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T CELLS FROM TUMOR-IMMUNE MICE NONSPECIFICALLY EXPANDED IN VITRO WITH ANTI-CD3 PLUS IL-2 RETAIN SPECIFIC FUNCTION IN VITRO AND CAN ERADICATE DISSEMINATED LEUKEMIA IN VIVO[1]

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The therapeutic efficacy of adoptive immunotherapy of cancer has been shown to positively correlate with the dose of tumor-immune T cells transferred. Therefore, the success of this therapy is critically dependent on the ability to procure large numbers of functionally active T cells. Previous studies in animal models have shown that the limited therapeutic efficacy of a small number of immune T cells can be greatly enhanced by expansion of T cells in vitro to greater numbers before transfer in vivo. Optimal regimens for T cell expansion in vitro have generally employed the use of intermittent stimulation of the TCR with specific Ag followed by exogenous IL-2. The use of IL-2 alone does not provide for requisite episodic up-regulation of IL-2R. Stimulation of the invariant CD3 portion of the TCR/CD3 complex with antibody to CD3 (anti-CD3) represents an alternative method of up-regulating IL-2R and has been used to nonspecifically induce the growth of Ag-specific T cell lines and clones long-term in vitro with maintenance of function and specificity. The current study examined whether resting T cell populations containing small numbers of memory tumor-specific T cells could be rendered more effective in tumor therapy by nonspecific expansion in vitro with anti-CD3 plus IL-2. Spleens from C57BL/6 mice previously immunized to FBL-3, a syngeneic virus-induced leukemia, were nonspecifically stimulated with anti-CD3 plus IL-2. The resultant T cells were expanded in number, were nonlytic to FBL-3 but retained the ability to become lytic upon specific stimulation by FBL-3, and were effective in specific tumor therapy. The Ag-specific anti-tumor immune function declined on a per cell basis after each cycle of anti-CD3-induced T cell expansion. However, the approach resulted in a substantial increase in total T cell number and an overall net increase in the function of the effector T cell population. Thus, stimulation of tumor-immune T cell populations with anti-CD3 plus IL-2 represents a nonspecific method for expanding the number of specific effector T cells for cancer therapy.

A variety of animal models has been developed in which established malignancies can be eradicated by use of adoptively transferred T cells specifically immune to the tumor (1–4). In general, therapeutic efficacy is directly proportional to the number of immune T cells transferred. Thus, successful adoptive immunotherapy with tumor-immune T cells is critically dependent on the availability of adequate numbers of tumor-specific T cells. One approach for procuring large numbers of Ag-specific T cells for in vivo therapy that has been effective in animal models is to grow immune T cells in vitro in response to intermittent stimulation with tumor cells or tumor Ag (5–7). However, this approach has not yet been broadly applicable for culturing human tumor-specific T cells, in part because of intrinsic problems of using tumor cells as stimulator cells, such as the potential presence of tumor-infiltrating suppressor or inhibitory cells, tumor-derived suppressor factors, and inappropriate expression of tumor Ag or accessory molecules. Moreover, human tumor Ag recognizable by T cells have still not been well characterized. They may be unique for each tumor and thus would not readily be available in purified form to be used for T cell stimulation. Therefore, methods of growing tumor-reactive T cells in vitro by nonspecific means that do not require the use of tumor cells or tumor Ag have been studied in recent years. The "standard" culture method employed is the use of high doses of IL-2, i.e., 1000 U/ml. As an example, IL-2 has been used as a nonspecific stimulus to activate and expand T cells in vitro that have infiltrated tumor tissues (8–10). The use of IL-2 as the sole stimulus for T cell proliferation has resulted in the generation or enrichment of lymphocyte populations reactive with tumor, but has also yielded T cells dependent on exogenous IL-2 for continual survival and growth (11). Therefore, this approach selects against T cells capable of secreting IL-2 and proliferating in response to tumor, properties that are requisite for optimal efficacy in cellular therapy. In pursuit of a more effective, yet still generally applicable nonspecific way for propagating tumor-reactive T cells in culture for use in adoptive immunotherapy, the current study examined the use of an antibody to CD3 as the primary stimulus for T cell proliferation (12, 13).

