AUGMENTATION OF THE ANTI-TUMOR THERAPEUTIC EFFICACY OF LONG-TERM CULTURED T LYMPHOCYTES BY IN VIVO ADMINISTRATION OF PURIFIED INTERLEUKIN 2*

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Adoptively transferred cells immune to tumor-associated antigens have often been studied as a potential approach to experimental tumor therapy in animals (1). Although the lymphoid cells used have been largely derived by immunization in vivo, attempts have been made to enhance the therapeutic efficacy of immune cells by using techniques for secondary sensitization to tumor in vitro (2–6). The purpose of secondary sensitization in vitro of cells primed in vivo is to bypass mechanisms that limit effective sensitization in vivo and thus increase the quantity of effector cells (7–9).

The utility of in vitro sensitization has been limited by inefficient expansion of effector cells (10), in part because of progressive cell loss during culture (3, 4). However, culture with interleukin 2 (IL-2)[1] can potentially overcome these problems. Activated T lymphocytes can be induced to proliferate and expand numerically in vitro by IL-2 and can be maintained continuously, even without the continued presence of the activating antigen (11–15). By repeatedly supplementing cultures with IL-2, lymphocytes cytotoxic to tumors can be grown in vitro to large numbers (12, 16, 17).

We recently examined the efficacy of such long-term cultured T lymphocytes in the adoptive therapy of FBL-3, a syngeneic transplantable Friend virus-induced leukemia. In the model used, denoted as adoptive chemoimmunotherapy, advanced disseminated tumor can be successfully treated with a combination of cyclophosphamide (CY) plus cells from immune mice (18). The CY has a direct tumoricidal effect (18) as well as a potential facilitating role on host tumor immunity (19), and the therapeutic efficacy of donor cells is mediated by specifically immune T lymphocytes

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; CY, cyclophosphamide; FCS, fetal calf serum; IEF, isoelectric focusing; IL-2, interleukin 2; MST, median survival time; SDS, sodium dodecyl sulfate.

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(20, 21). In prior studies, donor lymphocytes were derived from mice immunized to FBL-3 and tested directly in therapy without culture (18). Recently, similar in vivo immunized cells have been cultured long-term with IL-2 after specific activation by secondary sensitization to FBL-3 in vitro and have been shown to be specifically cytotoxic to FBL-3 and able to mediate specific adoptive chemoimmunotherapy (21).

Such long-term cultured T lymphocytes are exquisitely dependent upon repeated exposure to exogenous IL-2 in vitro for both proliferation and survival in vitro (14). The current studies were performed to determine whether the in vivo efficacy of long-term cultured T lymphocytes might also be augmented by exogenously administered IL-2. The results demonstrated that although IL-2 had no detectable intrinsic antitumor activity when used alone or with CY, it significantly enhanced the therapeutic efficacy of long-term cultured T lymphocytes in adoptive chemoimmunotherapy.

Materials and Methods

Mice. 8- to 12-wk-old C57BL/6 mice were obtained from Simonsen Laboratory, Gilroy, CA.

Tumor. FBL-3 is a transplanted ascitic Friend virus-induced leukemia of C57BL/6 origin that possesses tumor-associated surface antigens that cross-react with other FMR tumors (22, 23).

Preparation of Concanavalin A (Con A)-stimulated Cell Supernatants. Conditions for the preparation of Con A supernatants used for cell culture were similar to those of Lafferty et al. (24) and have been previously described (21). Spleen cells from 6- to 12-wk-old BALB/c mice were removed, pooled, minced, forced through stainless steel mesh, and brought up to a concentration of 1×10^8 cells/ml in RPMI supplemented with Hepes, 2-mercaptoethanol, glutamine, sodium pyruvate, nonessential amino acids, antibiotics, and Con A at a concentration of 5 μg/ml. 3 ml of this cell suspension was incubated in a horizontal 75-cm^2 flask at 37°C for 2 h, which allowed the formation of a cell monolayer. The monolayers, washed three times to remove excess Con A and nonadherent cells, were incubated at 37°C for 18 h with 30 ml of the supplemented serum-free media. After incubation, the supernatants were decanted, centrifuged, passed through an 0.5-μm Nalgene filter, dialyzed, concentrated 10-fold, resterilized by millipore filtration, and stored at 15°C.

