against multiple tumor specimens, other variable levels of lytic activity again were observed. For three of the four tumor specimens, lysis by activated autologous PBM was higher than that by activated normal donor PBM (Table 2). In two of four cases, lysis by allogeneic NSCLC patient LAK was higher than that by LAK from a normal donor, but less than by autologous LAK. Significant differences in the synergistic effect of IL-2 and TNF among the three sources of PBM ($P < 0.1$) were noted. Thus, we found that significant antitumor activity could be generated using either autologous or allogeneic lymphocytes (normal donor or cancer-bearing patient).

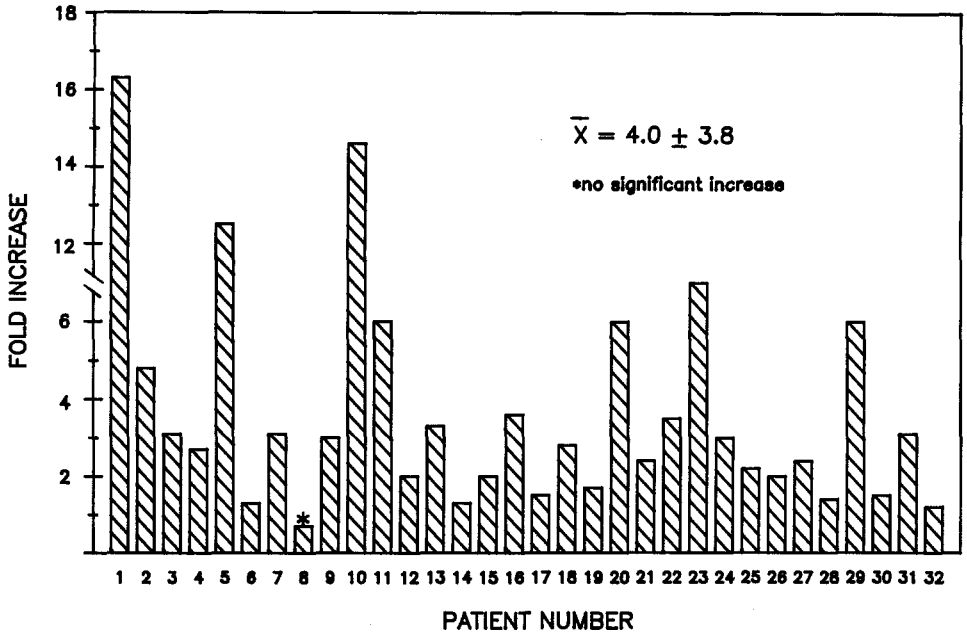

Fig. 2. Increase in lytic activity (-fold) with addition of TNF over IL-2 alone for fresh NSCLC tumor. Normal donor PBM were activated with 100 U/ml IL-2 in the presence or absence of 500 U/ml TNF for 5 days in AIM-V. Oncolytic activity of both effector types against each fresh tumor was measured as described in Materials and methods. Lytic activity, obtained by induction with IL-2 and TNF, was divided by that of IL-2 alone for each tumor target to obtain the increase (-fold). Induction of LAK activity was significantly increased with the addition of TNF over IL-2 alone against all 32 specimens except that from patient 8 (two-tailed paired *t*-test, $P < 0.05$)

Table 1. Increase in induction of lymphokine-activated killing (LAK) with tumor necrosis factor-α (TNF) plus interleukin-2 (IL-2) compared with IL-2 alone against fresh non-small cell lung cancer tumor cells, by histological type

Increase with TNF over IL-2 alone (-fold)	Number by histological cell type			
	Adeno-carcinoma	Squamous cell	Large cell	Meso-thelioma
<2	4	3	0	0
>2[a]	15	6	1	1
>3[a]	9	5	1	1

[a] Significance between adenocarcinoma and squamous cell groups, $P = 0.10$

Table 2. Lysis of fresh non-small cell lung cancer tumor by autologous[a] and allogeneic[b] LAK

Target	Effector origin	Lytic activity (LU)/10^6 effectors	
		IL-2[b]	IL-2+TNF[b]
Patient 4	Normal donor	0.72	2.24
	Autologous	1.21	1.72
	Patient 11	2.20	5.10
Patient 11	Normal donor	1.05	3.61
	Autologous	2.12	5.56
Patient 21	Normal donor	10.92	16.35
	Autologous	2.54	3.03
	Patient 11	4.30	5.44
Patient 31	Normal donor	3.18	6.90
	Autologous	43.57	67.29
	Patient 11	25.09	40.81