CD3 is a complex of polypeptides that are linked to the TCR heterodimer and may be involved in transmembrane signaling events leading to T cell activation after initial triggering of the TCR by specific Ag (14). At present, the

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most effective method of growing T cells in culture with retention of specificity is by repetitive stimulation with specific Ag, which is presented as processed fragments bound to MHC class I or class II molecules and which induces T cell proliferation via the TCR/CD3 complex (15). Stimulation of the TCR/CD3 complex with antibodies to CD3 has been shown to provide a means of mimicking the normal pathways of T cell activation (16, 17). Thus, presuming that tumor-specific T cells reside in vivo at sites of tumor deposition, growth of tumor-infiltrating T cells in response to anti-CD3 could potentially allow procurement of large numbers of tumor-specific T cells under standardized conditions from a variety of tumor types in the absence of tumor cells or purified tumor Ag as specific stimuli.

Recently, we reported that an antibody to CD3 could be used to generate virus-specific human T cells in vitro (18). Such clones could be expanded in number with retention of Ag specificity in response to anti-CD3 stimulation for over 3 mo in culture. In the current investigation anti-CD3 was used in an animal model for inducing growth of tumor-immune T cells from a nonenriched spleen cell population that contained tumor-specific T cells as well as a preponderance of normal tumor nonreactive $CD3^+$ T cells. T cells from mice that had been immunized against FBL-3, a Friend virus-induced erythroleukemia were expanded in vitro in response to nonspecific stimulation with anti-CD3 and tested for specific in vivo function in therapy against disseminated FBL. Previous work in our laboratory had demonstrated that spleen cells from mice immune to FBL could be grown in vitro in response to intermittent stimulation with FBL tumor cells (5). The T cells grown in vitro in response to specific stimulation were expanded in number, retained specificity and function against FBL in vitro, and displayed normal function in vivo as defined by their ability to distribute widely, proliferate in response to specific stimulation by FBL, mediate tumor-specific therapy, and persist long-term as functional memory T cells (5). In parallel studies T cells immune to FBL could be grown in vitro nonspecifically in response to IL-2 as the major stimulus for proliferation (as opposed to Ag), but became dependent on IL-2 and failed to survive in vitro or in vivo without exogenous IL-2, thus possessing limited therapeutic efficacy in vivo. The current study shows that tumor-specific T cells from spleens of FBL-immune mice nonspecifically expanded in vitro with anti-CD3 plus low-doses of IL-2 can retain specific function against tumor in vitro and can eradicate disseminated leukemia in vivo. The use of anti-CD3 plus IL-2 was more effective than the use of IL-2 alone in inducing T cell expansion and function. Despite the ability of anti-CD3 expanded T cells to function in an Ag-specific manner, such T cells showed a gradual decline in anti-tumor activity both in vitro and in vivo with repeated cycles of anti-CD3 stimulation. However, despite the decreases in specific anti-tumor function on a per cell basis, anti-CD3 stimulation resulted in a substantial increase in total T cell number and an overall net increase in effector T cell numbers available for in vivo therapy. It is possible, therefore, that not all functional subsets of immune T cells were equally expanded with anti-CD3 in vitro. One identifiable problem for the method used was an enrichment of predominantly $CD8^+$ T cells. It has been previously demonstrated that the generation of an optimal $CD8^+$ T cell cytotoxic response against FBL requires help from immune $CD4^+$ cells (19, 20). Therefore, methods that enhance the growth of both $CD8^+$ and $CD4^+$ populations in response to anti-CD3 stimulation are being pursued in an attempt to maintain the functional activity of both subsets of immune T cells through successive cycles of antibody stimulation. The implications of using antibody-expanded T cells for adoptive immunotherapy of cancer are discussed.

