# Interleukin 7 Promotes Long-Term In Vitro Growth of Antitumor Cytotoxic T Lymphocytes with Immunotherapeutic Efficacy In Vivo

By David H. Lynch and Robert E. Miller

*From the Department of Immunobiology, Immunex Research and Development Corp., Seattle, Washington 98101*

## Summary

A major obstacle to the effective use of adoptive immunotherapeutic treatment of cancer is the difficulty of obtaining tumor-reactive lymphocytes in either sufficient numbers or with appropriate in vivo function to make such an approach feasible. Previous studies have shown that antitumor cytotoxic T lymphocytes (CTL) with in vivo efficacy can be generated in vitro from lymphoid cells obtained from lymph nodes that drain the anatomical site of a tumor. Results presented here demonstrate that inclusion of interleukin 7 (IL-7) into the medium in which such CTL are cultured can support their growth in vitro for prolonged periods of time in the absence of repeated stimulation with either tumor stimulator cells or tumor antigen. More importantly, antitumor CTL propagated in medium containing IL-7 have retained both their antigenic specificity and their ability to reject tumors in vivo subsequent to intravenous injection. Parallel cultures of antitumor CTL similarly cultured in medium containing only IL-2 could only be maintained for 5–6 wk, after which the number and proportion of viable cells that were recoverable from such cultures progressively decreased. Phenotypic analysis of CTL maintained after extended culture (i.e., 22 mo) in medium containing IL-7 demonstrated them to be $CD3^+4^-8^+$ T cells. These cells were also found to express lymphocyte function associated 1, intercellular adhesion molecule 1, and Mel-14 cell interaction molecules. The data also demonstrate that these CTL do not require the presence of antigen-presenting cell populations to mount a proliferative response to tumor stimulator cells. Cells in these cultures were also demonstrated to produce IL-2 after stimulation with irradiated tumor cells, thereby indicating that these CTL have become independent of the requirement for $CD4^+$ helper cells to survive and function either in vitro or in vivo. Collectively, the findings that IL-7 can beneficially augment the generation, and propagate the long-term growth, of antitumor CTL from lymph nodes draining a tumor site may have profound implications for promoting the immunotherapeutic treatment of cancer in humans.

A major obstacle to the effective use of adoptive immunotherapeutic treatment of cancer is the difficulty of obtaining tumor-reactive lymphocytes in either sufficient numbers or with appropriate in vivo function to make such an approach feasible. Experimental attempts in murine systems to solve these problems have included the use of donors actively immunized with either irradiated tumor cells or tumor cells mixed with potent adjuvants as sources of primed tumor-reactive lymphoid cells for expansion in vitro (1–9). However, in the human situation, the luxury of syngeneic donors who can be immunized and from whom tumor-reactive T cells can be obtained and propagated for subsequent immunotherapeutic use does not exist.

The observation that solid tumor masses are often infiltrated by T cells (10–12) suggested the possibility that such tumor-infiltrating lymphocytes (TIL)[1] might be enriched for tumor-reactive T cells that could be expanded in vitro using recombinant cytokines such as IL-2 (13–18). However, even short-term culture of tumor-reactive T cells in growth-promoting concentrations of IL-2 enhances the generation of T cells that are dependent upon exogenous IL-2 for growth and survival in vitro and optimal function in vivo. Results from several studies have demonstrated the need for continuing treatment of the recipients with IL-2 after the infusion of antitumor effector cells in order to promote optimal function in vivo (7, 8, 19–22). As has previously been shown,

[1] *Abbreviations used in this paper:* DLN, draining lymph node; HSA, heat stable antigen; ICAM-1, intercellular adhesion molecule 1; TIL, tumor-infiltrating lymphocyte.


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administration of doses of IL-2 sufficient to maintain proliferation and effector function of transferred TIL can lead to significant toxicity. Further, even when using cultured TIL as a source of tumor-reactive lymphocytes it has been estimated that $>2 \times 10^{11}$ cells would be required to effectively treat patients with metastatic disease (23). These deficiencies continue to provide a major impetus for the development of techniques to enhance the in vivo efficacy of antitumor CTL generated in vitro.

LN that drain the anatomical site of a tumor contain increased numbers of lymphocytes, and these cells can differentiate into antitumor CTL during a short in vitro culture period (24, 25). We have previously demonstrated that intravenous injection of relatively modest numbers of antitumor CTL generated from such draining lymph nodes (DLN) are able to mediate tumor rejection in a systemic fashion and do not require ancillary cytokine treatment in order to perform this function (26). In addition, we have recently evaluated the effects of a number of cytokines and combinations of cytokines to augment the generation of therapeutically effective antitumor CTL from these DLN. In this regard, IL-7 was demonstrated to be substantially more potent than either IL-2 or IL-4 (27).

During the course of these studies we noted that addition of IL-7 to culture medium promoted the long-term growth and maintenance of antitumor CTL in vitro. The studies presented here were performed to characterize the CTL maintained in vitro for extended periods of time under such conditions and to evaluate their potential use in the immunotherapeutic treatment of cancer. The results clearly demonstrate that antitumor CTL can be maintained in vitro for prolonged periods of time in the absence of repeated stimulation with either tumor-stimulator cells or tumor antigen. More importantly, antitumor CTL propagated in medium containing IL-7 retain both their antigenic specificity and their ability to reject tumors in vivo subsequent to intravenous injection.

## Materials and Methods

*Mice.* Female C57BL/10J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were age-matched (10–12-wk-old) at the onset of each experiment.

*Tumors.* B10.2 and B10.5 are fibrosarcomas of B10 origin and have been described previously (26, 27). Tumor cell lines were maintained in vitro in $\alpha$-modified MEM containing 5% FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 $\mu$g/ml streptomycin. The cultures of tumor cells were routinely screened for the presence of mycoplasma using a detection system (Genprobe, San Diego, CA), and determined to be mycoplasma free.

*Tumor Immunization and Generation of Antitumor CTL.* Mice were injected in the hind footpads with $2–4 \times 10^6$ viable syngeneic tumor cells. DLN were aseptically excised 8–12 d later and dissociated into a single cell suspension. Cell cultures were established (in the absence of added tumor stimulator cells) in upright 25 $cm^2$ tissue culture flasks at $1.5 \times 10^6$ viable cells/ml (20 ml/flask) in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, $5 \times 10^{-5}$ M 2-ME, 50 $\mu$g/ml streptomycin, and 50 U/ml penicillin at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After 4 d in culture, the resulting CTL were subcultured in 24-well tissue culture plates at a concentration of $5 \times 10^5$ cells/ml (2 ml/well) in medium supplemented with IL-2 (5 ng/ml), IL-7 (10 ng/ml), or a mixture of IL-2 and IL-7 (2 and 10 ng/ml, respectively) with irradiated (10,000 rad) tumor stimulator cells. Unless otherwise noted, the subcultures were then maintained by weekly feeding with the appropriate medium in the absence of additional tumor cell stimulation. Cytotoxic activity of CTL was determined in a 6-h $^{51}Cr$-release assay. Percent specific $^{51}Cr$ release was calculated as $100 \times [(E - M)/(NP - M)]$, where $E$ = cpm released in the presence of effector cells, $M$ = cpm released in the presence of medium alone, and $NP$ = cpm released in the presence of 1% NP-40. LU determinations were based on E/T titration curves in which one LU was defined as the fraction of the initial culture causing 30% lysis of tumor target cells.