[a] Blood from NSCLC patients obtained 2 weeks after surgical resection

[b] Concentrations used: Il-2, 100 U/ml; TNF, 500 U/ml

Sequence and schedule dependence of IL-2 and TNF for LAK induction

It was observed from these series of experiments that enhanced lymphocyte-mediated lytic activity to NSCLC was directly related to the sequence of cytokine addition and to the time interval between administration. From a series of five experiments, the addition of TNF first, then IL-2 in activating PBM in vitro consistently enhanced LAK activity from three- to fivefold over that obtained either with the simultaneous addition of the two or with the reverse sequence (Il-2 then TNF). There was a minimal effect seen using 100 U/ml IL-2 for the sequence experiments; therefore, lower concentrations were used to increase the sensitivity of the assay. Representative data of effectors tested against the Raji and NCI-H460(A) cell lines are shown in Fig. 3.

The time interval between addition of the two cytokines was also found to be critical and is also depicted in Fig. 3. Augmented activity was found in all five experiments when TNF preceeded IL-2 in culture by between 2 and 6, but the activity decreased beyond this time period. A similar phenomenon was also observed when TNF followed IL-2 in culture; however, the increase in lytic activity during this time interval was not as great as that brought about by the inverted sequence.

Discussion

Primary lung cancer continues to be the leading cause of cancer-related deaths in the adult population of the United States [17]. Despite current cancer treatment modalities, systemic metastases continue to be the leading cause of mortality from this disease [3]. Because combination therapy, consisting of surgery, chemotherapy, and irradiation, translates into low patient survival rates for advanced disease, other modes of therapy have been sought.

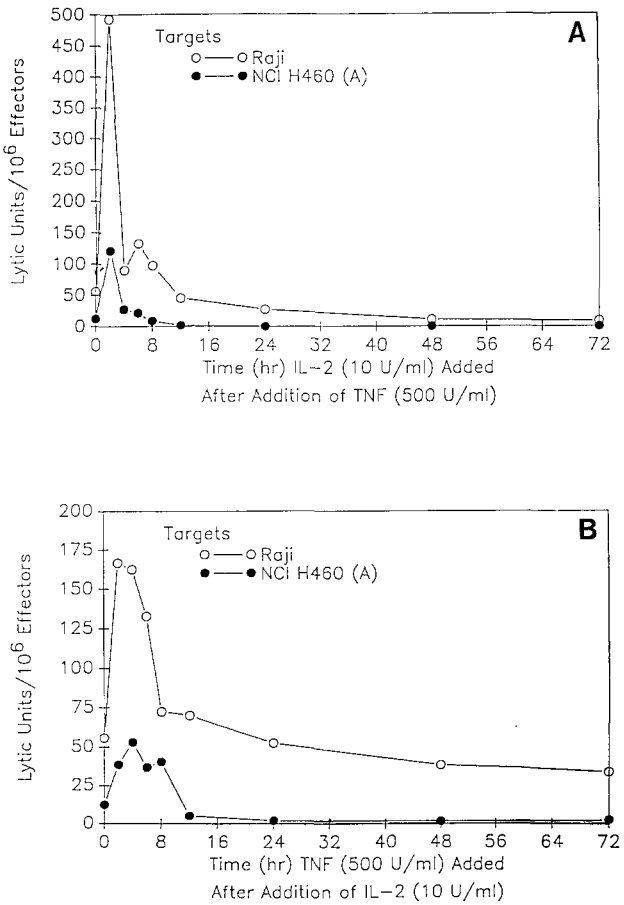

Fig. 3. Sequence and interval interdependence of IL-2 and TNF on cytolytic activity. IL-2 (10 U/ml) and TNF (500 U/ml) were added to PBM cultured in AIM-V in forward **(A)** and reverse **(B)** order at various increasing time intervals. Effector cytolytic activity (LU) for each of the cytokine combinations was measured after 5 days via a standard 4-h ^{51}Cr-release assay against Raji cell and cultured line NCI-H460(A) targets. Representative kinetics curves are depicted. Enhanced lytic activity was consistently observed in five out of five experiments when the addition of TNF preceeded IL-2 by 2–6 h