MATERIALS AND METHODS

Mice. Female C57BL/6 (H-2^b, Thy-1.2; denoted B6) and BALB/c (H-2^d) mice, 6 to 12 wk of age, were obtained from The Jackson Laboratory (Bar Harbor, ME).

To generate B6 mice specifically immune to FBL-3, donor mice were inoculated with 10^7 irradiated (12,000 rad γ-irradiation) FBL-3 cells i.p. for a total of two doses given 2 wk apart, and the immune spleen cells were harvested 2 to 6 wk later.

Tumor cell lines. FBL-3 (FBL), a Friend murine leukemia virus (F-MuLV)-induced leukemia of B6 (H-2^b) origin, was subcloned and maintained by in vivo serial i.p. transplantation in syngeneic mice for use as an in vitro and in vivo stimulator cell, and maintained by in vitro passage for use as targets in cytotoxicity assays. EL4(G-)(EL-4), a chemically-induced thymoma antigenically unrelated to FBL-3, of B6 origin, was subcloned and maintained by in vitro passage. This cell line has been used as a specificity control in previous publications (1).

Reagents. Anti-CD3 mAb, 145-2C11, an IgG1 produced by a previously described hamster-mouse hybridoma (gift from Dr. Jeff Bluestone) recognizing the ϵ-chain of the murine CD3 complex (21). Antibody was affinity purified from culture supernatants by passage over a protein A-Sepharose column (Zymed, Burlingame, CA), and eluted with 0.2 M glycine, pH 2.8. The purified antibody was dialyzed extensively against PBS, sterilized by passage through an 0.2-μm filter, and stored at −70°C until use. Purity of each antibody preparation was assessed by gel electrophoresis, and concentration determined by OD_{280} by using a Beckman spectrophotometer. Human rIL-2, generously provided by Cetus Corp. (Emeryville, CA), was diluted in HBSS containing 1% pathogen-free syngeneic mouse sera.

FITC-conjugated anti-CD8 and anti-Thy-1.2 mAb and PE[3]-conjugated anti-CD4 mAb were purchased from Becton-Dickinson Immunocytometry Systems (Mountain View, CA) for use as staining reagents.

Generation and culture of anti-CD3 plus IL-2-stimulated cells from tumor-immune spleens. Splenocytes from mice previously immunized to FBL-3 were stimulated by culture with soluble anti-CD3 plus IL-2. For the first round of stimulation, 4×10^7 splenocytes were suspended in 20 ml media consisting of 50% RPMI 1640/50% Eagle's Hanks' amino acids supplemented with 10% selected FCS, 0.002 M mercaptoethanol, 100 U/ml penicillin, 100 U/ml streptomycin and fresh L-glutamine, and affinity-purified anti-CD3 mAb added at a final concentration of 0.5 to 1.0 μg/ml. This dose of antibody was initially determined to be optimal in inducing FBL-immune spleen cell proliferation in a [^{3}H]TdR uptake assay. After 2 days in culture, the cells were harvested, washed in HBSS to remove residual anti-CD3, and placed in fresh media supplemented with 20 U/ml IL-2. A second dose of IL-2, 20 U/ml, was added on day 4 of culture. For subsequent rounds of stimulation with anti-CD3, cells were harvested when they appeared to be resting (as judged by microscopic inspection) approximately on days 8 to 12 of culture, and viability was assessed by trypan blue dye exclusion. A total of 2×10^6 recovered viable cells were restimulated with anti-CD3 mAb (0.5 μg/ml final concentration) in the presence of 5×10^6 irradiated (3300 rad γ-irradiation) B6 spleen cells as fillers in 20 ml media. As with the initial round of stimulation, the cells were again harvested, washed, and placed in fresh IL-2-containing media on day 2, with a second IL-2 supplementation on day 4. Cultures were split and fresh media (without supplemental IL-2) was added as necessary to maintain cell density at approximately 1 to 2×10^6 cells/ml. Cells were routinely harvested for assays on days 8 to 12 after addition of anti-CD3.