Preparation of Purified IL-2. IL-2 for in vivo therapy was derived from a radiation-induced murine splenic T cell lymphoma denoted as LBRM (26). IL-2 from cultured LBRM cells was purified biochemically as detailed elsewhere (25). The cells were cloned by a limiting dilution procedure, and the work described here used LBRM clone 5A4. To produce IL-2 for purification, LBRM cells were seeded in Falcon T75 tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) in medium supplemented with 10% fetal calf serum (FCS) and were grown to confluency. Culture medium was removed and replaced with serum-free medium containing 1% phytohemagglutinin (Gibco Laboratories, Grand Island Biological Company, Grand Island, NY) for 20 h. Culture supernatants were then removed, and the cells were removed by centrifugation at 10,000 *g* for 15 min. The resulting supernatant was then concentrated by ammonium sulfate precipitation.

For biochemical purification of IL-2, all chromatography was performed at 4°C by using sterile buffers. Concentrated supernatants containing IL-2 activity were dialyzed against 0.04 M NaCl-Hepes buffer (pH 7.2) and then fractionated by using DEAE-Sephacel, as detailed elsewhere (27, 28). The ionic strength of the fractions collected was determined by using a conductivity meter and then assayed for activity. Column fractions containing biologic activity were pooled and fractionated by gel filtration by using a 2- × 90-cm column of AcA54 (LKB Produkter, Sweden) equilibrated in 0.9% saline, as detailed elsewhere (27, 28).

Flat-bed isoelectric focusing (IEF) was performed in horizontal layers of Sephadex (27). Samples were prepared in 100 ml solution containing 1% glycine, 0.1% aspartic acid, and 2% amphylines (pH 3–10; LKB Produkter, Sweden). The sample was mixed with 5 gm Ultradex (LKB Produkter), and the gel suspension was spread in a gel tray. The tray was electrophoresed on a cooling tray for 20–24 h under a constant current of 7 mA, during which time the voltage

increased from 100 to 1,000 volts (27). After electrophoresis, the gel was sectioned into 30 portions, and the pH and IL-2 activity in each was determined. After flat-bed IEF, active fractions were pooled, concentrated by lyophilization and electrophoresis on a preparative slab, mono-Tris biline sodium dodecyl sulfate (SDS)-polyacrylamide gel system (29). Electrophoretic elution of a stained protein band (Rf corresponding to 26,000 mμ) yielded purified IL-2 used in these studies.

Before use in in vivo trials, electro-elated IL-2 was diluted to a concentration of 100 U IL-2/ml in 0.9% NaCl containing 1 μg/ml polyethylene glycol to aid in protection of the molecule during subsequent in vitro manipulation associated with injection.

Purified IL-2 has no interferon, macrophage/granulocyte colony-stimulating factor, interleukin 1, or burst promoting activity (30).

Assay for IL-2 Activity. The amount of IL-2 activity present in supernatant samples was determined in a standard microassay (14) based upon the IL-2-dependent proliferation of CTLL-2, a cytotoxic T cell line (12). Briefly, CTLL cells were cultured in 200-μl volumes in flat bottomed microplate wells in Clicks medium (Altick Associates, Hudson, WI) supplemented with 10% FCS, 300 μg/ml L-glutamine, 25 mM Hepes buffer, 16 mM $NaHCO_3$, 50 U/ml penicillin, and 50 μg/ml streptomycin. Each well contained 4,000 CTLL cells together with a $\log_2$ dilution (ranging from 0.1 to 50% by volume) of a putative IL-2-containing sample. After a 24-h incubation (37°C in a humidified atmosphere of 5% CO_2 and air) the microplate wells were pulsed with 0.5 Ci of [^{3}H]thymidine. Cultures were harvested 4 h later onto glass fiber filter strips, and [^{3}H]thymidine incorporation was determined via liquid scintillation. Results were quantified by probit analysis (14). IL-2 activity was expressed in units per ml by comparing experimental probit data with that obtained from assay of a standard IL-2 sample assigned a value of 1 U/ml (48-h tissue culture medium conditioned by the 5 μg/ml Con A stimulation of rat splenocytes (10^6 cells/ml)).

In Vivo Immunization of Donor Mice to Tumor. C57BL/6 mice were immunized by two weekly inoculations of 2×10^7 FBL-3 irradiated with 10,000 rad. 6 wk after the second inoculation, spleen cells were removed and teased to form single cell suspensions. Viable nucleated cells were enumerated by trypan blue dye exclusion.