*Adoptive Immunotherapy of Tumors In Vivo.* To ensure that the anti-tumor immune responses being evaluated were solely due to the adoptively transferred cells, recipient mice were irradiated with 500 rad (using a $^{137}Cs$ source) to inhibit the generation of primary immune responses (28) and intravenously injected with cultured anti-tumor CTL. Mice that received either no cells or cultured lymphocytes obtained from regional LN of normal mice (CLN cells) served as controls. Mice were challenged with $5 \times 10^5$ tumor cells via intradermal injection in a midline ventral position in a total volume of 50 $\mu$l. Tumor challenges were initiated and completed within 1 h of the lymphoid cell transfer. Tumor size was calculated as the product of two perpendicular diameters of the tumors (as measured with calipers), and is expressed as the mean tumor size of all animals within a particular treatment group.

*Cytokines.* Recombinant murine IL-7 was expressed and purified to homogeneity as detailed elsewhere, and had a biological activity of $1.4 \times 10^5$ U/$\mu$g protein as determined by its ability to stimulate proliferation of 2B cells (29–31). Recombinant human IL-2 was expressed and purified to homogeneity as previously described, and had a biological activity of $2.4 \times 10^5$ U/$\mu$g as determined by its ability to stimulate proliferation of CTLL-2 cells (32–34).

*Antibodies.* The monoclonal rat anti-murine IL-2 Ab S4B6 (35) was graciously provided by Tim Mossman (University of Alberta, Edmonton, Alberta, Canada). Purified 11B11 Ab (36) was purchased from Verax Corp. (Lebanon, NH). FITC-labeled Abs to CD3 (clone 500A2), CD4 (clone L3T4), CD8 (clone 53–6.7), TCR-$\alpha/\beta$ (clone H57–597), TCR-$\gamma/\delta$ (clone GL-3), LFA-1 (CD11a, clone 2D7), intercellular adhesion molecule (ICAM-1) (clone 3E2), IL-2R $\alpha$ chain (clone 7D4), CD44 (Pgp-1, clone 1M7), and heat stable antigen (HSA) (clone J11d) were purchased from Pharmingen (San Diego, CA). Mel-14 (leukocyte cell adhesion molecule 1) was detected using a culture supernatant from the Mel-14 hybridoma (obtained from the American Type Culture Collection, Rockville, MD) followed by PE-conjugated anti-mouse Ig.

*Immunofluorescent Staining and Flow Cytometry.* 500,000 cells were preincubated for 30 min at 4°C with saturating concentrations of 2.4G2 mAb to block Fc receptors. The cells were washed once with PBS containing 0.1% BSA and 0.1% $NaN_3$, and then incubated for an additional 30 min at 4°C with saturating concentrations of the appropriate marker-specific Abs. The cells were then washed and analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA) using an argon laser that emitted visible light at 488 nm and 15 mW constant power. Data were collected on $10^4$ viable cells and analyzed using LYSYS II software (Becton Dickinson & Co.).

## Results

*In Vitro and In Vivo Function of Antitumor CTL Propagated in Cytokine-containing Medium.* To determine whether an-

titumor CTL could be maintained for extended periods of time in vitro in cytokine-containing media, lymphocytes from cultures of B10 anti-B10.5 DLN were recovered and recultured with irradiated tumor stimulator cells in either medium alone or medium containing IL-2, IL-7, or a combination of IL-2 and IL-7 ($10^6$ cells/culture). These cultures were subsequently passaged every 7–10 d in the appropriate medium in the the absence of further stimulation with irradiated tumor cells. Although the numbers of cells in all of the culture groups increased from the initial $10^6$ cells during the first week after restimulation, after 25 d the total number of viable cells recovered from cultures of cells maintained in medium alone had decreased to $<3 \times 10^4$. The number of cells recovered from cultures maintained in IL-2 had increased to $2 \times 10^6$ and cells cultured in IL-7 or IL-2 and IL-7 had increased to $8 \times 10^6$ and $11 \times 10^6$, respectively. After the removal of $10^6$ cells from each of these cultures for assessment of tumoricidal activity in vitro (discussed below), cultures of the remaining cells were maintained in fresh media containing the appropriate cytokine(s). After an additional 7 d of culture, CTL grown in IL-2 increased from $10^6$ to $5.6 \times 10^6$ cells, CTL grown in IL-7 increased from $7.0 \times 10^6$ to $17.5 \times 10^6$ cells, and CTL cultured in a combination of IL-2 and IL-7 increased from $10 \times 10^6$ to $32 \times 10^6$ cells. Thereafter, cells from cultures maintained in IL-2 decreased in both total number and viability until $<10^6$ were recoverable by day 60 of culture. In contrast, CTL cultured in either IL-7 alone or a combination of IL-2 and IL-7 slowly increased during this time period with average doubling times of 5–6 d.

Analysis of effector cell function of the CTL maintained in vitro for either 25, 32, or 53 d after their initiation demonstrated potent and specific tumoricidal activity in vitro (Fig. 1, *A*, *B*, and *C*, respectively). Flow cytometric analysis of these three cell populations at day 32 of culture revealed similar profiles in which 100% of the cells were Thy $1.2^+$, $CD3^+$ T cells (Fig. 2). Of these, 95% were $CD8^+$ and ~3–4% were $CD4^+$.

The ability of these populations of CTL to mediate tumor rejection in vivo was initially tested on day 32 of culture by intravenously injecting $2 \times 10^6$ antitumor CTL maintained in either IL-7 or IL-2 and IL-7 into 500 rad irradiated recipients, followed by bilateral tumor challenge with B10.5 and B10.2 tumor cells. Recoveries of cells from cultures maintained in IL-2 alone were too low to permit functional assessment in vivo. Mice that received anti-B10.5 CTL maintained in either IL-7 alone or a combination of IL-2 and IL-7 effectively rejected challenges of B10.5 tumor cells, but not B10.2 tumor cells (Fig. 3 *A*). Growth of the B10.2 tumor challenge cannot be ascribed to an inherent inability of this tumor to be rejected because B10.2 tumor cells were eliminated by anti-B10.2 CTL freshly generated from cultures of B10 anti-B10.2 DLN cells (Fig. 3 *B*).

During the course of these experiments we noted that the tumoricidal activity of the anti-B10.5 CTL slowly decreased as a function of increasing time in culture. For example, the lytic activity of CTL maintained in IL-2 and IL-7 could not be determined at day 25 (because plateau-level killing of target cells was observed even at the lowest E/T ratio tested), whereas

