In Vivo Enhancement of Antitumor Immunity by Interleukin-2-rich Lymphokines

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ABSTRACT

The ability of interleukin 2 (IL-2) to enhance in vivo antitumor immunity has been evaluated in the line 1 alveolar cell carcinoma (LI) model of BALB/c mice. A crude supernatant from phorbol myristate acetate exposed EL-4 cells rich in IL-2 plus other lymphokines (EL-4 IL-2), a concanavalin A-induced supernatant from murine splenocytes (Con A IL-2), and recombinant IL-2 (rIL-2) provided by Biogen were tested. Mice were immunized with a cloned population of LI cells (1×10^7 irradiated LI cells given s.c. in the left inguinal region) followed by s.c. injections of EL-4 IL-2, Con A IL-2, or rIL-2 given to the same site. Two immunizations of LI cells each followed by IL-2 administration were given prior to challenge with live LI cells s.c. on the right chest wall. Mice receiving EL-4 IL-2 survived significantly longer than those receiving LI cells only. Daily administration of EL-4 IL-2 for 7 days after the last LI immunization was significantly better than 3 days (P < 0.01) which in turn was significantly better than 1 day (P < 0.05). Among the doses tested (normalized in vitro to the Biologic Response Modifiers Program IL-2 standard) 404 units of IL-2 injection was optimal. The EL-4 IL-2 had to be injected adjacent to the site of LI cells; s.c. injection at a distant site or i.p. was not effective. When rIL-2 or Con A IL-2 was substituted for EL-4 IL-2, survival was not prolonged; however, if Con A IL-2 (low IL-2 levels) was supplemented with rIL-2 to 404 units of IL-2, it augmented immunity as well as 404 units of EL-4 IL-2. The data suggest that IL-2 is not the only lymphokine active in augmenting antitumor immunity induced by LI cells. Some preliminary experiments indicate that a multilymphokine approach may have potential clinical relevance.

INTRODUCTION

The recent body of experimental results describing the interactions of the immune system and the immunoregulatory lymphokines controlling it has provided new possibilities for tumor immunotherapy. One such lymphokine, IL-2, has received much attention because of its role in promoting the growth of T-lymphocytes (1), its ability to enhance natural killer activity (2), its ability to induce secretion of immune interferon (3), and most recently its ability to generate and maintain lymphokine activated killers (4). Our group, like many others, became interested in how IL-2 and other lymphokines might be used in vivo to improve on methods for generating tumor immunity. Our approach was based on recent findings in which IL-2 was used to boost an immune response against alloantigens (5–7).

Rosenberg et al. (5) demonstrated that concentrated IL-2 containing supernatants from EL-4 cells could markedly augment the generation of specific cytotoxicity. The IL-2 was not effective if given before or with the antigen challenges but clearly effective when given 2 to 5 days after antigen challenge.

Wagner et al. (6), working with trinitrophenyl-modified spleen cells, concluded that high concentrations of crude IL-2 preparations given locally with antigen were capable of boosting immunity. These in vivo experiments are consistent with the in vitro evidence that T-cells have a unique hormone receptor system requiring the continued presence of immune stimulation for IL-2 receptor expression (8). In the absence of stimulation, the number of IL-2 receptors decreases.

The experimental direction we have tested is the administration of a cell vaccine of syngeneic tumor cells followed by the injection of IL-2 rich lymphokines to the same local site. The tumor model chosen was the line 1 alveolar cell carcinoma (LI) which we had previously shown to be a weakly immunogenic tumor (9). Our results demonstrate that IL-2 rich lymphokines administered adjacent to a cell vaccine of irradiated LI cells augmented the immunity induced by the cells. Optimal dose, schedule, and sources of lymphokines are discussed.

MATERIALS AND METHODS

Animals. Eighteen- to 20-g female BALB/c J and BALB/c By J mice (The Jackson Laboratory, Bar Harbor, ME) were used.

Tumor Lines. The line 1 alveolar cell carcinoma arose spontaneously in a 12-month-old unirradiated female BALB/c mouse in the laboratory of Yuhas (10) from whom the tumor was obtained. The tumor was adapted to tissue culture and cloned by limiting dilution in laboratory to yield the clone designated L1/C1. This clone is maintained in culture in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% FBS (JR Scientific, Inc., Woodland, CA) and Eagle's minimal essential medium nonessential amino acids (0.1 mm) (Grand Island Biological Co.). The cells were grown in large batches and frozen in liquid nitrogen. Tumor cells were not used beyond 15 passages after the initial cloning date to minimize change that may occur through antigenic drift. EL-4 cells, a murine thymoma cell line, were a gift from Alfred E. Chang (Surgery Branch, NIH, Bethesda, MD). They were maintained in RPMI 1640 (Grand Island Biological Co.) supplemented with 10% FBS. HT2 cells, an IL-2-dependent murine cell line, were a gift from David Scott (Cancer Center, University of Rochester, Rochester, NY). They were maintained in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) supplemented with 10% FBS, 2-mercaptoethanol (5 × 10^-5 M) (Fischer Chemical Co., Rockland, IL), and a 1:10 dilution of Con A IL-2 (11).