Our current study was undertaken to assess in vitro LAK activity in a histological spectrum of human primary lung tumors. All 7 cultured lines and 32 consecutively obtained fresh solid tumors had varying degrees of lytic sensitivity by LAK. The addition of TNF to IL-2 in the lymphocyte culture significantly augmented effector cytolysis of 31 of the fresh tumors (including the poorly sensitive specimens) and all cultured cell lines. Incubation of PBM with medium or TNF alone did not result in measurable oncolytic activity. Optimal induction of LAK was obtained using 100 U/ml IL-2 and 500 U/ml TNF against both fresh and cultured targets; the use of higher levels of IL-2 did not significantly increase lytic activity. This indicated that the synergistic effect of IL-2 and TNF for LAK induction results in effectors cytotoxic to cultured cell lines and fresh solid tumors. This combination for LAK induction has already been described in murine tumor models [19, 31]. No apparent correlation was found between the degree of tumor lysis when PBM were activated with IL-2 (in the presence of absence of TNF) and the histological tumor type or stage of disease at the time of surgical resection. Adenocarcinoma lesions seemed to be more susceptible to effector lysis ($P = 0.10$) when IL-2 and TNF were used for activation (Table 2).

The mechanism by which TNF augments IL-2-induced lymphocyte cytotoxicity is not well understood, although the role of TNF as an immunomodulator for cytotoxic lymphocytes has been well documented [20, 22]. Neither the incubation of PBM with TNF alone for five days nor the addition of TNF directly to tumor targets in a cytotoxic assay yielded evidence of tumor lysis (Yang et al., unpublished observations). This might further support evidence that TNF exerts its role on the LAK effector population as previously described [22]. Others have observed that TNF not only induces its own receptor, but also augments the high-affinity IL-2 receptor expression on activated T cells [26]. TNF is also known to stimulate cell proliferation; however, equivalent cell numbers were recovered using IL-2 alone and with TNF (data not shown).

Evidence for endogenous TNF production has been previously described [22] and supported by in vivo observations [27]. The synergistic effect of the IL-2/TNF combination is dependent on the dose of IL-2 (unpublished data) and may be explained by the endogenous production of other cytokines. This augmentation may ultimately, therefore, be shown to occur by an indirect pathway.

The interesting observations from this study on the sequence and interval dependence of IL-2 and TNF for LAK induction further support the above observations. The importance in first priming lymphocytes with TNF suggests a greater up-regulation of both TNF and IL-2 receptors than with the simultaneous addition of both cytokines, thus leading to enhanced LAK induction when IL-2 is added. The time interval of 2–6 h may be critical in the activation of these receptors by IL-2, which may down-regulate after this time period has passed. How the reverse sequence of adding IL-2 first and then TNF also enhances LAK induction is an enigma, but the production of other immunoregulators may play a predominant role in this situation.

As we have shown in this study, LAK activity may be generated against fresh tumors by both allogeneic and autologous PBM. Although lytic activity is generated from both populations, a specific effector function of autologous lymphocytes to lyse host tumor may still exist, which will require much detailed analysis and perhaps LAK cloning.

Although the degree of lysis of NSCLC cultured cells was markedly higher than that of fresh tumor cells, we studied in vitro cytolysis of fresh tumor in an effort to simulate in vivo interactions in the tumor environment. The rationale for using fresh tumor was based on the premise that it contains all naturally occuring heterogeneous tumor cell populations. Unlike fresh tumor targets, tumor cells growing in culture are likely to undergo antigenic and phenotypic modulation and, therefore, increase their susceptibility to LAK lysis. The lysis of fresh specimens in vitro by LAK activated with IL-2 alone has been shown in other tumor systems [1, 7, 10, 12, 15], but no extensive studies have been done against human NSCLC. Thus, the use of fresh tumor specimens for in vitro studies may be more reliable than the use of cultured cells and provide a more reasonable basis for proposed clinical therapy.

In summary, we showed that fresh human primary NSCLC tumors are sensitive to LAK lysis in vitro, and

that this effect can be potentiated with the addition of TNF. There was, however, no correlation between clinical parameters (previous therapy, histological tumor type, stage of disease) and the degree of lytic activity. Kinetics data revealed that the sequence and interval of cytokine addition influenced LAK induction, with enhanced oncolytic activity observed with TNF followed by IL-2. These findings demonstrated an alternative pathway for enhancement of LAK activity in vitro and suggested possible treatment applications for patients with NSCLC.

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