ANTIGEN-DRIVEN LONG TERM–CULTURED T CELLS PROLIFERATE IN VIVO, DISTRIBUTE WIDELY, MEDIATE SPECIFIC TUMOR THERAPY, AND PERSIST LONG-TERM AS FUNCTIONAL MEMORY T CELLS

By MARTIN A. CHEEVER,* DIANNE BRITZMANN THOMPSON,*
JAY P. KLARNET,* AND PHILIP D. GREENBERG*‡

From the *Division of Oncology, Department of Medicine; and the ‡Department of
Microbiology/Immunology, University of Washington, Seattle, Washington 98195; and the Fred
Hutchinson Cancer Research Center, Seattle, Washington 98104

Primary sensitization in vivo results in an increase in the number of antigen-reactive effector T cells and an augmented cell-mediated response. Repeated immunization can further increase the number of antigen-reactive effector T cells, but eventually a plateau of responsiveness is reached; mediated in part by a complex network of specific and nonspecific regulatory systems that limit the clonal expansion of antigen-reactive T cells. By selectively expanding the number of antigen-reactive T cells in vitro and then adoptively transferring such cultured T cells into the host, it has become possible to augment in vivo cell-mediated immunity to a variety of viral (1, 2), tumor (3–8), transplantation (10), and tissue antigens (11, 12), and to potentially achieve a higher degree of responsiveness than can be generated by active in vivo immunization.

Immune T cell lines for use in vivo have been grown in vitro using two overlapping but distinct approaches that allow for the derivation of IL-2-dependent or antigen-driven T cell lines (reviewed in 13). IL-2-dependent T cell lines can be generated by activating T cells to express IL-2 receptors by specific antigen stimulation, then inducing proliferation of antigen-activated T cells by repeated supplementation of the culture media with IL-2 (i.e., after antigen activation, exogenous IL-2 serves as the major stimulus for proliferation). Alternatively, antigen-driven T cell lines can be generated by intermittent specific stimulation of immune T cells with antigen on filler cells without the addition of exogenous IL-2 (i.e., antigen is the major stimulus for proliferation, and presumably acts by inducing production of endogenous IL-2). The filler cells, usually irradiated syngeneic spleen cells, perform several potential functions, including antigen processing, antigen presentation, and secretion of growth and/or differentiation factors.

To determine the feasibility of using cultured T cells as reagents in vivo to

augment specific T cell immunity, we have extensively examined the immuno-biology of cultured T cells in models for the adoptive tumor therapy of antigenic murine leukemia. Previous studies (5) examined IL-2-dependent T cell lines in vivo and showed that T cells specifically cytotoxic to murine leukemia could be greatly expanded in number by culture with IL-2, and could be used for specific adoptive tumor therapy. However, such T cell lines were exquisitely dependent upon exogenous IL-2, and died rapidly in vitro or in vivo (14) without repeated supplementation with IL-2. Thus, these transferred cells were functionally limited in vivo without frequent administration of exogenous IL-2 (15). Moreover, the demonstrated inability of IL-2-dependent long term–cultured T cells to survive in vivo implied that such T cells would be inappropriate reagents for providing immunologic memory in vivo.

To overcome the problems of rapid donor T cell death in vivo and the inability of IL-2-dependent long term–cultured T cell lines to provide immunologic memory in vivo, we have developed and studied antigen-driven long-term cultured T cell lines. Antigen-driven T cell lines derived from B6 mice were generated reactive to FBL-3, a syngeneic Friend virus–induced leukemia. Such cultured T cells were greatly expanded in number and specifically cytotoxic to FBL-3 tumor. However, in distinction to IL-2-dependent T cell lines, the antigen-driven T cell lines produced IL-2 and proliferated in vitro in response to specific stimulation by irradiated FBL-3. Moreover, these T cells could rest in vitro for >1 mo on syngeneic filler cells without antigen stimulation and retain the capability of subsequently responding specifically to antigen; they therefore provided an in vitro equivalent of T cell memory.

In the current study, antigen-driven long term–cultured T cells were used in vivo as an adjunct to cyclophosphamide (CY)[1] for the therapy of disseminated FBL-3 leukemia in a model termed adoptive chemoimmunotherapy (16, 17). The results showed that tumor-specific, antigen-driven, long term–cultured T cells could proliferate rapidly in vivo, distribute widely, eradicate disseminated FBL-3 leukemia, survive long-term in vivo and provide specific immunologic memory long after adoptive transfer.

Materials and Methods

Mice. 6–8 wk-old C57BL/6 mice, denoted B6, were obtained from The Jackson Laboratory, Bar Harbor, ME. Breeding pairs of B6.PL(74NS) mice, established congenic to C57BL/6 mice for Thy-1 antigen and denoted as B6/Thy-1.1, were obtained from The Jackson Laboratory and were maintained in our colony.

Tumor. FBL-3 is a transplanted Friend virus–induced erythroleukemia of B6 origin that possesses tumor-associated surface antigens that crossreact with other FMR tumors (16).

Generation of Antigen-driven, Tumor-specific, Long Term–cultured T Cells. Antigen-driven long term–cultured T cells were derived from splenic T cells of mice immune to FBL-3. Donor mice were sensitized to FBL-3 in vivo by innoculation of 2×10^7 irradiated FBL-3 cells i.p., and immune cells were obtained 4–6 wk later (18).