Lymphokines from EL-4 Cells. IL-2-containing supernatants were prepared according to Farrar et al. (12) by harvesting 2-3 day-old EL-4 cultures and resuspending the cells to 10^6 cells/ml in RPMI 1640 supplemented with 1% FBS and phorbol-12-myristate-13-acetate, 10 ng/ml (Sigma Chemical Co., St. Louis, MO). Flasks containing approximately 250 ml were incubated for 48 h at 37°C (6% CO2, 95% humidity) at which time the supernatants were harvested, filtered, and assayed for IL-2 activity. Some batches were concentrated on an Amicon PM-10 filter (Amicon Corporation, Danvers, MA).

Recombinant IL-2. Human rIL-2 was a generous gift from the Biogen Research Co. (Cambridge, MA) and was reconstituted in 1.1 ml double distilled H2O as needed for each experiment. Each vial was then further diluted in M199 (Grand Island Biological Co.), M199 with 10% FBS, or Con A IL-2.

Lymphokines from Concanavalin A-induced Splenocytes. IL-2-containing supernatants were prepared according to Rosenberg et al. (11) with some modifications. Splenics of BALB/c J mice were aseptically removed, teased apart in M199, and counted. RBC were removed by taking aliquots of 10⁶ spleen cells and pelleting in 50-ml centrifuge
tubes. The pellet was resuspended in 20 ml of cold buffered ammonium chloride solution, shaken gently for 3 min on ice, and pelleted at 4°C for 10 min. The cells were centrifuged and washed twice. The final cell pellet was suspended in medium consisting of Dulbecco's modified Eagles' medium supplemented with 10% FBS, 2-mercaptoethanol, 1 mM sodium pyruvate (Grand Island Biological Co.), gentamycin, 100 µg/ml (Elkin-Sinn, Inc., Cherry Hill, NJ), and Con A, 5 µg/ml (Sigma Chemical Co.) to 10^7 cells/ml and incubated for 18–24 h at 37°C (6% CO₂, 95% humidity). Following incubation, the cells were centrifuged and the supernatant was depleted of Con A by adding preswollen Sephadex G-200 (Pharmacia Inc., Piscataway, NJ) at the ratio of 20 ml of Sephadex to each 80 ml of supernatant. The mixture was rocked for 1.5 h at 37°C. The Sephadex G-200 was spun out by centrifugation and the supernatant filter was sterilized. Con A IL-2 was kept at 4°C and used within 2 weeks (fresh Con A IL-2) or frozen immediately at –20°C and thawed as needed (frozen Con A IL-2).

Bioassy for IL-2 Activity. IL-2 was assayed as previously described (13). Serial 2-fold dilutions of a medium control, a standard IL-2 preparation obtained from the BRMP (Biological Resource Branch, NCI-FCRE, Frederick, MD) (defined as 500 units/ml), and our test samples were incubated with 10^6 HT-2 cells for 24 h at 37°C (6% CO₂, 95% humidity) followed by an 18-h pulse with [3H]thymidine (Amer sham Corporation, Arlington Heights, IL) (18–25 Ci/mmol; 2 µCi/20 µl/well). After the incubation period cells were harvested with a multiple automated sample harvester and uptake of [3H]thymidine was measured by liquid scintillation counting. The reciprocal of the dilution giving 50% of the maximum [3H]thymidine incorporation tentatively defined the number of units of IL-2 in each sample. The actual number of units was defined by the following equation.