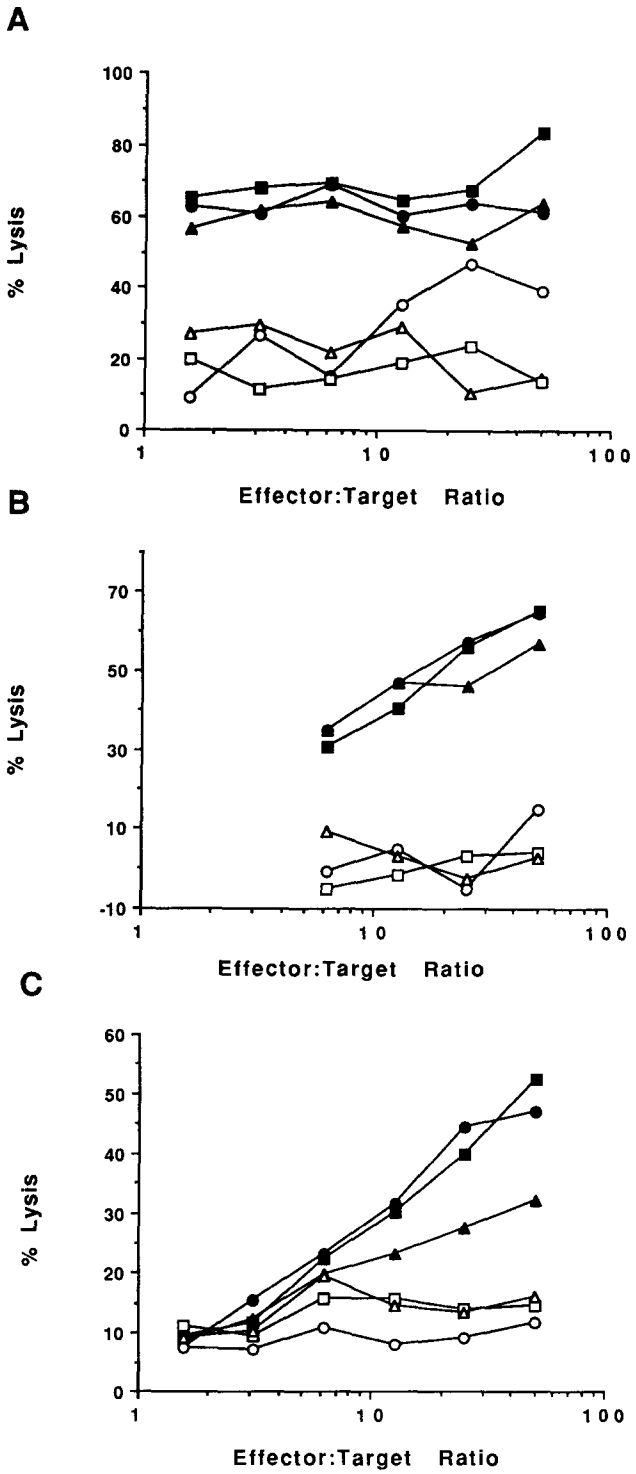


**Figure 1.** Specificity and lytic activity of anti-B10.5 CTL maintained in culture by cytokines. B10 anti-B10.5 CTL that had been maintained in continuous culture for 25 d (*A*), 32 d (*B*), or 53 d (*C*) by IL-2 (▲, △), IL-7 (●, ○), or a combination of IL-2 and IL-7 (■, □) were tested using either B10.5 (*solid symbols*) or B10.2 (*open symbols*) target cells.

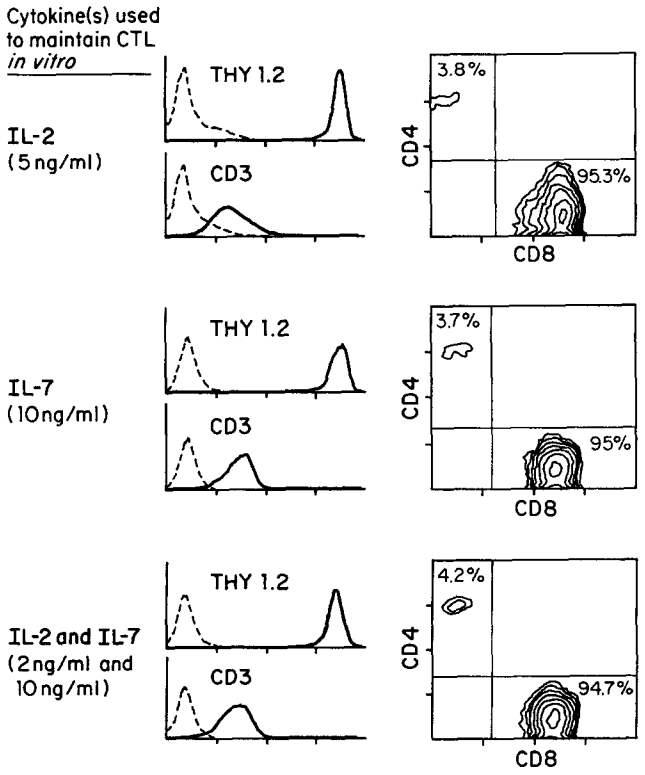


**Figure 2.** Flow cytometric analysis of anti-B10.5 CTL maintained in continuous culture for 32 d by either IL-2, IL-7, or a combination of IL-2 and IL-7. (*Dashed line*) background staining of cells using appropriately labeled control Abs.

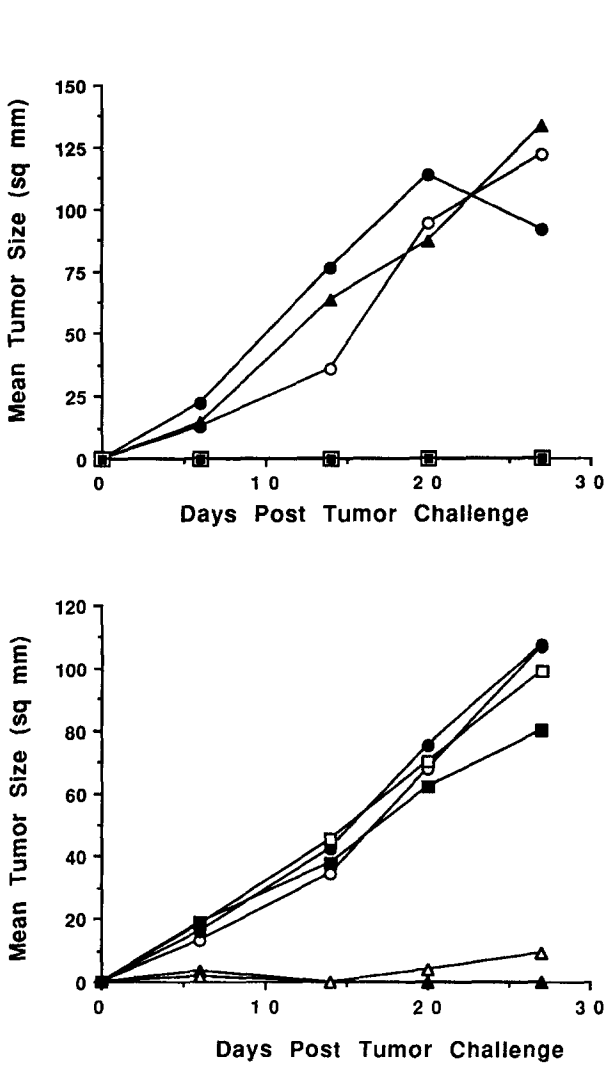


**Figure 3.** Specificity and in vivo efficacy of anti-B10.5 CTL maintained in vitro in medium containing IL-7. 500 rad-irradiated mice were intravenously injected with either no cells (○), 6 × $10^6$ cultured CLN (●), 2 × $10^6$ anti-B10.5 CTL maintained for 32 d in IL-7 (□), or IL-2 and IL-7 (■), 15 × $10^6$ cultured anti-B10.2 DLN cells (▲) or 7.5 × $10^6$ cultured anti-B10.2 DLN cells (△). Mice were then bilaterally challenged with B10.5 and B10.2 tumor cells. Growth of the B10.5 (*A*) and B10.2 (*B*) tumor challenges was monitored over the next 27 d. The number of mice bearing B10.5 tumors at the termination of the experiment was: mice receiving no cells, 4/4; mice receiving 6 × $10^6$ CLN cells, 4/4; mice receiving 2 × $10^6$ anti-B10.5 CTL maintained in medium containing IL-7, 0/4; mice receiving 2 × $10^6$ anti-B10.5 CTL maintained in medium containing IL-2 and IL-7, 0/4; and mice receiving 15 × $10^6$ anti-B10.2 DLN, 4/4. The number of mice bearing B10.2 tumors at the termination of the experiment was: mice receiving no cells, 4/4; mice receiving 6 × $10^6$ CLN cells, 4/4; mice receiving 2 × $10^6$ anti-B10.5 CTL maintained in medium containing IL-7, 4/4; mice receiving 2 × $10^6$ anti-B10.5 CTL maintained in medium containing IL-2 and IL-7, 4/4; mice receiving 15 × $10^6$ anti-B10.2 DLN, 0/4; and mice receiving 7.5 × $10^6$ anti-B10.2 DLN, 1/4.