Cell-surface staining. Cells were removed from culture, sedimented, and resuspended at 1 to 2×10^7 cells/ml in HBSS containing 0.1% Na azide and 5% FCS. All staining steps were performed at 4°C for 45 min, and the stained cells washed in azide-containing HBSS. For some experiments, the samples were preserved by the addition

[3] Abbreviations used in this paper: PE, phycoerythrin; CY, cyclophosphamide; LAK, lymphokine-activated killer cells.

of an equal volume of 2% paraformdehyde in PBS at 4°C for up to 1 wk before analysis. Thy-1.2$^+$ cells were labeled with FITC-conjugated anti-Thy-1.2 mAb. Percentages of CD8$^+$ and CD4$^+$ cells were determined by simultaneous staining with FITC-conjugated anti-CD8 mAb and PE-conjugated anti-CD4 mAb, respectively, followed by flow microfluorometry analysis on an Ortho Systems 50H cytofluorograph interfaced to a model 2150 computer (Ortho Diagnostics Systems, Westwood, MA).

In vitro proliferative assays. For in vitro assay of proliferative responses, spleen cells from mice previously immunized with FBL-3 were plated at 10^6 cells/well in 96-well, flat bottom plates (Costar, Cambridge, MA) in RPMI 1640 containing 10% FCS. Anti-CD3 mAb was added at the concentration indicated in each figure legend. Each well contained a 200 μl final volume, and samples were performed in triplicates. The plates were cultured at 37°C for 3 days, and 1.0 μCi of [^{3}H]TdR (20 mCi/mM, New England Nuclear, Boston, MA) was added to each well for the final 18 h of culture. All cultures were harvested onto glass fiber filter strips, and counted in a scintillation counter.

In vitro cytotoxicity assays. The cytotoxic activity of effector cells was assayed in a standard 4-h ^{51}Cr-release assay by using the indicated tumor targets, as previously published (1).

Adoptive chemoimmunotherapy model. This assay, previously described in detail, consists of treating mice bearing established disseminated FBL-3 leukemia with a combination of chemotherapy and adoptively transferred immune cells (5). Briefly, on day 0, host B6 mice are inoculated i.p. with 5×10^6 viable FBL-3 leukemia cells, a 100% lethal dose without therapy. By day 5, after the tumor is widely disseminated and present outside of ascites in spleen, lymph nodes, and peripheral blood, mice receive CY (180 mg/kg) i.p. 4 to 6 h before adoptive transfer of either fresh splenocytes from mice previously immune to FBL-3, or cultured anti-CD3-expanded immune cells i.p. CY was utilized to decrease tumor burden and to increase donor T cell proliferation and long-term survival in vivo in this model. In the absence of prior host immunosuppression with CY, immune T cells injected on day 5 into tumor-bearing mice are totally ineffective and exert no detectable therapeutic efficacy. Therapy groups contained a minimum of six mice/experiment, and control groups included mice receiving no therapy or CY alone.

RESULTS

Splenic T cells from tumor-immune mice can be expanded nonspecifically in vitro by using anti-CD3 plus IL-2. Using the optimal antibody concentration (0.5 μg/ml) previously determined, it was possible to grow and expand spleen cell number in vitro approximately 60-fold during a single 10-day cycle of anti-CD3 stimulation supplemented with two doses of exogenous IL-2 of 20 U/ml on days 2 and 4 (Table I). Similar degrees of T cell expansion could be obtained with additional cycles of exposure to anti-CD3 and IL-2. Anti-CD3 alone expanded spleen cells to a much lesser degree. Thus, expansion by anti-CD3, which is known to up-regulate IL-2R on T cells and induce secretion of endogenous IL-2, was limited by availability of endogenous IL-2 production. Exogenous IL-2 given alone could induce proliferation of some cells, but the combination of anti-CD3 followed by IL-2-induced greater expansion. As expected, cells expanded nonspecifically from FBL-immune spleens were greater than 95% T cells as detected by cell-surface staining with anti-Thy-1.2 antibody (Table II).