T cells were grown under conditions adopted from those described by Kimoto and Fathman (19). 4×10^7 lymphocytes from spleens of mice previously immunized to FBL-3 were specifically stimulated by culture for 12 d with 2×10^6 irradiated (12,000 rad γ radiation) FBL-3 tumor cells in 20 ml of RPMI 1640 media supplemented with 10% FCS,

---

[1] *Abbreviations used in this paper:* CY, cyclophosphamide; HEV, high endothelial venules.

2-ME, HEPES buffer, L-glutamine, and antibiotics on 8×10^7 irradiated (3,300 rad γ radiation) B6 spleen cells as filler cells. On day 12, cells were harvested and viability was determined using the vital stain fluorescein diacetate.

2×10^5 viable recovered T cells were restimulated with 10^5 irradiated FBL-3 on 5×10^6 irradiated filler cells in 2 ml media supplemented with 50% culture supernatant fluid from secondary mixed leukocyte cultures as described by Glasebrook and Fitch (20), denoted MLC-SF. Following a reculture period of 10–12 d, the expanded cells were again restimulated under similar conditions but with the addition of 10% MLC-SF to the media. The MLC-SF contained ~2–4 U of IL-2 per milliliter, and was used to circumvent culture crisis. No MLC-SF was added in subsequent restimulations, which were performed every 10–12 d. After 62 d of culture, the resultant T cells, expanded ~1,000-fold, were tested in vitro to confirm specific proliferative and cytolytic function, and tested in vivo in tumor therapy.

Interleukin 2. Specific activity of IL-2 was determined in vitro in a standard functional microassay measuring the capacity of IL-2 to induce the replication of an IL-2-dependent T cell line, as quantified by thymidine incorporation (21). The units of specific activity were adjusted to correspond to the activity of an IL-2 reference standard provided by the Biological Resources Branch of the Biological Response Modifiers Program of the National Cancer Institute.

Purified human recombinant IL-2 was provided by the Immunex Corp., Seattle, WA, and Hoffman-LaRoche, Inc., Nutley, NJ. The dose used in vivo, 2.4×10^3 U/d (U corresponding to the Reference Standard and equivalent to 80 U of functional activity in several of our previous publications [14, 15]), was previously shown to induce the in vivo proliferation (14, 22) and augment the function of tumor-specific IL-2-dependent T cells (15). This dose of IL-2 was below the level required to induce lymphokine-activated killer (LAK) cells in vivo (23), and in multiple previous experiments was shown to induce no detectable antitumor effect when used alone or as an adjunct to CY (15).

Model to Count Donor T Cells In Vivo. To identify and quantify donor T cells in vivo, mice congenic at the Thy-1 locus were used for therapy (14, 17, 22). Thus, B6/Thy-1.1 host mice were inoculated i.p. with long term–cultured T cells derived from B6 mice (which are Thy-1.2). Proliferation, distribution, and persistence of donor T cells was determined by enumeration of donor Thy-1.2$^+$ cells in host ascites, spleen, and mesenteric and axillary lymph nodes at subsequent time points. T cells were labeled with fluorescein-conjugated monoclonal anti-Thy-1.2 (Becton-Dickinson Monoclonal Center, Sunnyvale, CA) for counting by phase/fluorescence microscopy as previously detailed (14, 22). B6 mice are low responders to Thy antigens (24), and previous studies (17) have shown that no immune response to disparities at the Thy-1 allele is generated after adoptive transfer of donor T cells into congenic hosts, and that the transfer of specific T cell immunity from donor to host is equally effective regardless of whether the donor of immune T cells is syngeneic or congenic.

Therapy Model; Adoptive Chemoimmunotherapy. This assay, previously described in detail (5, 15–17), consists of treating mice bearing established disseminated FBL-3 leukemia with a combination of chemotherapy and adoptively transferred immune cells. On day 0, host mice are inoculated i.p. with 5×10^6 viable FBL-3 leukemia cells. By day 5 the tumor is disseminated and mice are treated with CY at dose of 180 mg/kg followed in 5 h by adoptively transferred donor cells. Without therapy, mice die of disseminated leukemia in ~2 wk. CY reduces the tumor burden and potentially ablates host suppressor T cells (9) and prolongs survival to ~4 wk. Therapy with immune cells alone on day 5 without CY has no detectable effect on survival. However, treatment with CY plus cells immune to FBL-3 prolongs survival and cures mice in a cell dose–dependent manner, with larger doses of immune cells inducing longer survival and a greater percentage of cures. For maximum efficacy, donor T cells must be capable of proliferating and surviving long term in the host (17, 25).

To be effective, the donor cell population must contain T cells specifically immune to FBL-3 tumor-associated antigens (i.e., populations of cells from immune donors depleted of T cells are ineffective, as are T cells immune to antigenically distinct syngeneic or

allogeneic tumors) (5, 16). In addition, immune effector T cells are restricted by gene products of the MHC (18).