Generation of Specifically Cytotoxic Long-Term Cultured T Lymphocytes for Adoptive Therapy. Lymphocytes from spleens of mice previously immunized to FBL-3, denoted as $C57_{\alpha FBL}$, were specifically activated by culture for 7 d with tumor and then nonspecifically expanded in number by subsequent culture with Con A supernatants containing IL-2 under conditions previously described (21). 60×10^6 stimulator cells ($C57_{\alpha FBl}$) were cultured for 7 d with 3×10^6 irradiated FBL-3 cells, $(FBL)_x$, in 20 ml of RPMI supplemented with FCS, 2-mercaptoethanol, and antibiotics, at 37°C in 5% CO_2 humidified atmosphere. By using simultaneous culture with a non-cross-reactive syngeneic tumor, EL-4(G-), and by serially examining culture cells for cytolytic reactivity in a standard 4-h chromium release assay, we previously demonstrated (21) that such secondary sensitization in vitro results in specific activation of tumor-specific cytotoxic cells.

After 7 d of culture, cells were washed, pooled, and reestablished in culture at a concentration of 1.5×10^5 cells/ml, under culture conditions as described above, but with the addition to media of concentrated Con A-stimulated cell supernatants at a 1:10 volume ratio. On day 10 and day 16 of culture, 8 ml of media was removed and replaced with fresh media at a concentration of 1.5×10^5 cells/ml. On day 19, cells were harvested, washed, and viable nucleated cells enumerated.

Such long-term cultured lymphocytes were expanded in number to ~700% and remained specifically cytotoxic to FBL-3. They were exquisitely dependent upon the presence of IL-2. Continued growth required repeated addition of supernatant from Con A-stimulated lymphocytes containing IL-2. Cells cultured without IL-2 supplementation died rapidly.

In Vivo Adoptive Chemoimmunotherapy Model. The assay for treating advanced disseminated FBL-3 with a combination of nonlethal, noncurative chemotherapy and adoptively transferred immune cells has previously been described (18, 20, 21). C57BL/6 mice inoculated with 5×10^6 FBL-3 on day 0 are treated on day 5. Treatment with CY prolongs the median survival time (MST) to ~4 wk but cures no mice. Therapy with immune cells alone on day 5 without CY has no detectable effect on survival. However, treatment with CY plus either noncultured

cells from mice immunized with FBL-3 or with similar cells after sequential activation in vitro and nonspecific expansion by culture with IL-2 prolongs survival and cures mice, depending on the dose of cells administered (21). Mice surviving to day 80 were considered cured because relapses are very rare after day 80. The specificity of adoptive chemoimmunotherapy with either noncultured or cultured immune cells has been confirmed in reciprocal specificity experiments (20, 21) using the adoptive chemoimmunotherapy of an antigenically distinct chemically induced tumor, EL-4(G-), in criss-cross therapy experiments.

Statistical Analysis. Survival curves were derived from cumulative experiments, with each data point representing the percentage of mice surviving on that day. The significance of the observed differences in survival times between groups was determined by a rank test designed for the analysis of multiple samples with censored observations (31). Two sample differences were confirmed using a generalized Wilcoxon test (32).

Results

To determine whether exogenously administered IL-2 could enhance the therapeutic efficacy of long-term cultured T lymphocytes in adoptive tumor therapy, mice bearing disseminated FBL-3 were treated with CY and long-term cultured T lymphocytes plus purified IL-2. Cells for therapy were derived from cultures of spleen cells obtained from C57BL/6 mice immunized in vivo with FBL-3, activated in vitro by culture for 7 d with irradiated FBL-3, and nonspecifically expanded in vitro to day 19 by repeated exposure to partially purified supernatants from Con A-stimulated lymphocytes containing IL-2. These long-term cultured T lymphocytes, denoted as $C57_{\alpha FBL}$ (cultured), were highly cytotoxic to FBL-3, but not EL-4(G-), an antigenically distinct C57BL tumor (Table I). Recipient specificity of cytotoxicity has previously been confirmed (21).