TABLE I

Splenic T cells from tumor-immune mice can be nonspecifically expanded with anti-CD3 plus IL-2[a]

Anti-CD3 Antibody (μg/ml) (Day 0)	IL-2, (U/ml) (Days 2 and 4)	Expansion of Thy 1.2$^+$ Cells by Day 8
0.5	None	3×
0.5	10	12×
0.5	20	50×
0.5	50	62×
0.5	200	67×
None	200	5×

[a] Spleen cells from mice immune to FBL were stimulated in vitro with anti-CD3 at 0.5 μg/ml for 48 h, washed, and IL-2 was added at the indicated concentrations on days 2 and 4. The total number of viable cells was enumerated on day 8 of culture, and the percentage of Thy-1.2$^+$ cells determined by staining with FITC-conjugated anti-Thy-1.2 antibody as analyzed by flow microfluorometry.

Splenic T cells from tumor-immune mice expanded with anti-CD3 plus IL-2 retain specific function in response to tumor in mixed lymphocyte-tumor culture and are predominantly CD8$^+$ in phenotype. Previous work has shown that noncultured splenic T cells from mice previously immunized to FBL are not directly lytic to FBL tumor cells, but CD8$^+$ CTL can be generated by specific stimulation in vitro with irradiated tumor (11, 19, 20). Ability to generate this response normally requires priming and involves both immune CD8$^+$ T cells and immune CD4$^+$ helper/inducer T cells (19). The current study above showed that T cells in spleens of mice immune to FBL could be nonspecifically expanded by culture with anti-CD3 plus IL-2. In order to determine whether such cells retained the ability to become specifically cytolytic to FBL, T cells that had undergone one, two, or three cycles of antibody-induced expansion were then placed in mixed lymphocyte-tumor culture for an additional 5 days followed by testing for tumor-specific cytotoxicity against a specific (FBL) and an antigenically unrelated syngeneic tumor target (EL-4) in a standard 4-h ^{51}Cr release assay. As shown in Table III, the population of T cells expanded nonspecifically for one and two cycles with anti-CD3 plus IL-2 retained the ability to lyse FBL specifically after resensitization with tumor in vitro. The cytotoxicity was not caused by residual anti-CD3 binding to FcR on target cells because unrelated FcR-positive targets were not lysed (data not shown). The percent lysis at a given E:T ratio decreased with each additional cycle of in vitro expansion by anti-CD3. However, despite the apparent decrease in frequency of tumor-reactive T cells in the viable cell population after each stimulation, there was an overriding increase in the total number of T cells obtained with each cycle. Thus, there remained an overall 10- to 15-fold net increase in total lytic units available within the anti-CD3 expanded population. Although more T cells were required to achieve the same level of cytolytic activity, the total number of T cells available far exceeded that need. As a control, splenic T cells grown in the same dose of IL-2 alone were present only in small numbers and did not display significant cytolytic function against either tumor target (data not shown). Thus, maintenance of specific immune function required stimulation by anti-CD3 in addition to IL-2.

It was not possible to elicit significant tumor lysis even at the highest E:T ratio tested (80:1) if the spleen cells had undergone three or more cycles of anti-CD3 expansion in vitro (Table III). Previous studies in the FBL system have shown that CD4$^+$ and CD8$^+$ T cells are both required for the generation of cytolytic function against FBL in mixed lymphocyte-tumor culture (19). The percentage of CD4$^+$ T cells declined after each cycle of anti-CD3 stimulation (Table II). Therefore, the marked decrease in CD4$^+$ T cells might account for the reduced ability to generate specific CD8$^+$ CTL from the cultured populations. However, the fall in CD4$^+$ T cells from the second to third cycle of only 7.5% to 6.7% might not by itself fully explain the substantial decrease in lytic potential from cycle 2 to cycle 3 (42.6% lysis vs 3.5% lysis) and alternative expla-