Results

Generation of Antigen-driven Long Term–Cultured T Cell Lines. Cultured T cells specifically reactive to FBL-3 leukemia were derived from cultures of spleen cells obtained from B6 mice immunized in vivo with FBL-3 and induced to proliferate in vitro by intermittent stimulation with irradiated FBL-3 on filler cells. By the time of therapy on day 62 of culture, the donor T cells had expanded in number ~1,000-fold. Phenotypically, the cultured T cells were 40–50% Lyt-2$^+$ and 50–60% L3T4$^+$ with no apparent overlapping populations. Functionally, the T cells were able to proliferate in vitro in response to irradiated FBL-3 on syngeneic filler cells, but failed to proliferate on either syngeneic or allogeneic filler cells alone. When stimulated with irradiated FBL-3 on syngeneic filler cells, IL-2 was produced and could be measured in culture supernatants at 36 h of culture at a level of ~2–4 U of IL-2.

In addition to expressing proliferative function, the T cell lines were specifically cytotoxic to FBL-3 in a standard 4-h chromium-release assay as previously described (5) for IL-2-dependent T cell lines. Specificity controls for cytolytic reactivity included syngeneic and allogeneic Con A–induced lymphoblasts and EL-4, an antigenically irrelevant syngeneic tumor in experiments similar to those described in Table III (data not presented).

Antigen-driven Long Term–cultured T Cells Proliferate Rapidly In Vivo and Distribute Widely. B6/Thy-1.1 mice were injected with 5 × 10^6 viable FBL-3 cells on day 0, and on day 5, when tumor was disseminated, were treated with 180 mg/kg CY plus 3.3 × 10^6 cultured T cells derived from B6 mice (i.e. Thy-1.2). On day 11 (i.e. 6 d after cell transfer) donor T cells in host ascites, spleen, and mesenteric lymph nodes were identified by use of fluoresceinated antibody to Thy-1.2. The results (Fig. 1) showed that donor T cells had expanded in number in vivo approximately threefold, and comprised 24% of total ascitic cells, 4% of spleen cells, 6% of mesenteric lymph node cells, and 0.9% of axillary lymph node cells.

Injection of mice daily with 2.4 × 10^3 U of IL-2 i.p. greatly increased the already rapid rate of donor T cell growth (Fig. 1). By day 6 in vivo, IL-2 had increased the total number of donor T cells approximately eightfold, to 27% of total ascitic cells, 12% of spleen cells, 50% of mesenteric lymph node cells, and 3.8% of axillary lymph node cells.

Cultured T Cells Are Effective in Tumor Therapy. Cohorts of the above-treated mice were followed for survival (Fig. 2). Mice receiving no treatment had a median survival time of 12 d, and all died by day 13. Therapy on day 5 with CY alone prolonged the median survival time to day 24, but all mice died. By contrast, therapy on day 5 with CY plus 3.3 × 10^6 or 4.0 × 10^7 cultured T cells alone or followed by 2.4 × 10^3 U of IL-2 daily for 6 d cured 100% of mice. Thus, as an adjunct to CY, even a small dose of tumor-specific long term–cultured T cells were curative in tumor therapy.

Cultured T Cells Persist Long Term In Vivo in Cured Mice. The cured mice were killed 120 d after cell transfer and examined for donor T cells (Table I).
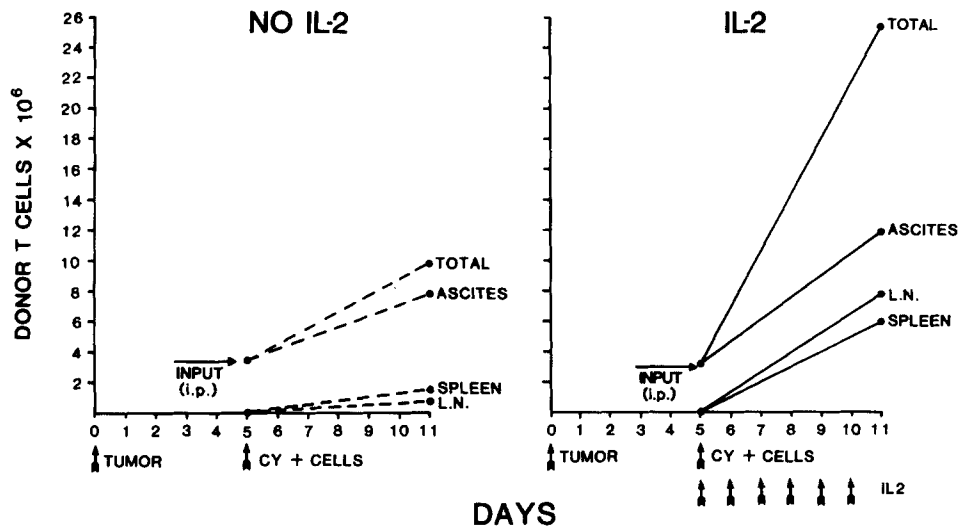
FIGURE 1.   Antigen-driven long term–cultured T cells proliferate and distribute widely in vivo. On day 0, B6/Thy-1.1 host mice were injected i.p. with 5 × 10⁶ viable FBL-3 tumor cells. On day 5, when tumor was disseminated, mice were treated with adoptive chemoimmunotherapy using CY (180 mg/kg) plus 3.3 × 10⁶ B6 (i.e. Thy-1.2) antigen-driven long term–cultured T cells alone or followed by IL-2 injected i.p. daily at a dose of 2.5 × 10³ U/day. The long term–cultured T cells were specifically immune to FBL-3 and were generated by culture for 62 d with proliferation induced by intermittent stimulation with FBL-3. On day 11 of therapy (i.e. day 6 after cell transfer) donor T cells within host ascites, spleen, and mesenteric lymph nodes were identified with fluoresceinated antibody to Thy-1.2 and counted by phase/fluorescence microscopy. The data points represent the means of six mice from three experiments.
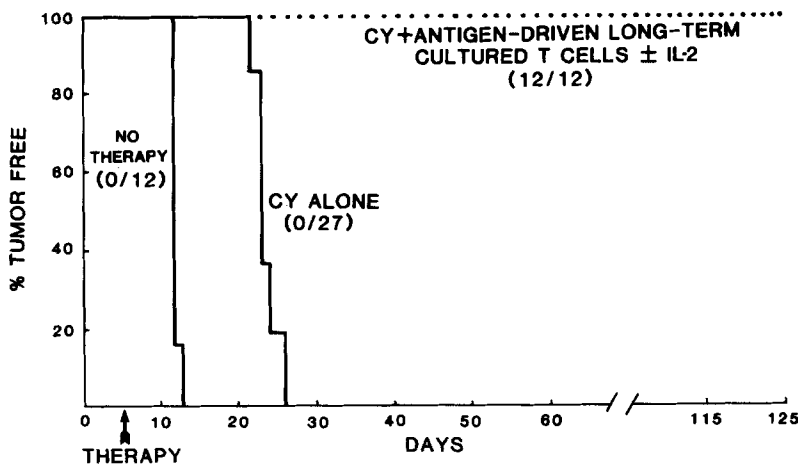