at day 32 there was 29.4 LU/$10^6$ cells and at day 53 there was 19.6 LU/$10^6$ cells. To determine whether restimulation of these CTL with irradiated tumor cells would increase their cytolytic activity, 5 × $10^6$ effector cells that had been maintained for 53 d in medium containing IL-2 and IL-7 were cultured either with or without irradiated B10.5 tumor cells and the number and lytic activity of these CTL assessed 4 d later using either B10.5 or B10.2 target cells. Effector cells cultured in the absence of tumor stimulator cells increased to 7.7 × $10^6$ compared with 15.7 × $10^6$ for cells cultured with tumor stimulator cells. Cytolytic activity of the CTL also increased from 20 to 50 LU/$10^6$ cells, respectively (Fig. 4).

*Effects of Cytokines and Tumor Stimulator Cells on Proliferative Responses of Antitumor CTL.* We next sought to determine the effect of various cytokines in the presence or absence of irradiated tumor stimulator cells on the proliferation of CTL grown for 5 mo in vitro. Culture of CTL in medium containing IL-2 caused increased levels of [$^3$H]TdR incorporation compared with CTL cultured in the medium alone, and substantially increased the levels of [$^3$H]TdR incorporation by the antitumor CTL in the presence of tumor stimulator cells compared with that induced by tumor stimulator cells alone (Table 1). A slight increase in the proliferative response induced by B10.5 tumor stimulator cells was detected in cultures to which IL-4 had been added, suggesting that IL-4 could also act as a costimulatory molecule for antigen-specific stimulation of these cells. However, no direct stimulatory effect of IL-4 on these CTL was detected and dose–response studies have indicated that the costimulatory effect

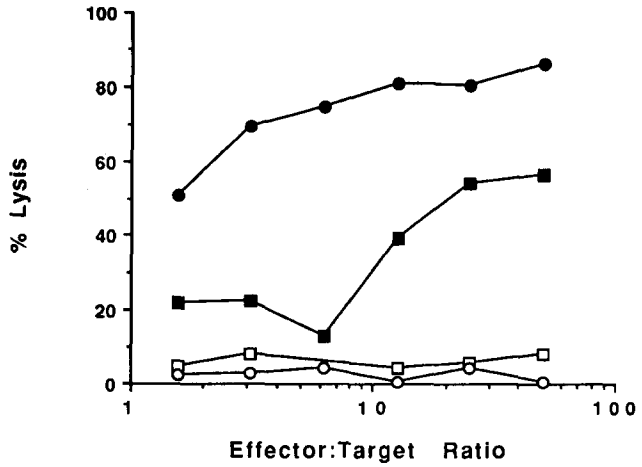


**Figure 4.** Effect of restimulation of anti-B10.5 CTL maintained in medium containing IL-2 and IL-7 with irradiated tumor cells on cytolytic activity and specificity in vitro. Anti-B10.5 CTL maintained in vitro in cytokine-containing medium were cultured for four additional days in either the absence (■, □) or presence (●, ○) of irradiated B10.5 tumor cells and cytolytic activity assessed using either B10.5 (*solid symbols*) or B10.2 (*open symbols*) target cells.

of IL-4 is ~50-fold weaker than that mediated by IL-2 (data not shown). None of the other cytokines tested, including IL-7, led to significant increases in proliferation over that seen in the presence of medium alone.

*In Vivo Efficacy of Antitumor CTL Maintained in Medium Containing IL-2 and IL-7.* To determine the efficacy of antitumor CTL maintained in culture for extended periods of time, CTL that had been maintained in vitro in the absence of tumor cell stimulation for 6 mo were intravenously injected into 500 rad mice that were intradermally challenged with B10.5 tumor cells. In this experiment, intravenous injection of even the lowest number of CTL tested ($10^5$) was sufficient to mediate complete elimination of the tumor challenge (Table 2). The mice in each of the treatment groups that received the antitumor CTL were monitored for an additional 138 d after the termination of the experiment and found to be completely free of detectable tumors.

**Table 2.** *Low Numbers of Anti-B10.5 CTL Maintained in IL-7 Effectively Mediate Rejection of Tumors at Distal Skin Sites*

| Group | No. of cells transferred | No. TBA/ No. challenged | Mean tumor size ($mm^2$ ± SEM) |
|---|---|---|---|
| A | None* | 4/4‡ | 104.5 ± 14.6 |
| B | $3 \times 10^6$ | 0/4 | 0 ± 0 |
| C | $1 \times 10^6$ | 0/4 | 0 ± 0 |
| D | $3 \times 10^5$ | 0/4 | 0 ± 0 |
| E | $1 \times 10^5$ | 0/4 | 0 ± 0 |

* Intravenous injection of anti-B10.5 CTL maintained long-term in medium containing IL-7.
‡ Number of tumor-bearing animals/number challenged 23 d after intradermal injection with B10.5 tumor cells.

A similar experiment was performed using B10 anti-B10.2 CTL that had been maintained in vitro for 7 mo in the absence of tumor cell restimulation (Fig. 5). In this experiment, the intradermal challenge of B10.2 cells was completely rejected in all mice that received either $3 \times 10^6$ anti-B10.2 CTL. Although small tumors were detected during the first week after tumor challenge in three of the four mice that received $10^6$ CTL, they were all found to be tumor free by day 13. The tumor challenge also grew transiently in all four of the mice that received $3 \times 10^5$ CTL. However, the tumors were ultimately rejected in three of the four mice in this group, and grew more slowly in the 4th mouse compared with controls. Intravenous injection of $5 \times 10^4$ CTL proved to be insufficient to mediate tumor rejection since

**Table 1.** *Proliferative Responses of Anti-B10.5 CTL Maintained in IL-7 to Cytokines and Tumor Stimulator Cells*

| Cytokine added | Concentration | Stimulation: None | Stimulation: Irradiated B10.5* |
|---|---|---|---|
| None | - | 1,165.3 ± 248.8‡ | 23,528.6 ± 1,066.4§ |
| IL-1α | 10 ng/ml | 542.0 ± 24.0 | 14,654.0 ± 2,906.1 |
| IL-2 | 10 ng/ml | 10,004.0 ± 379.4 | 140,550.3 ± 4,904.2 |
| IL-4 | 10 ng/ml | 1,342.7 ± 147.7 | 63,098.6 ± 3,806.8 |
| IL-6 | 15,000 U | 484.0 ± 49.2 | 22,179.3 ± 2,450.0 |
| IL-7 | 10 ng/ml | 1,955.0 ± 511.3 | 30,215.3 ± 945.5 |
| IL-2 and IL-7 | 2 and 10 ng/ml | 10,026.0 ± 562.1 | 127,853.3 ± 5,504.7 |

* 2,000 rad-irradiated tumor cells. Mean cpm [$^3$H]TdR incorporation of the irradiated B10.5 tumor cells on day 3 of culture was 5,482 ± 1,310.
‡ Mean cpm [$^3$H]TdR incorporation ± SEM of triplicate wells on day 3 of culture.
§ Delta cpm [$^3$H]TdR incorporation ± SEM of triplicate wells containing antitumor CTL + irradiated tumor cells minus the mean cpm [$^3$H]TdR incorporation of irradiated B10.5 cells in medium alone on day 3 of culture.