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\text{Standardized units of IL-2 in test batch} = \frac{500 \text{ units/ml in defined standard}}{\text{tentatively defined units of IL-2 in BRMP standard}} \times \text{tentatively defined units of IL-2 in test batch}
\]

Immunizations. Two immunizations with L1/C1 cells were given at days 1 and 8 or at days 1 and 15. The cells were removed from liquid nitrogen, thawed rapidly, irradiated with 15,000 rads by exposure to a ^131Cs source, and washed twice in serum-free M199. The cells were diluted to 10^5 cells/ml in M199 and injected s.c. in the left inner thigh (day 1). Immunizations of 10^6 irradiated L1/C1 cells were begun on day 2 and were given weekly for 2–3 weeks to the right lower abdomen. EL-4 IL-2 was given at a dose of 404 units QD immediately adjacent to the vaccine site beginning on day 3 and continuing for 13–20 days. Radiation was used to slow the growth of the tumor and was given by exposing only the tumor bearing limb to a ^137Cs source while the mouse was tranquilized and in a restraining device. One thousand rads were given on day 4 or days 4 and 11. Mice were sacrificed when the tumor reached a diameter of 1 cm.

Statistical Analysis. The effectiveness of each immunization protocol was evaluated by comparing survival times of mice following tumor challenge. Survival comparisons were performed via the Wilcoxon and the Generalized Savage (Mantel-Cox) method after their estimation by the product limit method. When essentially the same comparison was made in more than one experiment the overall P for the comparison was obtained using logit (14) and Fisher's methods for combining independent tests.

RESULTS

Previous studies with the L1 tumor used an in vivo grown tumor and reported the 50% lethal dose at 470 cells for unimmunized mice and at 9 × 10^4 cells for immunized mice (5 weekly immunizations with 10^6 irradiated L1 cells and 3.5 µg of Corynebacterium parvum) (9). The tumor was adapted to tissue culture and dilution cloned to yield the population designated L1/C1. When mice were immunized with 10^6 irradiated L1/C1 cells (CV) weekly twice and challenged 1 week later, the calculated 50% lethal dose by regression analysis was 11,064 cells. The 95% confidence interval for this mean value was 8,293–14,763 cells. This same dose range was usually 100% lethal for unimmunized mice. In all but one experiment illustrated in this paper (Fig. 5) mice immunized with L1/C1 cells demonstrated prolonged survival compared to unimmunized controls.

In order to augment the immunity induced by L1/C1 cells further, we investigated the addition to this protocol of daily injections of a crude lymphokine preparation containing a high titer of IL-2 (EL-4 supernatant). Other lymphokines known to be present in this supernatant include colony stimulating factor, macrophage activating factor, B-cell growth factor and interferon (15). Each batch of EL-4 lymphokines was quantified on the basis of IL-2 content and standardized to the IL-2 standard of the BRMP. A typical batch had approximately 404 units of IL-2/ml and usually required concentrating 10–40 times before use.

Fig. 1 illustrates the results of our first experiment in which survival of mice immunized with CV plus a medium control was compared with survival of mice receiving CV plus EL-4 lymphokines at various doses and schedules. Although groups of mice receiving 1616 units of EL-4 IL-2 BID days 2–4 and 16–18 (D) using 12 mice/group. Lymphokines were given s.c. adjacent to CV. Challenge (3.3 × 10^6 cells) was done on day 22.

Fig. 1. Survival curves for groups of mice receiving no treatment (C), CV on days 1 and 15 plus medium control QD days 2–4 and 16–18 (E), CV plus 808 units EL-4 IL-2 QD days 2–4 and 16–18 (G), and CV plus 1616 units EL-4 IL-2 BID days 2–4 and 16–18 (H) using 12 mice/group. Lymphokines were given s.c. adjacent to CV. Challenge (3.3 × 10^6 cells) was done on day 22.
each CV (mean survival = 37.58 ± 5.28 (SE) days) and 808 units QD 3 days following each CV (mean survival = 36.00 ± 3.07 days) appeared to have improved survival, only the latter was statistically significant (P = 0.0263) when compared to mice receiving the CV plus medium control (mean survival = 28.55 ± 1.79 days). Mice receiving CV plus 808 units QD 1 day following each CV had a mean survival of 27.00 ± 2.58 days (data not shown).

The next two experiments explored different doses of EL-4 lymphokines given in the schedule, which resulted in the most tumor free survivors in Fig. 1: following each CV with lymphokines BID for 3 days. Figs. 2 and 3 illustrate the results for doses of IL-2 from 40.4-1616 units. In both experiments 404 units significantly prolonged the survival of mice when compared to groups of mice receiving the CV without additional lymphokines (P = 0.0175 and 0.0174, respectively); in addition, although not statistically significant when considered individually, the 2 doses higher (Fig. 2) and the 2 doses lower than 404 units (Fig. 3) also resulted in prolonged survival.