FIGURE 2.   Therapy of disseminated FBL-3 leukemia with antigen-driven long term–cultured T cells. On day 0, B6/Thy-1.1 hosts were inoculated i.p. with 5 × 10⁶ viable FBL-3 leukemia cells and left untreated, treated on day 5 with 180 mg/kg CY, or treated on day 5 with CY plus 3.3 × 10⁶, or 4.0 × 10⁷ antigen-driven long term–cultured T cells immune to FBL-3 (day 62 of culture) either alone (six mice) or followed by 2.5 × 10³ U of IL-2 daily i.p. for 6 d (six mice). The numbers represent surviving mice per total.

TABLE I

Enumeration of Donor T Cells Persisting 120 d In Vivo in Cured Mice

Treatment of hosts*	Percent of donor T cells in host lymphoid organs‡				Total recoverable donor T cells§	
	Ascites	Spleen	Lymph nodes		Total number	Percent of input
			Mesenteric	Axillary		
No IL-2	0.40	0.59	0.93	0.27	0.96×10^6	2.4
IL-2	1.10	7.19	5.37	1.69	17.60×10^6	44.0

B6/Thy-1.1 mice were inoculated with 5×10^6 viable FBL-3 cells i.p. on day 0 and treated on day 5 with CY + 4.0×10^7 FBL-specific antigen-driven long term–cultured T cells (grown to day 62 in vitro) alone or followed by IL-2.

* After adoptive tumor therapy, mice received either no exogenous IL-2 or received IL-2 at a dose of 2.4×10^3 U i.p. daily for 6 d.

‡ On day 125, mice were killed and host lymphoid organs were examined for the presence and phenotype (Table II) of donor T cells. The number of donor T cells is expressed as a percent of total cells in host lymphoid organs, and as an absolute number of donor T cells recoverable in the host lymphoid organs examined.

§ The total number of donor T cells recoverable is presented as a percent of the number of T cells used for therapy on day 5 (i.e., 4.0×10^7 cultured T cells). The data represent the means of two experiments.

In mice treated with 4.0×10^7 donor T cells without IL-2, donor T cells comprised 0.6% of total spleen cells and 0.99% of total mesenteric lymph node cells. Treatment with IL-2 for 5 d after cell transfer increased the recoverable donor T cells to 7% of total spleen cells and 5% of total mesenteric lymph node cells. The total number of recoverable donor T cells was 2.4% of the original number injected in mice not receiving IL-2 and 44% in mice receiving IL-2. As previously reported (17) for therapy with noncultured immune cells, the total number of nucleated cells and the percentage of T cells (i.e. donor plus host) in lymphoid organs of cured mice were equivalent to normal mice (data not presented).

Cultured Lyt-2⁺ T Cells and L3T4⁺ T Cells Survive Equivalently In Vivo. On the day of cell transfer, ~53% of cultured donor T cells were Lyt-2⁺, and the remaining donor T cells were L3T4⁺. Examination of the phenotype of donor T cells recoverable after 120 d in vivo revealed (Table II) that both Lyt-2⁺ and L3T4⁺ cultured T cells survived long term in vivo, both were present in all lymphoid organs examined, and both survived in approximately the same proportion. However, there was a moderate preference for survival of L3T4⁺ T cells in ascites, and for Lyt-2⁺ T cells in spleen and lymph nodes. Although injection of exogenous IL-2 after cell transfer greatly increased numbers of donor T cells surviving 120 d (Table I), IL-2 did not appreciably influence the phenotype of persisting donor T cells (Table II). Concurrent counts of host T cells revealed that 10% of host T cells in spleen and 7–13% of host T cells in lymph nodes were Lyt-2⁺ (Table II). Thus, the phenotype of host T cells in treated mice was equivalent to that of normal mice.