$C57_{\alpha FBL}$ (cultured) continue to proliferate in vitro and expand in number when repeatedly exposed to IL-2 in vitro. However, when cultured without IL-2, <10% remains viable at 72 h. When the proliferative capacity of $C57_{\alpha FBL}$ (cultured) is

TABLE I

*Effect of γ Irradiation on the Cytolytic Reactivity of Long-Term Cultured T Lymphocytes**

Radiation dose to cytotoxic effector cells	Percent specific lysis			
	FBL-3		EL-4(G-)	
	20:1	5:1	20:1	5:1
rad				
0	85	74	17	15
300	88	71		
1,200	83	67		
4,800	62	34		
9,600	29	12		

* Spleen cells from C57BL/6 mice that had been immunized in vivo with FBL-3 were cultured for 7 d with irradiated FBL-3 and then further cultured to day 19 in media supplemented with IL-2. Cytotoxicity against FBL-3 and EL-4(G-) was determined on day 19 in a 4-h chromium release assay at effector-to-target ratios of 20:1 and 5:1. Effector cells were irradiated with variable doses at a rate of 230 rad/min with a $^{137}CsCl$ gamma irradiator immediately before testing.

inhibited by γ irradiation with 1,200 rad, >95% of cells die within 72 h, despite culture with IL-2.

Before examining the in vivo effects of exogenous IL-2 on adoptively transferred long-term cultured T lymphocytes, the radiation sensitivity of long-term cultured T lymphocytes was determined both in vitro and in vivo to establish a potential role for donor cell proliferation in vivo. $C57_{\alpha FBL}$ (cultured) were treated with variable doses of γ irradiation and tested for cytolytic reactivity in a 4-h chromium release assay (Table I) and for efficacy in tumor therapy (Fig. 1). Cytolytic reactivity of long-term cultured T lymphocytes was inversely related to the dose of radiation. However, at a dose of 1,200 rad, which significantly inhibited cell proliferation, $C57_{\alpha FBL}$ (cultured) remained highly cytotoxic to FBL-3. To determine whether cytotoxic effector cells must be capable of proliferating to be effective in vivo, mice were treated with CY plus $C57_{\alpha FBL}$ (cultured) that had been irradiated with 1,200 rad, and results were compared to therapy with CY and nonirradiated $C57_{\alpha FBL}$ (cultured).

The cumulative data of two therapy experiments are presented in Fig. 1. Mice inoculated with 5×10^6 FBL-3 on day 0 and given no treatment had an MST of 11 d, and all died by day 16. Therapy on day 5 with CY alone (180 mg/kg) prolonged the MST to day 24. As an adjunct to CY, therapy with nonirradiated $C57_{\alpha FBL}$ (cultured) had a significant dose-dependent effect on survival. Thus, 2.5×10^6 $C57_{\alpha FBL}$ (cultured) prolonged the MST to day 38 ($P < 0.01$), 5×10^6 $C57_{\alpha FBL}$ (cultured) prolonged the MST to day 49, and 10×10^6 $C57_{\alpha FBL}$ (cultured) further prolonged survival and cured 11 of 16 mice. In contrast, therapy with CY plus 5×10^6 irradiated $C57_{\alpha FBL}$ (cultured) prolonged the MST to only day 30 and thus was significantly ($P < 0.01$) more effective than therapy with CY alone but less effective ($P < 0.01$) than therapy with one-half the dose of nonirradiated cells. These findings are consistent with the assumption that the ability of long-term cultured T lymphocytes to proliferate in vivo is not an absolute requirement for efficacy in therapy but is necessary for optimum therapeutic benefit and imply that the therapeutic efficacy of long-term cultured T lymphocytes might be augmented if the in vivo proliferation of such cells was enhanced or prolonged by exogenous IL-2.

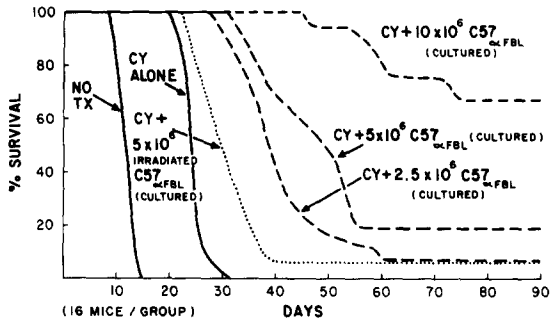

FIG. 1. Efficacy of irradiated long-term cultured T lymphocytes in adoptive chemoimmunotherapy of FBL-3. C57BL/6 mice inoculated intraperitoneally with 5×10^6 FBL-3 on day 0 received either no therapy (no Tx), treatment on day 5 with cyclophosphamide (CY alone) at a dose of 180 mg/kg, or therapy with CY plus variable doses of spleen cells from mice immunized in vivo with FBL-3, and subsequently secondarily sensitized in vitro and numerically expanded by culture through day 19 with IL-2, denoted as CY + $C57_{\alpha FBL}$ (cultured). Cells were tested directly from culture or immediately after 1,200 rad irradiation. The cumulative data from two experiments are presented.