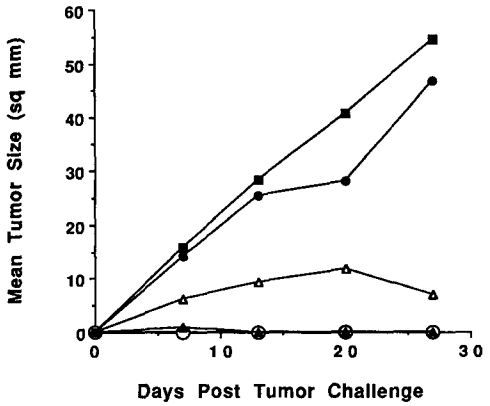


**Figure 5.** Low numbers of anti-B10.2 CTL maintained in IL-7 effectively mediate tumor rejection at distal skin sites. 500 rad-irradiated mice were intravenously injected with either no cells (■), $3 \times 10^6$ (○), $10^6$ (▲), $3 \times 10^5$ (△), or $5 \times 10^4$ (●) anti-B10.2 CTL that had been maintained in vitro for 7 mo by passage in medium containing IL-2 and IL-7. The number of tumor-bearing animals/the number challenged at the termination of the experiment was: mice receiving no cells, 4/4; mice receiving $3 \times 10^6$ CTL, 0/4; mice receiving $10^6$ CTL, 0/4; mice receiving $3 \times 10^5$ CTL, 1/4; and mice receiving $5 \times 10^4$ CTL, 4/4.

progressive tumor growth was observed in all of the mice challenged in this group.

*Antitumor CTL Maintained Long-Term in Medium Containing IL-2 and IL-7 Mediate Tumor Rejection In Vivo.* Although it had been determined that CTL cultured for relatively short (2–6 mo) periods of time could mediate tumor rejection in vivo, it was not known whether this function would be gradually lost as a function of time in culture in the absence of specific stimulation with tumor antigen. Thus, the ability of these CTL to mediate tumor rejection in vivo was tested at various times by intravenously injecting 500 rad-irradiated mice with CTL that had been maintained under a variety of conditions followed by an intradermal challenge with B10.5 tumor cells. antitumor cytotoxic activity of these CTL was also measured using a $^{51}Cr$-release assay. Not only were antitumor CTL from cultures that had been restimulated with tumor antigen at various times during their culture history able to specifically eliminate a tumor challenge in vivo, but so were CTL that had been maintained in continuous culture in the absence of tumor stimulation for as long as 31 mo (Table 3). The data also demonstrate that cryogenic storage of these cells does not result in loss of ability to mediate tumor rejection in a systemic fashion. Interestingly, the in vitro cytotoxic activity of the cell lines tested was not predictive of their ability to mediate tumor rejection in vivo since cells without detectable tumoricidal activity in short-term in vitro assays were still able to mediate tumor rejection in vivo.

*Proliferation of CTL to Tumor Stimulator Cells Is Dependent on Endogenously Produced IL-2.* The ability of antitumor CTL maintained in medium containing IL-2 and IL-7 to proliferate in response to stimulation by tumor cells was evaluated by culture of these CTL in the presence of medium alone or irradiated B10.5 tumor cells, in the presence of either S4B6 (anti-IL-2), 11B11 (anti-IL-4), or a combination of S4B6 and 11B11 Abs. Proliferative responses of these CTL to stimulation with B10.5 tumor cells was inhibited with anti-IL-2, but not anti-IL-4, Abs (Fig. 6). Nor was the inhibitory effect mediated by the anti-IL-2 Abs enhanced by addition of anti-IL-4 Abs. Thus, the proliferative responses induced in these CTL are dependent upon endogenously produced IL-2. Further, although IL-4 can act as a cofactor to promote proliferative responses induced by tumor-stimulator cells, its presence is neither required nor sufficient.

*Kinetics of Cytokine Production after Stimulation with Tumor Cells In Vitro.* Anti-B10.5 CTL maintained in culture for 23 mo in medium containing IL-2 and IL-7 were cultured either alone or with irradiated B10.5 or B10.2 stimulator cells. Culture supernatants from replicate wells were harvested 24, 48, 72, or 96 h after culture initiation. Analysis of cytokines present in the supernatants revealed significant levels of IL-2 produced by CTL stimulated with B10.5 tumor cells during the first 24 h (Fig. 7 *A*). Lower levels of IL-2 were also detected in supernatants collected after 48 h of culture, but not after 72 or 96 h, suggesting that the IL-2 being produced was also being consumed by the CTL. This interpretation is consistent with the finding that anti-IL-2 Abs completely inhibited the proliferative response of the CTL to B10.5 stimulator cells. The possibility that the IL-2 detected in these supernatants was produced by $CD4^+$ cells is not supported since no $CD4^+$ cells could be detected by FACS® analysis. Nor was IL-2 detected in supernatants from CTL cultured either in medium alone or with B10.2 stimulator cells.

High levels of IFN-$\gamma$ were also produced by the CTL after stimulation with B10.5, but not B10.2, tumor cells (Fig. 7 *B*). In contrast to the situation observed with IL-2 production, however, the levels of IFN-$\gamma$ detected remained relatively high throughout the course of the experiment. Thus, either the IFN-$\gamma$ produced was not consumed to any significant degree by the CTL or the CTL continued to produce this cytokine for extended periods of time after stimulation with the B10.5 tumor cells. As with IL-2, no production of IFN-$\gamma$ was detected in cultures of CTL cultured with B10.2 tumor cells or in medium alone. No IL-4 or TNF-$\alpha$ was detected in supernatants from CTL cultured with either B10.5 or B10.2 stimulator cells at any of the time points tested.

*Flow Cytometric Analysis of Antitumor CTL Maintained Long-Term in Medium Containing IL-7.* Flow microfluorometric analysis of anti-B10.5 CTL maintained for 22 mo in medium containing IL-2 and IL-7 determined them to be $CD3^+4^-8^+$ T cells expressing TCR-$\alpha/\beta$, but not TCR-$\gamma/\delta$, receptors (Fig. 8). These CTL also express CD-44, ICAM-1, LFA-1, and Mel-14, but not HSA, cell surface determinants. Low levels of IL-2R ($\alpha$ chain) were also found to be expressed by the majority of these cells. Thus, the data regarding the cell surface phenotype of the antitumor CTL maintained in medium containing IL-7 are consistent with these cells being mature memory cells with the ability to traffic through, and extravasate from, the vasculature at the site of a tumor challenge.