Cultured T Cells Provide Immunologic Memory In Vivo. Donor T cells surviving 120 d in vivo were tested for persistence of specific proliferative and cytolytic function. To assess proliferative function, spleen cells from cured mice were

TABLE II

Phenotype of Donor T Cells Persisting 120 d In Vivo in Cured Mice

Source tissue	Lyt-2$^+$ T cells (%)*			
	Donor		Host	
	No IL-2	IL-2	No IL-2	IL-2
Ascites	44	46	30	23
Spleen	68	74	10	8
Mesenteric lymph node	75	75	13	12
Axillary lymph node	66	66	7	6

See Table I for details of therapy.
* The number of donor Lyt-2$^+$ T cells is expressed as a percent of total donor T cells, and the number of host Lyt-2$^+$ T cells is expressed as a percent of total host T cells.

placed into culture and stimulated for 5 d with irradiated FBL-3 or with irradiated syngeneic B6 spleen cells, and the growth of donor T cells was determined by counting donor T cells using fluorescence-tagged mAb against Thy-1.2. The results showed that donor T cells in spleens of cured mice increased 18–25% when stimulated by FBL-3, and decreased 43–81% when cultured without FBL-3. Both donor L3T4$^+$ T cells and Lyt-2$^+$ T cells proliferated equivalently.

To assess cytolytic function, spleen cells from cured mice were similarly cultured but tested for ability of such antigen-stimulated cells to mediate specific cytolytic reactivity in a standard 4-h chromium-release assay. The results showed that spleen cells from cured mice became specifically cytotoxic to FBL-3 after stimulation by FBL-3 (Table III), and that cytotoxicity was mediated by persistent donor T cells (i.e. Thy-1.2$^+$) as opposed to host T cells (i.e. Thy-1.1$^+$).

Discussion

One major question concerning the use of cultured T cells in vivo to augment specific T cell immunity was whether such T cells could survive and function long term in vivo (7, 14, 35). Culture conditions for generating antigen-driven T cells, as described previously (13, 19, 20), allow for the generation in vitro of immune T cells that can rest in vitro on syngeneic filler cells (38), and therefore may represent an in vitro functional equivalent of memory T cells. The current study extended these in vitro findings and showed that tumor-specific T cells cultured in vitro for 62 d and greatly expanded in number could proliferate in vivo, distribute widely to host lymphoid organs, mediate specific tumor therapy, and persist long term in vivo as functioning memory T cells.

The immune spleen cells for therapy were activated to express IL-2 receptors by culture with irradiated FBL-3, then induced to proliferate and to grow long term in vitro by intermittent antigen stimulation every 10–12 d. The T cell lines generated under such conditions contained Lyt-2$^+$ T cells and L3T4$^+$ T cells, were specifically cytotoxic to FBL-3, and were able to secrete IL-2 and proliferate in vitro in response to antigen-stimulation. It is likely that different T cell subsets were responsible for mediating cytotoxicity and for producing IL-2, as previously reported (26) for similar alloreactive T cell lines generated under similar condi-

Table III

Antigen-driven Long Term–cultured T Cells Become Specifically Cytotoxic In Vitro in Response to Antigen after 120 d In Vivo

Responder	Stimulator cells*	Depletion of donor or host T cells after culture‡	Percent chromium release (E/T ratio of 20:1)§		
			FBL	B6	EL-4
Spleen cells from mice cured with cul-	B6	—	14	1	4
tured T cells	FBL	—	63	−2	1
		Anti-Thy-1.2 + C'	10	—	—
		Anti-Thy-1.1 + C'	67	—	—
Spleen cells from mice cured with cul-	B6	—	30	5	4
tured T cells plus IL-2	FBL	—	55	0	1
Spleen cells from B6 mice immunized	B6	—	9	−5	−1
with irradiated FBL cells	FBL	—	34	−4	−2
Normal B6 spleen cells	B6	—	10	−4	−1
	FBL	—	8	−1	0

B6/Thy-1.1 mice bearing disseminated FBL-3 were cured in adoptive chemoimmunotherapy using CY plus 4.0×10^7 FBL-specific, antigen-driven, long term–cultured T cells (cultured 62 d) either alone or followed by IL-2 for 6 d (see Fig. 1 for details).
* On 120 d after cell transfer, mice were killed and spleen cells were placed into culture and stimulated for 5 d with irradiated syngeneic B6 spleen cells, or irradiated FBL-3.
‡ Effector populations from culture were tested untreated or after depletion of donor T cells with anti-Thy-1.2 + C' or depletion of host T cells with anti-Thy-1.1 + C', using reagents previously described (28).
§ After culture with B6 or FBL, resultant cells were tested for cytolytic reactivity in a standard 4-h chromium-release assay against the targets of FBL-3, B6 Con A blasts, and EL-4 (an antigenically distinct non-crossreacting syngeneic B6 tumor). The cytotoxicity represents the mean cytotoxicity from three experimental mice.

tions, since previous studies in the FBL-3 system (using noncultured spleen cells from mice immune to FBL-3) have shown that CTL are of the Lyt-2$^+$ phenotype and require IL-2-producing Lyt-2$^-$ T cells for proliferation (27, 28).