TABLE II

Splenic T cells from tumor-immune mice expanded with anti-CD3 plus IL-2 are predominantly CD8[a]

Source of Cells	Conditions	Day of Culture	% Thy 1.2$^+$	% CD4$^+$	% CD8$^+$
FBL-Immune Spleen	Not cultured	D.0	21.8	13.8	10.1
FBL-Immune Spleen	One cycle of anti-CD3 + IL-2	D.10	97.3	7.5	91.7
FBL-Immune Spleen	Two cycles of anti-CD3 + IL-2	D.20	98.6	6.7	92.4

[a] Spleen cells from mice immune to FBL were stimulated in vitro with anti-CD3 at 0.5 μg/ml for 48 h, washed, and IL-2 was added at 20 U/ml on days 2 and 4. The noncultured spleen cells and the cells after expansion with either one (day 10) or two (day 20) cycles of anti-CD3 antibody were stained with FITC-conjugated anti-Thy-1.2 and anti-CD8 antibodies and PE-conjugated anti-CD4 antibody and analyzed by flow microfluorometry.

TABLE III

Splenic T cells from tumor-immune mice expanded with anti-CD3 plus IL-2 retain tumor-specific function in mixed lymphocyte-tumor culture[a]

Responder Population	Anti-CD3 + IL-2 Expansion	In Vitro Stimulator	% Lysis (E:T = 80:1)	
			FBL	EL-4
FBL-immune spleen	None	$(FBL)_x$	66.8	2.5
		None	16.2	5.5
FBL-immune spleen	One cycle	$(FBL)_x$	52.3	0.0
		None	0.0	0.0
FBL-immune spleen	Two cycles	$(FBL)_x$	42.6	4.4
		None	0.7	4.5
FBL-immune spleen	Three cycles	$(FBL)_x$	3.5	2.4
		None	0.0	4.7
BALB/c spleen	None	B6 spleen	67.5	58.5

[a] Spleen cells from mice immune to FBL were stimulated in vitro with anti-CD3 at 0.5 μg/ml for 48 h and washed, and IL-2 was added at 20 U/ml on days 2 and 4. For the second cycle of expansion with anti-CD3, the cells were harvested on days 8 to 10 of the preceding round, washed, and placed in fresh media, and IL-2 was added at 20 U/ml on days 2 and 4. Noncultured FBL-immune spleen cells, or cells harvested after indicated number of cycles of expansion in vitro with anti-CD3 were then placed in mixed lymphocyte-tumor culture with irradiated FBL tumor for 5 days. Harvested cells were then assayed in a standard 4-h ^{51}Cr release assay for cytotoxicity against FBL or unrelated tumor (EL-4) targets.

nations might be entertained (see *Discussion*).

Splenic T cells from tumor-immune mice expanded nonspecifically in vitro with the use of anti-CD3 plus IL-2 are effective in the eradication of disseminated FBL-3 leukemia in vivo. Disseminated FBL leukemia can be eradicated by use of immune T cells in combination with chemotherapy. The effect of immune T cells is tumor-specific and dependent both on the number of cells transferred as well as the ability of donor T cells to survive in vivo (1, 5). Survival of the mice can be used as a semi-quantitative endpoint to measure therapeutic efficacy of donor T cells. In the current study the effector population for use in adoptive chemoimmunotherapy was T cells from spleens of FBL-immune mice expanded in vitro as described above by using anti-CD3 plus IL-2 for one, two, or three cycles. Control groups included mice receiving no therapy, CY treatment alone, or CY plus noncultured spleen cells from FBL-immune mice. Results shown in Figure 1 represent cumulative data from several experiments, and data are presented in Figures 1*A* and 1*B* separately for the sake of clarity in illustrating distinct observations. Mice receiving no treatment had a median survival of 12.3 days, whereas those receiving CY alone had a median survival of 23.3 days, with neither group having any long-term survivors (Fig. 1*A*). By contrast, therapy with CY plus T cells expanded in vitro with anti-CD3 plus IL-2 cured 100% of animals at a T cell dose of 10^7 cells/mouse, and 50% of mice at a dose of 5×10^6 T cells/mouse. When comparing the survival curve gen-