The cumulative data of two therapy experiments examining the in vivo efficacy of purified IL-2 are presented in Fig. 2. Mice inoculated with 5×10^6 FBL-3 on day 0 and given no treatment had an MST of 11 d. Treatment with IL-2 alone, 80 U/d, on days 5–9, had no effect on survival. Therapy on day 5 with CY alone (180 mg/kg) or with CY plus Il-2 on days 5–9 prolonged the MST to day 24. Thus, IL-2 had no detectable anti-tumor effect either by itself or as an adjunct to CY.

Therapy on day 5 with CY plus 5×10^6 $C57_{\alpha FBL}$ (cultured) further prolonged the MST to day 38, but no mice were cured. In contrast, therapy on day 5 with CY and 5×10^6 $C57_{\alpha FBL}$ (cultured) plus 80 U/d of IL-2 on days 5–9 cured 11 of 16 mice. Thus, as an adjunct to CY, long-term cultured T lymphocytes were effective in tumor therapy, and their efficacy was significantly ($P < 0.01$) augmented by inoculation of exogenous IL-2.

Because exogenous IL-2 promotes continued proliferation and prolonged survival of long-term cultured T lymphocytes in vitro, exogenous IL-2 may augment the therapeutic efficacy of such cells by performing a similar function in vivo. This mechanism has not yet been established by quantitating in vivo proliferation of cultured cells in response to IL-2. However, if exogenously administered IL-2 augments the in vivo therapeutic efficacy of long-term cultured cells by stimulating continued clonal expansion in vivo, then the efficacy of IL-2 should be abolished by irradiating donor cells to prevent further proliferation. Thus, the ability of IL-2 to augment the therapeutic efficacy of irradiated $C57_{\alpha FBL}$ (cultured) was examined (Fig. 3). Mice inoculated with FBL-3 on day 0 and given no treatment all died by day 13. Treatment with CY alone prolonged the MST to day 24. Therapy with CY plus 5×10^6 $C57_{\alpha FBL}$ (cultured), which had been γ irradiated with 1,200 rad immediately before therapy, prolonged the MST to day 29, whereas therapy with CY and an equal number of irradiated $C57_{\alpha FBL}$ (cultured) plus IL-2 prolonged the MST to day 31 but was not significantly different ($P = 0.56$). By contrast, therapy with CY and 5×10^6 nonirradiated $C57_{\alpha FBL}$ (cultured) prolonged MST to day 38, and therapy with CY and 5×10^6 nonirradiated $C57_{\alpha FBL}$ (cultured) plus IL-2 prolonged the MST to day 47 and was significantly more effective ($P < 0.01$). Thus, the in vivo efficacy of

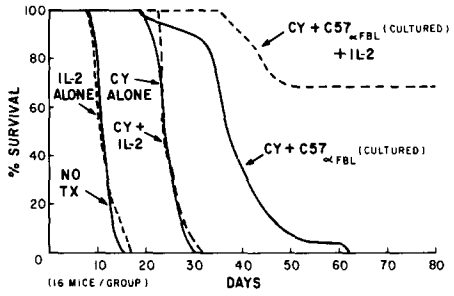

FIG. 2. Efficacy of purified IL-2 in vivo as an adjunct to long-term cultured T lymphocytes in adoptive chemoimmunotherapy. C57BL/6 mice inoculated intraperitoneally with 5×10^6 FBL-3 on day 0 received either no therapy [no Tx], treatment on days 5–9 with IL-2, 80 U/day (IL-2 alone), treatment on day 5 with cyclophosphamide at a dose of 180 mg/kg (CY alone), treatment with CY on day 5 plus IL-2 on days 5–9 (CY + IL-2), treatment on day 5 with CY and 5×10^6 long-term cultured T lymphocytes immune to FBL-3 (CY + $C57_{\alpha FBL}$ [cultured]), or treatment on day 5 with CY plus $C57_{\alpha FBL}$ (cultured) plus IL-2 on days 5–9 (CY + $C57_{\alpha FBL}$ [cultured] + IL-2).