Within the two experiments in Figs. 1 and 2 we also examined whether the lymphokines need to be given adjacent to the CV or could be given i.p. or at a distant site s.c. The mean survival time for mice receiving 808 units of EL-4 IL-2 s.c. adjacent to the CV was 36.00 ± 3.07 days, whereas the mean survival time of mice receiving the same dose and schedule of lymphokines given i.p. was 25.83 ± 1.81 days (data not shown). The difference between these 2 survival curves was statistically significant (P = 0.0164). Fig. 4 illustrates the difference of giving 1616 units of EL-4 IL-2 s.c. adjacent to the CV (mean survival = 43.67 ± 4.17 days) compared to the same dose and schedule of lymphokines given i.p. was statistically significant (P = 0.0263) when compared to mice receiving CV plus medium control BID days 2-4 and 16-18 (D), CV plus 1616 units EL-4 IL-2 BID days 2-4 and 16-18, adjacent to CV (•), and CV plus 1616 units EL-4 IL-2 BID days 2-4 and 16-18, opposite leg to CV (x) using 10-12 mice/group. IL-2 was given s.c. adjacent to CV. Challenges (3.3 x 10^6 cells) was done on day 22.

shorten survival even below that of mice receiving CV plus medium control (mean survival = 37.42 ± 3.98 days).

In a separate experiment (data not shown) we also compared groups of mice receiving no treatment, CV only, EL-4 IL-2 only, and CV plus EL-4 IL-2. The mean survival time for each group was as follows: control, 15.33 ± 0.64 days; CV only, 21.14 ± 3.51 days; EL-4 IL-2 only, 14.79 ± 0.71 days; and CV plus EL-4 IL-2, 34.07 ± 6.35 days. These results demonstrate that the EL-4 IL-2 alone does not induce immunity to L1/C1 cells by itself but only augments the immunity induced by L1/C1 cells.

Fig. 5 illustrates the results of the last experiment investigating the dose and schedule of lymphokine administration. Once daily injections were again compared with BID injections plus an additional group of mice which received the lymphokines QD for 7 consecutive days. Although the challenge in this experiment was potent and a suboptimal dose of EL-4 IL-2 was used, mice receiving BID injections of lymphokines had significantly prolonged survival (mean survival = 22.43 ± 2.01 days) compared to mice receiving CV plus medium control (mean survival = 17.86 ± 0.71 days) (P = 0.0197). More importantly, mice receiving 7 days of lymphokines had significantly prolonged survival (mean survival = 32.36 ± 2.57 days) compared to the CV control group (P = 0.0000), CV plus IL-2 BID (P = 0.0076), and CV plus IL-2 QD (mean survival = 21.14 ± 2.13 days) (P = 0.0030).

The final immunoprophylaxis experiments in this study investigate whether rIL-2 alone, a Con A-induced supernatant from murine splenocytes (Con A IL-2) or Con A IL-2 supplemented with rIL-2 to 404 units could be substituted for the EL-4 lymphokine preparation. Fig. 6 illustrates the results for the doses 808, 4,040 and 16,160 units of rIL-2 and Fig. 7 for 404 units of rIL-2. Recombinant IL-2 appeared detrimental
The fresh Con A IL-2 was also tested alone. Fig. 8 illustrates these results. Only mice receiving fresh Con A IL-2 supplemented with rIL-2 (mean survival = 28.58 ± 3.64 days) had a prolonged survival compared to mice receiving CV alone (mean survival = 21.33 ± 2.72 days).

To test the potential clinical relevance of this approach, we used a therapeutic protocol of challenging the mice prior to therapy. Because L1/C1 cells have the cell growth kinetics of an aggressive tumor, we needed to delay the onset of rapid growth to allow time for the immunizing procedure; therefore, we added one or two treatments of radiation to the leg tumor. Fig. 9 illustrates our first experiment in the therapeutic setting. The lymphokine/vaccine approach, when combined with one radiation treatment (mean survival = 33.25 ± 1.51 days) significantly prolonged survival compared to controls (mean survival = 14.25 ± 0.16 days; \( P = 0.0000 \)), CV plus EL-4 IL-2 (mean survival = 17.75 ± 0.84 days; \( P = 0.0000 \)), and radiation only (mean survival = 24.0 ± 1.35 days; \( P = 0.0004 \)). Fig. 10 illustrates the results of giving 2 radiation treatments, increasing the number of treatments with the CV from 2–3, and extending EL-4 IL-2 treatments from 13–20 days. Three weeks of immunization and 2 radiation treatments resulted in a 50%
tumor free survival rate and prolonged survival (mean survival = 50.75 ± 2.84 days) compared to controls (mean survival = 13.88 ± 0.35 days; \( P = 0.0000 \)), 2 weeks of radiation only (mean survival = 34.17 ± 3.65 days; \( P = 0.0035 \)), and 2 weeks of immunization and 2 radiation treatments (mean survival = 45.25 ± 4.58 days).