To test the potential function of antigen-driven long term–cultured T cells in vivo, cultured cells were used for the therapy of disseminated FBL-3 as an adjunct to CY. 6 d after cell transfer, donor T cells were greatly increased in number. The proliferation of donor T cells in vivo was presumably in response to endogenous production of IL-2 by donor helper/inducer T lymphocytes as described for in vitro–stimulated T cells (29), and implies that the IL-2-producing Th subset grown in vitro was able to survive adoptive cell transfer and function in vivo.

The demonstration that antigen-driven donor T cells could distribute widely and could be found in larger numbers in host ascites, spleen, and local-regional lymph nodes as well as in distant lymph nodes was surprising, since previous studies (30–34) have shown that cultured T cells traffic abnormally in vivo, localize poorly, and are unable to migrate into peripheral lymph nodes. The inability of cultured T cells to traffic and to home in other studies is presumably multifactorial, but included the major problem of being unable to proliferate in

vivo (7, 14, 32, 35), a problem seemingly circumvented by use of the culture systems described herein.

The mechanism by which cultured T cells gained entry in large numbers into mesenteric and axillary lymph nodes in this study is uncertain, because cultured T cells have been shown (33, 36) to lack receptors for adherence to high endothelial venules (HEV); and adherence of lymphocytes to HEV is the described first step necessary for interendothelial cell migration of lymphocytes into the parenchyma of lymph nodes. In the current study, donor T cells may have reexpressed receptors for HEV in vivo and gained access to axillary lymph nodes mediated by specific adherence, or, alternatively, entry of cultured T cells into the local-regional lymphatic compartment may have occurred by direct egress of i.p.-injected donor T cells into the collecting lymphatics in the omentum (reviewed in 34), and entry into distant axillary lymph nodes may have occurred as a random event with subsequent rapid proliferation of donor T cells in response to FBL-3 antigen within the lymph node. Regardless of the mechanisms allowing cell traffic, our results, in comparison to previous studies using IL-2-dependent T cells in vivo (14, 35), show that culture conditions play a major role in determining T cell survival and distribution patterns in vivo.

FBL-3 specific antigen-driven long term–cultured T cells proved to be extremely effective in tumor therapy. However, the mechanism of tumor eradication was not determined. The cultured donor T cell population contained both tumor-specific CTL and Th. Thus, it is possible that either subset alone was responsible for therapeutic efficacy, or alternatively, that a collaborative interaction between subsets in vivo was required. Previous studies using noncultured T cells from immune mice in the same therapy model established that the noncytolytic Lyt-2⁻ Th subset can eradicate disseminated FBL-3 alone without the participation of CTL (27, 28), presumably by secreting lymphokines and initiating a delayed-type hypersensitivity reaction upon specific stimulation by tumor in vivo. A similar antitumor effect might have been mediated by the cultured Th subset in the current experiment. However, since Th subsets vary greatly in requirements for in vitro growth (37), it is as yet unknown whether the functional subset(s) of Th required for tumor eradication by this subset alone survive long-term culture. In the current studies, tumor-specific donor CTL may have contributed significantly to therapeutic outcome, however, it is highly unlikely that this subset, which presumably lacks the capacity to secrete IL-2 and proliferate in response to tumor stimulation, could mediate tumor elimination alone without IL-2 supplied either by collaborating Th or by exogenous administration.

The use of cloned T cells in therapy might potentially provide evidence as to the mechanism of tumor eradication with antigen-driven T cell lines. However, it is unknown whether disseminated syngeneic tumor can be eradicated by individual T cell clones, or whether the progeny of a single cell can produce the requisite lymphokines in the proper proportions to mediate the potentially complex set of interactions between donor and host cells required for tumor eradication (28). Moreover, the limited reactivity of a single clone to a single antigenic moiety might prove inadequate for therapy, due to either tumor heterogeneity or antigenic modulation. In the single published study (6) using T

cell clones to treat FBL-3, cloned T cells capable of proliferating in vitro in response to FBL-3 had a demonstrable but limited effect in vivo, and cured no mice.

The administration of exogenous IL-2 for six consecutive days after cell transfer increased the already rapid rate of proliferation of donor T cells in vivo and resulted in a greatly increased number of donor T cells surviving long term. Previous studies (14) using noncultured T cells from mice immune to FBL-3, failed to show that IL-2 could increase the rapid growth rate of noncultured antigen-stimulated T cells in vivo. The ability of exogenous IL-2 to increase the rapid in vivo growth of cultured T cells, in contrast to noncultured T cells, may reflect an inadequate production of endogenous IL-2 by cultured T cells to support maximal proliferation, or alternatively, that T cells activated in vitro may be more responsive to exogenous IL-2 than cells activated in vivo.

The expression of cell-mediated immunity in vivo is, in part, a reflection of the number of immune T cells present. Our results show that in vitro culture can be used to increase the number of antigen-reactive T cells in vivo. The extent to which models for the therapy of antigenic murine leukemia can be generalized to man is uncertain. However, this approach may prove to be applicable to several tumors already identified to be immunogenic, including HTLV$^+$ leukemias (39), idiotype- or clonotype-positive B and T cell malignancies (40), and tumors expressing unique membrane determinants such as might result from point mutation of an oncogene (41). In addition, this approach may prove applicable to the therapy of human viral, bacterial, fungal, and parasitic infectious diseases for which cellular immunity is limiting.