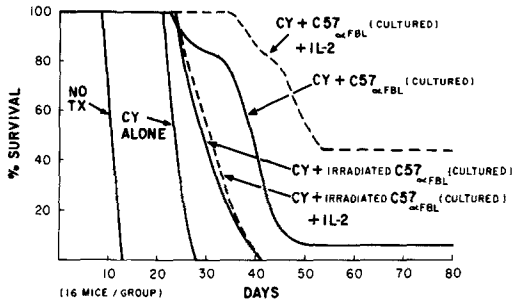

FIG. 3. Efficacy of purified IL-2 as an adjunct to irradiated long-term cultured T lymphocytes in adoptive chemoimmunotherapy of FBL-3. C57BL/6 mice inoculated on day 0 with FBL-3 received either no therapy (no Tx), treatment on day 5 with cyclophosphamide (CY alone), treatment on day 5 with CY plus nonirradiated long-term cultured T lymphocytes immune to FBL-3 (CY + $C57_{\alpha FBL}$ (cultured)), treatment on day 5 with CY and $C57_{\alpha FBL}$ (cultured) plus IL-2 on days 5–9 (CY + $C57_{\alpha FBL}$ [cultured] + IL 2), treatment on day 5 with CY and $C57_{\alpha FBL}$ (cultured) that had been irradiated with 1,200 rad immediately before therapy (CY + irradiated $C57_{\alpha FBL}$[cultured]), or treatment with CY and irradiated $C57_{\alpha FBL}$ (cultured) on day 5 plus IL-2 on days 5–9 (CY + irradiated $C57_{\alpha FBL}$ [cultured] + IL-2).

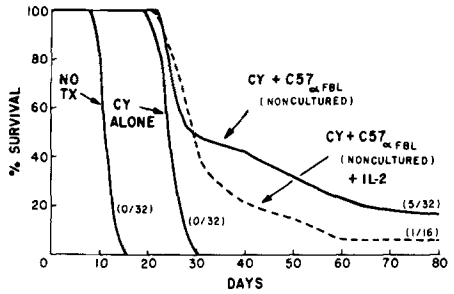

FIG. 4. Efficacy of purified IL-2 as an adjunct to noncultured immune cells in adoptive chemoimmunotherapy of FBL-3. C57BL/6 mice inoculated with FBL-3 on day 0 received either no therapy (no Tx), treatment on day 5 with cyclophosphamide (CY alone), treatment on day 5 with CY and noncultured immune cells (CY + $C57_{\alpha FBL}$ [noncultured]), or treatment on day 5 with CY and $C57_{\alpha FBL}$ (noncultured) plus IL-2 on days 5–9 (CY + $C57_{\alpha FBL}$ [noncultured] + IL-2). Fractions represent the number of mice surviving in the total cumulative group of two experiments.

exogenous IL-2 in adoptive chemoimmunotherapy required that the effector cell be capable of proliferating in the host.

In the preceding experiments, cells from mice immunized in vivo were grown in vitro by culture with IL-2 and then used for tumor therapy. These long-term cultured cells were exquisitely dependent upon IL-2 for survival in vitro, and their efficacy in vivo was augmented by exogenous IL-2. We subsequently examined whether exogenously administered IL-2 could augment the therapeutic efficacy of adoptively transferred noncultured donor lymphocytes that were specifically immune to tumor but not immediately dependent on exogenous IL-2 for survival (Fig. 4). Mice inoculated with 5×10^6 FBL-3 on day 0 and not treated died by day 16. Therapy on day 5 with CY alone prolonged the MST to day 24. Therapy with CY plus 5×10^6 noncultured immune spleen cells, denoted as $C57_{\alpha FBL}$ (noncultured), prolonged the MST to day 30 and cured 5 of 32 mice ($P < 0.01$). Therapy with CY plus 5×10^6 $C57_{\alpha FBL}$ (noncultured) plus 80 U/d of IL-2 on days 5–9 similarly prolonged the MST

to day 30 and cured 1 of 16 mice. Thus, although noncultured immune cells were effective in tumor therapy, their efficacy was not augmented by exogenous IL-2.

Discussion

Both noncultured immune cells and long-term cultured immune T lymphocytes are capable of mediating specific adoptive tumor therapy. At the time that they are used for therapy, noncultured immune cells are not a rapidly proliferating cell population (33) and are not directly cytotoxic to tumor (4). In contrast, long-term cultured T lymphocytes are rapidly proliferating and are directly cytolytic to tumor. Noncultured immune cells must be capable of proliferation in the host to be effective in therapy and must survive in the host for a minimum of 2 wk for maximum efficacy in therapy (34). Long-term cultured T lymphocytes are capable of lysing tumors at the time of therapy; thus, their ability to function in therapy need not necessarily require either further proliferation in the host or prolonged survival in the host. However, because their therapeutic efficacy is cell-dose dependent, any technique that induces numerical expansion of donor cells in vivo should augment their efficacy in vivo. Because the ability of long-term cultured cells to proliferate and survive, at least in vitro, is exquisitely dependent upon repeated exposure to exogenous IL-2, their ability to proliferate and survive in vivo, and thus their effectiveness in therapy, is likely to be dependent upon the availability of either endogenous or exogenous IL-2.