DISCUSSION

Concentrated, unpurified EL-4 supernatants containing a high titer of IL-2 augmented the immunity induced by irradiated L1/C1 cells. This effect was dependent on both the dose and schedule of lymphokine administration. In experiments testing doses from 40.4-404 units (Fig. 3) and 404-1616 units (Chart 2), 404 units of EL-4 lymphokines titrated on IL-2 content was optimal.

The results of various schedules of lymphokine administration show the need for repeated daily injections. Administration of lymphokines for 7 days following the last CV was significantly better than 3 days (Fig. 5, \( P = 0.0030 \)) which in turn was significantly better than 1 day (\( P = 0.0312 \), data not shown).

In addition to dose and schedule, another important parameter to augmenting immunity induced by L1/C1 cells was the need for the lymphokines to be injected adjacent to the CV and not i.p. or at a distant site. Injection of the EL-4 IL-2 alone without CV was also of no benefit. These findings suggest that the lymphokine augmentation observed is mediated by lymphocytes which are in close contact with CV (perhaps at the lymph nodes draining the vaccine site) or perhaps an effect of the lymphokines directly on the tumor cells and not by a generalized systemic effect.

Since an EL-4 supernatant contains many lymphokines, we tested the ability of rIL-2 and Con A IL-2 to substitute for the supernatant. The experiments were done with human rIL-2 obtained from Biogen standardized to the same BRMP standard as our EL-4 supernatants. In no experiments did human rIL-2 augment the immunity induced by L1/C1 cells, either when tested at the optimal dose for EL-4 lymphokines (Fig. 7) or at 3 higher doses (Fig. 6). In fact, rIL-2 in M199 appeared to detract from the CV effect. Although no independent comparisons were significant, a pooling of Ps for mice receiving rIL-2 in M199 compared to mice receiving the CV alone results in a significant value (\( P = 0.0500 \)).

We can only speculate on the reason why rIL-2 in M199 or EL-4 IL-2 given in a distant site from CV is detrimental to L1/C1-induced immunity. Suppressor cells may be selectively generated in the absence of other lymphokines or in the absence of antigen. We are interested in pursuing whether these suppressor cells are generated and if so, whether they are specific for L1/C1-induced immunity or are acting nonspecifically.

One potential problem with our initial rIL-2 experiments (Fig. 6 and 2 other experiments not shown) was that we diluted the rIL-2 in M199 without a protein source. Hefeneider et al. (7) suggested that a carrier protein was needed to ensure the stability of rIL-2. The survival curves in Fig. 7 illustrate that although rIL-2 prepared with FBS was better than rIL-2 without serum there was still no improvement in survival over CV without rIL-2. A second potential problem was the possibility that rIL-2 is not completely soluble in aqueous solutions as suggested by Matory et al. (16); however, our results with Con A IL-2 demonstrate that the problem was not with rIL-2. When rIL-2 was used to supplement low levels of IL-2 in Con A IL-2, results comparable to EL-4 IL-2 were obtained. This finding suggests that IL-2 alone is insufficient to augment L1/C1-induced immunity and that additional lymphokines are required.

Because of our interest (17) in augmenting the immunity induced by autologous cell vaccines in patients with metastatic cancer using lymphokines, we are currently attempting to define the lymphokines responsible for enhancing immunity and testing the relevance of this approach in a therapeutic model. Finke et al. (18) has shown that the generation of cytotoxic T-lymphocytes in vitro requires a differentiation/activation signal distinct from IL-2. This factor, however, is thought to be macrophage derived and would therefore not be present in our EL-4 supernatants. Wagner et al. (19) has also postulated a factor distinct from IL-2 needed for the generation of cytotoxic lymphocytes but its origin is only speculative; it was found in a concanavalin A-induced supernatant of murine splenocytes but not in an interleukin 1-rich macrophage supernatant. We are currently trying to obtain other purified murine lymphokines to combine with rIL-2 to determine which are relevant in this model.

Our first two studies using CV and lymphokines in a therapeutic protocol show the clinical relevance of this approach. Our own studies (16) and a recent study by Hoover et al. (20) demonstrate that autologous cells plus adjuvants are capable of inducing systemic immunity. Local administration of lymphokines following vaccinations with autologous cells may augment the immunity induced by these cells and thereby increase the response rate.

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REFERENCES

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