*Elimination of Established Tumors by CTL Maintained In Vitro in Medium Containing IL-7.* Finally, to determine whether

**Table 3.** *Anti-B10.5 CTL Maintained In Vitro for Extended Periods of Time in Medium Containing IL-7 Mediate Specific Tumor Rejection In Vivo*

| Expt. | Cells injected* | Culture history | $LU_{30}$/ $10^6$ cells | Tumor challenge: B10.5 No. TBA/ no. chal‡ | Tumor challenge: B10.5 Mean tumor size ($mm^2$ ± SEM) | Tumor challenge: B10.2 No. TBA/ No. chal‡ | Tumor challenge: B10.2 Mean tumor size ($mm^2$ ± SEM) |
|---|---|---|---|---|---|---|---|
| 1§ | None | – | – | 5/5 | 139.9 ± 33.7 | 5/5 | 162.9 ± 10.5 |
| | αB10.5 CTL | Continuous, 11.5 mo | <0.1 | 0/5 | 0 ± 0 | 5/5 | 172.3 ± 28.7 |
| | αB10.5 CTL | Continuous, 11.5 mo<br>Restimulated 1× @ 2 mo. | <0.1 | 0/5 | 0 ± 0 | 5/5 | 170.0 ± 43.7 |
| | αB10.5 CTL | Frozen @ 2 mo<br>Liquid $N_2$ storage, 9 mo<br>Restimulated 1× @ thaw<br>Cultured 12 d | 40.8 | 0/5 | 0 ± 0 | 5/5 | 226.7 ± 15.6 |
| 2 | None | – | – | 5/5 | 67.9 ± 15.9 | NT‖ | |
| | αB10.5 CTL | Continuous, 17 mo | 15.4 | 0/5 | 0 ± 0 | NT | |
| | αB10.5 CTL | Continuous, 17 mo<br>Restimulated 1× @ 2 mo | <0.1 | 0/5 | 0 ± 0 | NT | |
| | αB10.5 CTL | Frozen @ 2 mo<br>Liquid $N_2$, 9 mo<br>Restimulated 1× @ thaw<br>Continuous, 6 mo | 39.6 | 0/5 | 0 ± 0 | NT | |
| 3 | None | – | – | 5/5 | 58.1 ± 8.5 | NT | |
| | αB10.5 CTL | Continuous, 31 mo | NT | 1/5 | 7.2 ± 1.4 | NT | |

* The number of CTL injected into each recipient IV was $10^6$ (Expt. 1), $2 \times 10^6$ (Expt. 2).
‡ Number of tumor-bearing animals/number challenged at the termination of the experiment. The observation periods for the three experiments reported were 30, 31, and 27 d, respectively.
§ Mice were bilaterally challenged with both B10.5 and B10.2 tumor cells by intradermal injection.
‖ Not tested.

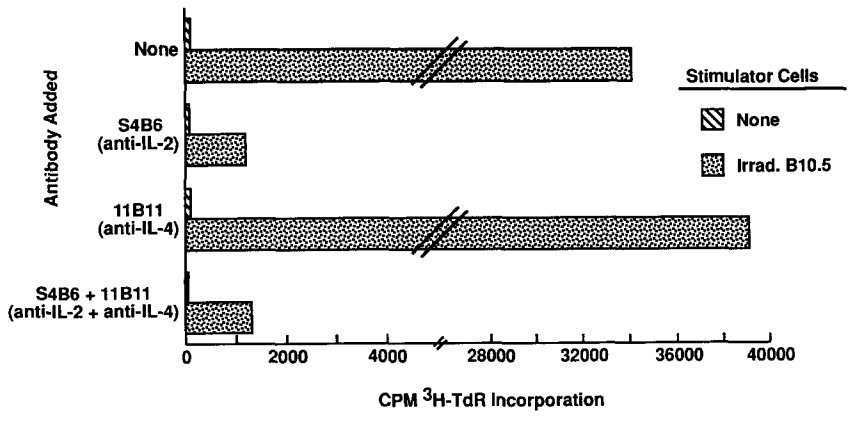


**Figure 6.** Proliferation of anti-B10.5 CTL to tumor stimulator cells is dependent upon endogenously produced IL-2. Anti-B10.5 CTL that had been maintained in vitro in medium containing a combination of IL-2 and IL-7 were cultured either in medium alone (*hatched bars*) or with irradiated B10.5 tumor cells (*stippled bars*) in either the absence or presence of anti-IL-2 and/or anti-IL-4 Abs, as indicated. Proliferation was assessed by [$^3$H]thymidine incorporation 48 h after culture initiation.