The in vivo distribution and availability of endogenous IL-2 is unknown because it is not readily detectable in serum by bioassay, and no radioimmunoassay for its presence has been reported. However, because the availability of endogenous IL-2 might limit the therapeutic efficacy of adoptively transferred long-term cultured T lymphocytes, the current studies were performed to determine whether exogenously administered IL-2 could augment the in vivo efficacy of such long-term cultured T lymphocytes. The results demonstrated that although IL-2 had no detectable intrinsic anti-tumor activity when used alone or with CY, it significantly augmented the therapeutic efficacy of long-term cultured T lymphocytes in adoptive chemoimmunotherapy.

In the present studies, supernatants from Con A-stimulated mouse spleen cells that had been only partially purified were used for cell culture in vitro, and purified IL-2 was used for therapy in vivo. Con A supernatants contain not only IL-2 but other lymphokines and monokines, such as colony-stimulating factors, interleukin 1 and immune interferon, as well as cell breakdown products and small amounts of residual lectin (24). Because the end point of culture was the growth of activated T lymphocytes, and because Con A supernatants supported growth well, the presence of these impurities was acceptable. However, such crude supernatants are inappropriate for testing the effect of IL-2 in vivo because the presence of impurities would render results uninterpretable. In fact, in preliminary experiments, partially purified Con A supernatants had a small direct anti-tumor effect in therapy. Thus, to determine the pharmacological anti-tumor effect of IL-2 in vivo, we used purified IL-2 that had been produced by the lymphoma cell line LBRM (26) and isolated by successive ammonium sulfate precipitation, gel exclusion chromotography (G-100), ion exchange chromotography (DEAE), preparative IEF, and SDS-poly acrylamide gel electropho-

resis (electroelution). This preparation migrates as a single band upon secondary SDS-poly acrylamide gel electrophoresis and contains no known functional activity other than those postulated to be associated with IL-2. It has been functionally screened and is negative for interferon, colony-stimulating factor, interleukin 1, and burst-promoting activity (30).

The mechanism by which IL-2 functions in vivo to augment the efficacy of long-term cultured T cells has not yet been established. However, mechanisms extraneous to, or independent of, the intrinsic functional properties of IL-2 were rendered unlikely by the use of purified IL-2. Because the effector cell for therapy is a T lymphocyte and because IL-2 provides for T cell growth in vitro, it is likely that exogenous IL-2 promotes in vivo growth of adoptively transferred cultured cells. This assumption is supported by the finding that IL-2 does not enhance the therapeutic efficacy of irradiated long-term cultured cells that are cytotoxic in vitro to tumor and modestly effective in therapy in vivo but that are incapable of further proliferation in the host. These data, however, do not rule out the possibility that radiation of donor cells inhibits a donor cell function vital to therapy other than the ability to proliferate, such as the ability to home to sites of tumor or the ability to survive in the host independent of the ability to proliferate. Thus, the final proof of donor cell growth in vivo in response to IL-2 will require actual quantitation of either donor cells or donor cell divisions in vivo.

Exogenous IL-2 augmented the in vivo efficacy of long-term cultured T lymphocytes but not the efficacy of noncultured immune cells. Augmentation of long-term cultured cells might simply reflect an exquisite dependence upon IL-2 for survival. Although the reasons for the inability of IL-2 to augment the in vivo efficacy of noncultured immune cells have not been determined, they may relate to multiple factors, such as donor cell subtype, the time of IL-2 administration, or the presence of serum inhibitors to IL-2 function after therapy with noncultured spleen cells but not long-term cultured T lymphocytes.

The predominant cell responsible for the therapeutic efficacy of noncultured immune cells is an immune Lyt 1^+2^- T cell that is not cytotoxic at the time of therapy (35). The mechanism by which these cells promote in vivo eradication of established FBL-3 is unknown but must reflect one or several of the T effector functions characterized by the subset—helper cells for antibody responses, initiators of DTH responses, and amplifiers of CTL responses. The inability of exogenous IL-2 to augment the efficacy of noncultured immune cells does not further define which of these mechanisms are operative. Helper cells for antibody responses and initiators of DTH responses grow in conditioned media containing IL-2 (36, 37). Although there is no evidence that amplifiers of CTL responses grow in response to IL-2, the cells that they amplify grow in response to IL-2. Thus, exogenous IL-2 could potentially augment the efficacy of any of these cell populations.