Summary

Mice bearing disseminated syngeneic FBL-3 leukemia were treated with cyclophosphamide plus long term–cultured T cells immune to FBL-3. The cultured T cells for therapy had been induced to grow in vitro for 62 d by intermittent stimulation with irradiated FBL-3. At the time of therapy, such antigen-driven long term–cultured T cells were greatly expanded in number, proliferated in vitro in response to FBL-3, and were specifically cytotoxic. Following adoptive transfer, donor T cells persisting in the host were identified and counted using donor and host mice congenic for the T cell marker Thy-1. The results show that antigen-driven long term–cultured T cells proliferated rapidly in vivo, distributed widely in host lymphoid organs, and were effective in tumor therapy. Moreover, the already rapid in vivo growth rate of donor T cells could be augmented by administration of exogenous IL-2.

When cured mice were examinedd 120 d after therapy, donor L3T4$^+$ T cells and donor Lyt-2$^+$ T cells could be found in large numbers in host ascites, spleen, and mesenteric and axillary lymph nodes. The persisting donor T cells proliferated in vitro, and became specifically cytotoxic in response to FBL-3, demonstrating that antigen-driven long term–cultured T cells can persist long term in vivo and provide immunologic memory.

References

1. Lukacher, A. E., V. L. Brachiale, and T. J. Braciale. 1984. In vivo effector function of influenza virus–specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* 160:814.

2. Byrne, J. A., and M. B. A. Oldstone. 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocyte choriomeningitis virus: clearance of virus in vivo. *J. Virol.* 51:682.

3. Kedar, E., and D. W. Weiss. 1983. The in vitro generation of effector lymphocytes and their employment in tumor immunotherapy. *Adv. Cancer Res.* 38:171.

4. Fernandez-Cruz, E., B. Halliburton, and J. D. Feldman. 1979. In vivo elimination by specific effector cells of an established syngeneic rat Moloney virus–induced sarcoma. *J. Immunol.* 123:1772.

5. Cheever, M. A., P. D. Greenberg, and A. Fefer. 1981. Specific adoptive therapy of established leukemia with syngeneic lymphocytes sequentially immunized in vivo and in vitro and non-specifically expanded by culture with interleukin 2. *J. Immunol.* 126:1318.

6. Rosenstein, M., and S. A. Rosenberg. 1984. Generation of lytic and proliferative lymphoid clones to syngeneic tumor: in vitro and in vivo studies. *J. Nat. Cancer Inst.* 72:1161.

7. Binz, H., M. Fenner, R. Engel, and H. Wigzell. 1983. Studies on chemically induced rat tumors. II. Partial protection against syngeneic lethal tumors by cloned syngeneic cytotoxic T lymphocytes. *Int. J. Cancer.* 32:491.

8. Yamasaki, T., H. Handa, J. Yamashita, Y. Watanabe, Y. Nambe, and M. Hanaoka. 1984. Specific adoptive immunotherapy with tumor-specific cytotoxic T-lymphocyte clone for murine malignant gliomas. *Cancer Res.* 44:1976.

9. North, R. J. 1982. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J. Exp. Med.* 155:1063.

10. Engers, H. D., A. L. Glasebrook, and G. D. Sorenson. 1982. Allogeneic tumor rejection induced by intravenous injection of Lyt-2$^+$ cytotoxic T lymphocyte clones. *J. Exp. Med.* 156:1280.

11. Bianchi, A. T. J., H. Hooijkaas, R. Benner, R. Tees, A. A. Nordin, and M. H. Schreier. 1981. Clones of helper T cells mediate antigen-specific, H-2-restricted DTH. *Nature (Lond.).* 290:62.

12. Zamvil, S., P. Nelson, J. Trotter, D. Mitchell, R. Knobler, R. Fritz, and L. Steinman. 1985. T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination in mice. *Nature (Lond.).* 317:355.

13. Fathman, C. G., and F. W. Fitch. 1983. Long-term culture of immunocompetent cells. *In:* Fundamental Immunology. W. E. Paul, editor. Raven Press, New York. 781–795.

14. Cheever, M. A., P. D. Greenberg, C. Irle, J. A. Thompson, D. L. Urdal, D. Y. Mochizuki, C. S. Henney, and S. Gillis. 1984. Interleukin 2 administered in vivo induces the growth of cultured T cells in vivo. *J. Immunol.* 132:2259.

15. Cheever, M. A., P. D. Greenberg, A. Fefer, and S. Gillis. 1982. Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by in vivo administration of purified interleukin 2. *J. Exp. Med.* 155:968.

16. Fefer, A., A. B. Einstein, M. A. Cheever, and J. R. Berenson. 1976. Models for syngeneic adoptive chemoimmunotherapy of murine leukemias. *Ann. N. Y. Acad. Sci.* 276:573.

17. Greenberg, P. D., and M. A. Cheever. 1984. Treatment of disseminated leukemia with cyclophosphamide and immune cells: tumor immunity reflects long-term persistence of tumor-specific donor T cells. *J. Immunol.* 133:3401.

18. Greenberg, P. D., M. A. Cheever, and A. Fefer. 1981. H-2 restriction of adoptive immunotherapy of advanced tumors. *J. Immunol.* 126:2100.