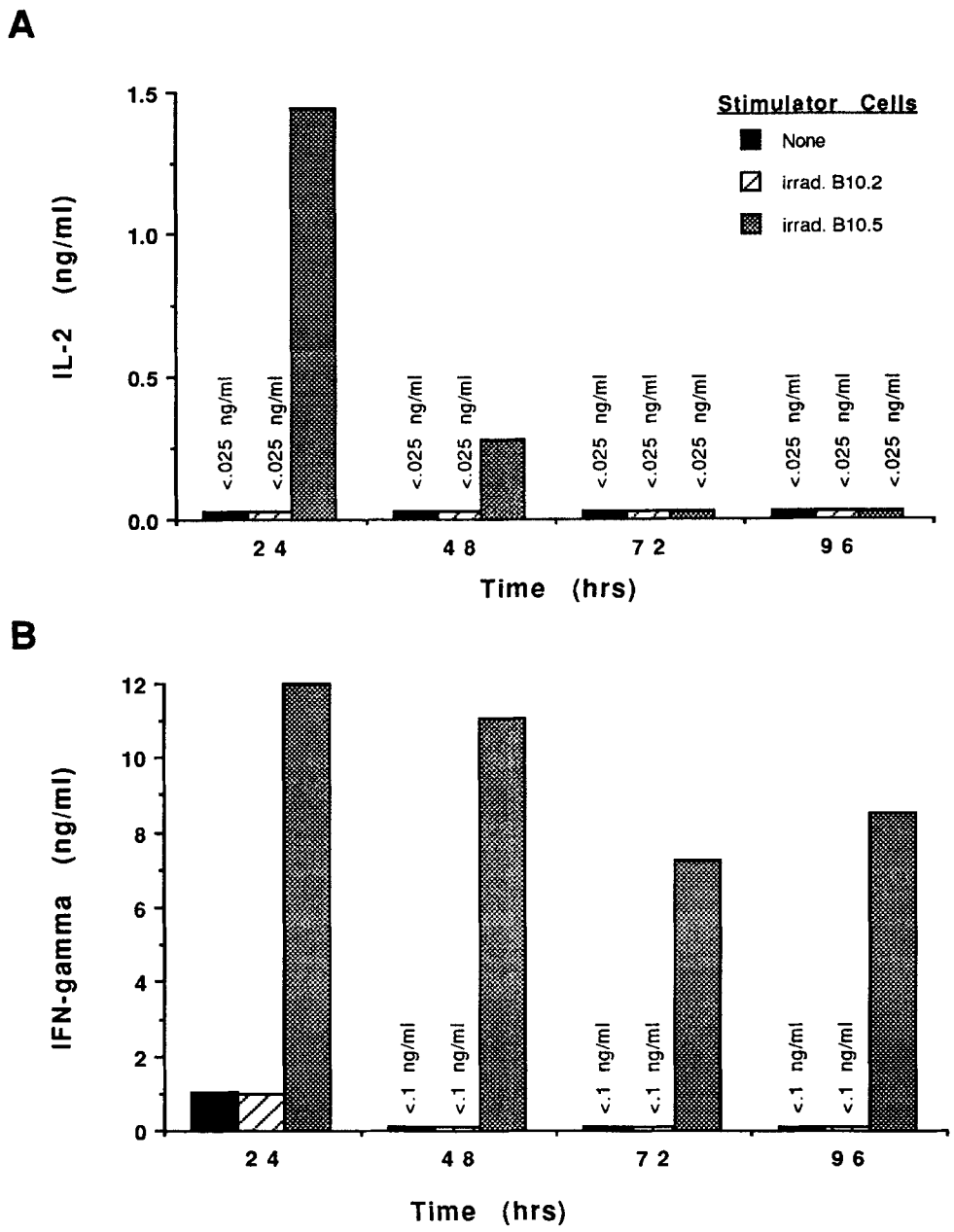


**Figure 7.** Kinetics of cytokine production by CTL after stimulation with irradiated tumor cells. Anti-B10.5 CTL that had been maintained in vitro in medium containing IL-2 and IL-7 were cultured either in medium alone, or with irradiated B10.2 or B10.5 tumor cells, as indicated. Culture supernatants from replicate wells were harvested 24, 48, 72, and 96 h after culture initiation and assayed for cytokine activity.

antitumor CTL maintained in vitro in medium containing IL-7 could be used to immunotherapeutically eliminate established tumors in vivo, tumors were established in groups of mice by intradermal injection with $5 \times 10^5$ B10.5 tumors cells before adoptive transfer with antitumor CTL. 3 d after tumor challenge, the mean size of the tumors was ~11 $mm^2$. On the fourth day after tumor challenge, groups of mice received either no cells or $3 \times 10^6$, $10^6$, or $5 \times 10^5$ anti-B10.5 CTL. The CTL used in this experiment had been maintained in continuous culture in medium containing IL-7 for 11 mo, and then had been cryogenically stored for 33 mo in liquid nitrogen. Before use in this experiment, the CTL were thawed and cultured for an additional 2 mo in medium containing IL-2 and IL-7. The tumor challenges were completely rejected in 75% of the mice that received either $3 \times 10^6$ or $10^6$ CTL (Fig. 9). Mice that received only $5 \times 10^5$ CTL continued to grow the tumor challenge, but at a slower rate than control mice that received no CTL. Thus, antitumor CTL maintained in vitro for extended periods of time in medium containing IL-7 are also capable of eliminating established tumors in vivo.

## Discussion

The studies presented here were conducted to determine whether IL-7 could promote the long-term growth of an-

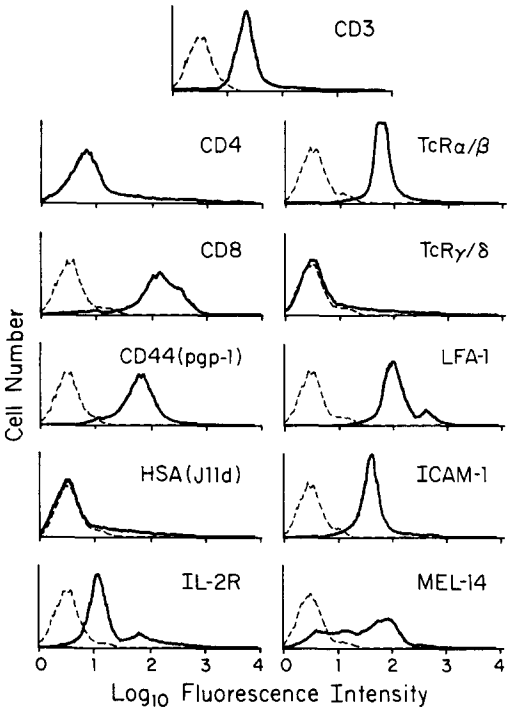


**Figure 8.** Flow cytometric analysis of anti-B10.5 CTL maintained for 22 mo in medium containing IL-2 and IL-7. (*Dashed lines*) background staining of cells using appropriately labeled control Abs.

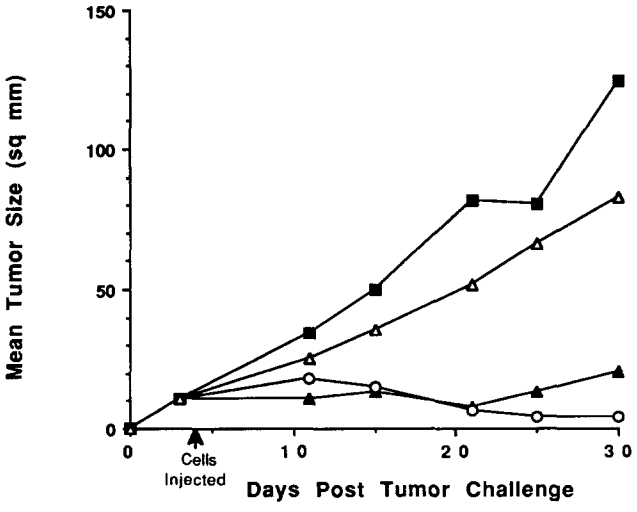


**Figure 9.** Antitumor CTL maintained in medium containing IL-7 mediate rejection of established tumors in vivo. Tumors were established in 500 rad-irradiated mice by intradermal injection with $5 \times 10^5$ B10.5 tumor cells 4 d before intravenous injection with either no cells (■) or $3 \times 10^6$ (▲),$10^6$ (○), or $5 \times 10^5$ (△) CTL. The CTL used in this experiment had been maintained in continuous culture in medium containing IL-2 and IL-7 for 11 mo followed by cryogenic storage for 33 mo. The CTL were thawed and cultured for an additional 2 mo in medium containing IL-2 and IL-7 immediately before use in this experiment. The number of tumor-bearing animals/the number challenged at the termination of the experiment was: mice receiving no cells, 4/4; mice receiving $3 \times 10^6$ CTL, 1/4; mice receiving $10^6$ CTL, 1/4; and mice receiving $5 \times 10^5$ CTL, 4/4.

titumor CTL in vitro. The results not only demonstrate that tumor-specific CTL can be maintained in vitro in IL-7, but that repeated restimulation of the cells is not necessary for their maintenance. Parallel cultures of antitumor CTL similarly cultured in medium containing only IL-2 could only be maintained for 5–6 wk, after which the number and proportion of viable cells that were recoverable from such cultures progressively decreased. In contrast, cell cultured in either IL-7 or a combination of IL-2 and IL-7 grew at a slower pace, yet cell viability remained consistently high (>98%). Further, these CTL maintained the ability to specifically proliferate upon stimulation with tumor cells. More importantly, the effector cells were able to effectively reject tumor challenges in vivo in a systemic fashion after intravenous injection.

The lytic activity of antitumor CTL maintained in culture with IL-7 was found to slowly decrease as a function of increasing time in culture. Although the reasons for this phenomenon are not understood, a substantial increase in tumoricidal activity was detected after a short in vitro restimulation of the CTL with tumor cells (Fig. 4). The in vitro cytolytic activity of CTL cultured for extended periods of time (i.e., 11–31 mo) was also found to be somewhat variable (Table 3). However, the data also clearly indicate that the cytolytic activity of effector cell populations cannot be used as reliable predictors of in vivo efficacy, since cells with no apparent cytotoxic activity (as measured in a short-term $^{51}Cr$-release assay) were still able to mediate tumor rejection in vivo. This observation is in agreement with the results of previous studies by both ourselves (27) and others (21) who have demonstrated that in vitro assays of tumoricidal activity cannot be used as reliable predictors of therapeutic activity in vivo.