The inability of exogenous IL-2 to augment noncultured immune cells might reflect timing of IL-2 administration rather than donor T cell subtype. As an example, if the critical immune cell for adoptive chemoimmunotherapy of established tumor is an amplifier of cytotoxic cells, one might expect that tumor eradication would be delayed for a period of time after adoptive transfer (34), during which time expansion of the pool of cytotoxic effector cells may occur. During the early phase after therapy, endogenous IL-2 or IL-2 produced by donor cells might be sufficient to support

maximum in vivo clonal expansion of anti-tumor CTL, and exogenous IL-2 would be ineffective. However, during the latter phase after therapy, after significant clonal expansion of cytotoxic cells occurred in vivo, endogenous or donor cell IL-2 might be deficient in relationship to the expanded numbers of cytotoxic cells bearing IL-2 receptors or even depleted by such cells (38), and exogenous IL-2 might then be effective. This possibility has not yet been tested.

Another reason might be the presence of inhibitors to IL-2 functioning in the sera of mice after therapy with CY and noncultured immune spleen cells but not after therapy with CY and long-term cultured T lymphocytes. Sera of normal mice contain high levels of an inhibitor to IL-2 that is induced by Lyt 23^+ cells but that is dramatically decreased by moderate doses of CY (39). Thus, in our models, serum inhibition to IL-2 activity is presumably low after therapy with CY alone. However, if noncultured immune spleen cells are capable of reconstituting CY-treated mice with inhibitor-inducing cells, normal or increasing levels of IL-2 inhibitor activity might be present at the time of IL-2 administration. By contrast, if long-term cultured immune cells contain no such normal inhibitor-inducing cells, serum inhibitor activity at the time of IL-2 administration would be low.

Recently, it has been demonstrated that in vivo inoculation of partially purified supernatants from Con A-stimulated spleen cells containing IL-2 can restore cytotoxic T cell responsiveness in athymic *nu/nu* mice (40) and augment in vivo generation of cytotoxic T cells to TNP-modified cells in the draining lymph node of normal mice (41). Although the level of reactivity in those experiments was extremely low and the IL-2 was not purified, the results suggested a potential in vivo role for IL-2 and offered the hope that IL-2 could be used to augment T cell-mediated tumor therapy. The current studies confirmed this possibility by demonstrating that purified IL-2 can augment donor T cell immunity in vivo after therapy with long-term cultured T lymphocytes. Although exogenous IL-2 was unable to augment the therapeutic efficacy of noncultured immune cells under the conditions tested, further examination of these models to determine the mechanisms responsible for allowing and/or disallowing exogenous IL-2 to function in vivo might offer the possibility that pharmacologic IL-2 can be used to control other T cell reactions in vivo.

Summary

Spleen cells from C57BL/6 mice immunized in vivo with a syngeneic Friend virus-induced leukemia, FBL-3, were specifically activated by culture for 7 d with FBL-3, then nonspecifically induced to proliferate in vitro for 12 d by addition of supernatants from concanavalin A-stimulated lymphocytes containing interleukin 2 (IL-2). Such long-term cultured T lymphocytes have previously been shown to specifically lyse FBL-3 and to mediate specific adoptive therapy of advanced disseminated FBL-3 when used as an adjunct to cyclophosphamide (CY) in adoptive chemoimmunotherapy. Because the cultured cells are dependent upon IL-2 for proliferation and survival in vitro, their efficacy in vivo is potentially limited by the availability of endogenous IL-2. Thus, the aim of the current study was to determine whether exogenously administered purified IL-2 could augment the in vivo efficacy of long-term cultured T lymphocytes.

Purified IL-2 alone or as an adjunct to CY was ineffective in tumor therapy. However, IL-2 was extremely effective in augmenting the efficacy of IL-2-dependent

long-term cultured T lymphocytes in adoptive chemoimmunotherapy. The mechanism by which IL-2 functions in vivo is presumably by promoting in vivo growth and/or survival of adoptively transferred cells. This assumption was supported by the findings that IL-2 did not enhance the modest therapeutic efficacy of irradiated long-term cultured cells that were incapable of proliferating in the host and was ineffective in augmenting the in vivo efficacy of noncultured immune cells that are not immediately dependent upon exogenous IL-2 for survival.

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