19. Kimoto, M., and C. G. Fathman. 1982. Immunization and long-term culture of murine immune lymph node cells. *In* Isolation, Characterization, and Utilization of T Lymphocyte Clones. C. G. Fathman and F. W. Fitch, editors. Academic Press, New York. 525–526.

20. Glasebrook, A. L., and F. W. Fitch. 1982. Cloning of alloreactive murine T cells. *In* Isolation, Characterization, and Utilization of T Lymphocyte Clones. C. G. Fathman and F. W. Fitch, editors. Academic Press, New York. 522–523.

21. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor: Parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.

22. Cheever, M. A., J. A. Thompson, D. E. Kern, and P. D. Greenberg. 1985. Interleuken 2 (IL 2) administered in vivo: Influence of IL 2 route and timing on T cell growth. *J. Immunol.* 134:3895.

23. Rosenberg, S. A., J. J. Mulé, P. J. Spiess, C. M. Reichert, and S. L. Schwarz. 1985. Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleuken 2. *J. Exp. Med.* 161:1169.

24. Zaleski, M., and J. Klein. 1978. Genetic control of the immune response to Thy 1 antigens. *Immunol. Rev.* 38:120.

25. Greenberg, P. D., M. A. Cheever, and A. Fefer. 1980. Detection of early and delayed anti-tumor effects following curative adoptive chemimmunotherapy of established leukemias. *Cancer Res.* 40:4428.

26. Glasebrook, A. L., and Fitch, F. W. 1980. Alloreactive cloned T cell lines I. Interactions between cloned amplifier and cytolytic T cell lines. *J. Exp. Med.* 151:876.

27. Greenberg, P. D., M. A. Cheever, and A. Fefer. 1981. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1$^+$,2$^-$ lymphocytes. *J. Exp. Med.* 154:952.

28. Greenberg, P. D., D. E. Kern, and M. A. Cheever. 1985. Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-1$^+$,2$^-$ T cells: tumor eradication does not require participation of cytotoxic T cells. *J. Exp. Med.* 161:1122.

29. Wagner, H., and Röllinghoff, M. 1978. T-T cell interaction during in vitro cytotoxic allograft responses. I. Soluble products from activated Ly-1$^+$ T cells trigger autonomously antigen-primed Ly-2,3$^+$ T cells to cell proliferation and cytolytic activity. *J. Exp. Med.* 148:1523.

30. Lotze, M. T., B. R. Line, D. J. Mathisen, and S. A. Rosenberg. 1980. The in vivo distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): Implications for the adoptive immunotherapy of tumors. *J. Immunol.* 125:1487.

31. Daily, M. O., C. G. Fathman, E. C. Butcher, E. Pillemer, and I. Weissman. 1982. Abnormal migration of T lymphocyte clones. *J. Immunol.* 128:2134.

32. Carroll, A. M., M. A. Palladino, H. Oettgen, and M. de Sousa. 1983. In vivo localization of cloned IL 2-dependent T cells. *Cell. Immunol.* 76:69.

33. Dailey, M. O., W. M. Gallatin, and I. L. Weisman. 1985. The in vivo behaviour of T

cell clones: altered migration due to loss of lymphocyte surface homing receptor. *J. Mol. Immunol.* 2:27.

34. de Sousa, M. 1981. Lymphocyte Circulation: Experimental and Clinical Aspects. John Wiley & Sons, New York.

35. Palladino, M. A., K. Welte, A. M. Carroll, and H. F. Oettgen. 1984. Characterization of interleukin 2 (IL 2)-dependent cytotoxic T cell clones. V. Transfer of resistance to allografts and tumor grafts requires exogenous IL 2. *Cell. Immunol.* 86:299.

36. Gallatin, W. M., I. L. Weissman, and E. C. Butcher. 1983. A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature (Lond.).* 304:30.

37. Conta, B. S., M. B. Powell, and N. H. Ruddle. 1985. Activation of Lyt-1$^+$ and Lyt-2$^+$ T cell cloned lines: stimulation of proliferation, lymphokine production, and self-destruction. *J. Immunol.* 134:2185.

38. Matis, L. A., S. K. Ruscetti, D. L. Longo, S. Jacobson, E. J. Brown, S. Zinn, and A. M. Kruisbeek. 1985. Distinct proliferative T cell clonotypes are generated in response to a murine retrovirus–induced syngeneic T cell leukemia: viral gp70 antigen–specific MT4$^+$ clones and Lyt-2$^+$ cytolytic clones which recognize a tumor-specific cell surface antigen. *J. Immunol.* 135:703.

39. Mitsuya, H., L. A. Matis, M. Megson, P. A. Bunn, C. Murray, D. L. Mann, R. C. Gallo, and S. Broder. 1983. Generation of an HLA-restricted cytotoxic T cell line reactive against cultured tumor cells from a patient infected with human T cell leukemia/lymphoma virus. *J. Exp. Med.* 158:994.

40. Miller, R. A., D. G. Maloney, R. Warnke, and R. Levy. 1982. Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *New Engl. J. Med.* 306:517.

41. Thor, A., P. Horan Hand, D. Wunderlich, A. Caruso, R. Muraro, and J. Schlom. 1985. Monoclonal antibodies define differential *ras* gene expression in malignant and benign colonic diseases. *Nature (Lond.).* 311:562.