Early in their culture history (ca 6 wk) a small percentage of the cells were found to be $CD4^+$, although the vast majority (95%) were identified as $CD8^+$ T cells. This led to the early speculation that the $CD4^+$ T cells might actually be tumor-specific helper cells that were required to promote the propagation of the antitumor CTL. However, phenotypic analysis of the CTL after extended culture (i.e., 22 mo) demonstrated them to be uniformly $CD3^+4^-8^+$ T cells. These cells were also found to express LFA-1, ICAM-1, and Mel-14 cell interaction molecules. These determinants no doubt play an integral role in the ability of the CTL to traffic through the circulation to the site of an intradermal tumor challenge to mediate tumor rejection.

It is also of interest to note that these CTL do not require the presence of APC populations to process and present tumor antigen to the CTL, indicating that the CTL recognize a tumor-specific antigen on the surface of the tumor cells themselves. Whether the tumor antigen is processed in some way by the tumor cells is not known. However, recognition of the tumor antigen by the CTL is most likely restricted by MHC class I determinants, since both proliferative and cytotoxic responses are inhibited by anti-CD8, but not anti-CD4, mAbs (our unpublished observations).

The anti-B10.5 CTL maintained long-term in medium containing IL-2 and IL-7 also express low levels of IL-2R. Although the CTL do not require the addition of exogenous

IL-2 to survive in vivo, it is clear that they require the presence of this cytokine to proliferate in response to tumor stimulator cells (Fig. 6). Similar results have also been obtained using CTL maintained in IL-7 alone (data not shown). The data also demonstrate that the cells in these cultures endogenously produce sufficient quantities of IL-2 for this purpose. Thus, these CTL have become independent of the requirement for $CD4^+$ helper cells to survive and function either in vitro or in vivo. Cells in these cultures also produce IFN-$\gamma$ in response to stimulation with tumor cells in vitro. However, preliminary studies indicate that IFN-$\gamma$ is not required for either proliferative or cytotoxic responses of these effector cells to tumors (data not shown). Whether the secretion of IFN-$\gamma$ in vivo plays a significant role in tumor rejection is not known at this time.

The existence of helper-independent antitumor CTL has been described previously (37). However, these cells not only required clonal activation before use, but also required ancillary IL-2 treatment of the recipients subsequent to adoptive transfer in order to demonstrate therapeutic efficacy (21). Such manipulations were not required to demonstrate tumor elimination in vivo by the antitumor CTL described here.

Although no attempt was made to clone the tumor-reactive T cells in these experiments, it seems highly likely that a significant selection for such cells has occurred. In this regard, it is somewhat surprising that continued culture of heterogeneous starting populations of lymphoid cells containing antitumor CTL in medium containing IL-7 tended to favor the outgrowth of effector cells that retained the ability to mediate tumor rejection in vivo. This should not be construed to mean that IL-7 necessarily selects for cell lines which will function in vivo. A more likely explanation is that IL-7 promotes the survival and slow growth of specifically activated T cells, and a high proportion of antitumor CTL generated from DLN are capable of functioning in vivo.

The potential implications of the results of this study for the immunotherapeutic treatment of cancer may be significant. To date, most adoptive immunotherapy trials have focused on the generation of extremely large numbers of tumoricidal cells. Methods that have been used to achieve this goal have included intermittent stimulation of lymphoid cells obtained from hyperimmunized donors with tumor cells or tumor antigen (6, 37, 38), nonspecific propagation with IL-2 (14–16), combinations of these two approaches (7–9, 20), or nonspecific stimulation with Ab to CD3 in combination with IL-2 (39, 40). Each of these approaches has attendant problems which have prevented them from being widely applicable to the immunotherapy of malignancy. For example, intermittent stimulation with tumor cells suffers from the problem that cells from spontaneously arising tumors are often difficult to propagate in vitro. Further, fresh tumors may contain infiltrating suppressor cells or may themselves produce suppressive factors (such as TGF-$\beta$). Expansion of tumoricidal lymphocytes with IL-2 (either alone or in combination with intermittent stimulation with tumor cells) tends to lead to the development of T cells dependent on exogenous IL-2 for continued growth and survival, both in vitro or in vivo (6, 38, 41). Finally, tumor-reactive T cells propagated with anti-CD3 and IL-2 show progressive decreases in both in vitro and in vivo antitumor reactivity with repeated cycles of stimulation (38). In contrast, the results of the studies presented here demonstrate that IL-7 promotes not only the propagation and maintenance of antitumor CTL for extended periods of time in vitro in the absence of repeated stimulation with either tumor cells or tumor antigen, but promotes the retention of in vivo function as well.

Data presented here demonstrate that antitumor CTL specific for tumors induced by either chronic exposure to UV irradiation (B10.5) or by the chemical carcinogen methylcholanthrene (B10.2) can be propagated in culture for extended periods of time using IL-7. In these experiments, we used antitumor CTL generated in vitro from the DLN of mice injected with viable syngeneic tumor cells. These cells were subsequently restimulated one time in vitro with irradiated tumor cells in medium containing IL-2, IL-7, or IL-2 and IL-7. However, the in vitro restimulation does not appear to be strictly required for the generation and maintenance of tumor-reactive cell lines since similar results have also been obtained using cultures of CTL generated from DLN in the absence of restimulation with tumor cells in vitro (our unpublished observations). Nor are the beneficial effects mediated by IL-7 necessarily limited to $CD8^+$ T cells, since antigen-specific $CD4^+$ cells have also been found to be maintained for extended periods of time (ca 12 mo) in IL-7. It is also interesting to note that although short-term culture of heterogeneous populations of lymphocytes from both mouse and humans in IL-7 promotes the generation of LAK (42, 43), such nonantigen-specific effector cells did not continue to proliferate in long-term cultures supplemented with IL-7. Indeed, after 4–5 wk of culture, only tumor-specific CTL continued to be maintained in cultures containing IL-7. The reasons for this phenomenon are not known, but have also been observed in populations of CTL maintained in IL-2 (38 and Fig. 1).

In conclusion, data presented here demonstrate that specific, antitumor CTL capable of mediating tumor rejection in vivo can be maintained in vitro for extended periods of time in the absence of repeated stimulation with irradiated tumor cells. Thus, with a continuously available source of antitumor effector cells, individuals with tumors could be treated (if need be) with several courses of CTL infusion. Further, with the increased specificity of the effector cell populations due to the absence of LAK activity, it seems likely that fewer cells might need to be infused compared with protocols currently in clinical use. Third, these cells can be cryogenically stored with no apparent loss in activity. Finally, such cells do not require the recipients to be treated with exogenous cytokines to promote or maintain function in vivo. Given the range of toxicities found to be caused by IL-2 administration in humans (44), avoidance of IL-2 therapy might be beneficial. Since IL-7 promotes many, if not all, of the same biological activities in both mouse and human, the use of IL-7 to beneficially augment the generation and propagation of antitumor CTL may have profound implications for promoting the immunotherapeutic treatment of neoplasia in cancer patients.

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Address correspondence to Dr. David H. Lynch, Department of Immunology, Immunex Research and Development Corp., 51 University Street, Seattle, WA 98101.

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