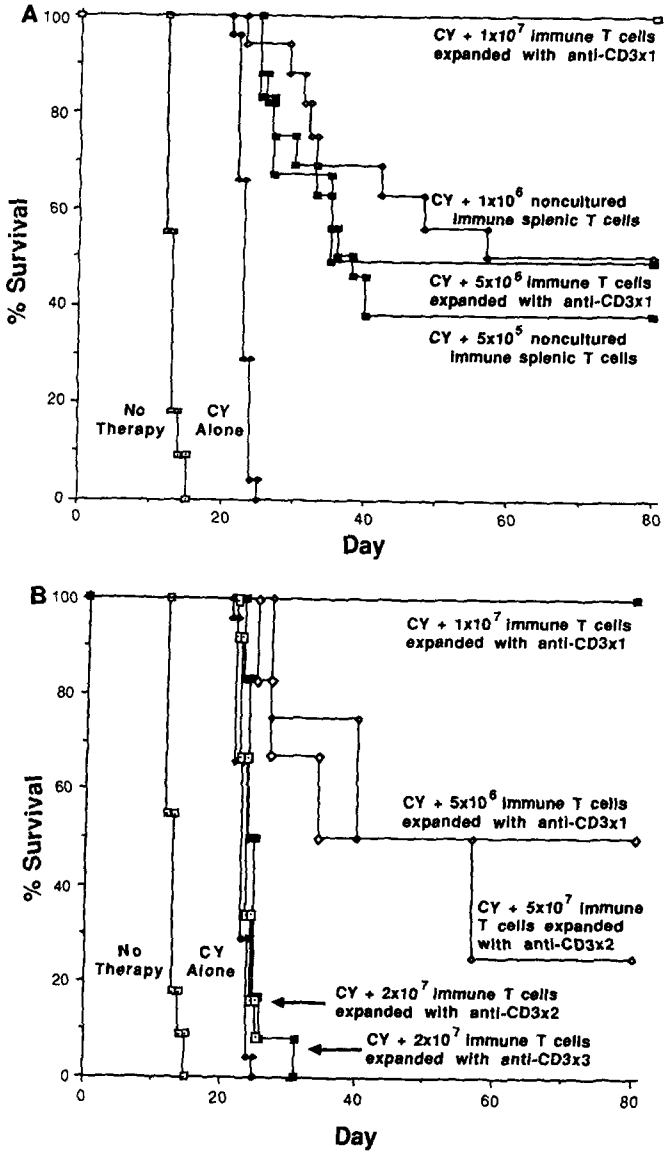

Figure 1. Splenic T cells from tumor-immune mice expanded nonspecifically in vitro with anti-CD3 plus IL-2 are effective in the in vivo therapy of disseminated FBL leukemia. On day 0, B6 mice were inoculated i.p. with 5×10^6 viable FBL leukemia cells and left untreated, treated on day 5 with 180 mg/kg CY, or treated on day 5 with CY plus the indicated number of noncultured FBL-immune spleen cells or T cells from FBL-immune mouse spleens after 1, 2, or 3 cycles of anti-CD3 expansion in vitro. The numbers represent the percent surviving mice from several separate experiments, with a total of at least 11 mice/group. Survival of animals was followed for 80 days. All cell doses are expressed as the number of T cells injected. *A*, different cell doses are compared between noncultured FBL-immune spleen cells and such cells after one cycle of in vitro anti-CD3 plus IL-2 expansion. *B*, different cell doses are compared between T cells stimulated with anti-CD3 in vitro for one, two, or three cycles.

erated by using noncultured FBL-immune spleen cells to that of T cells expanded by one cycle of anti-CD3 stimulation, an approximately fivefold higher dose of anti-CD3-expanded T cells was required to achieve a comparable 50% survival (i.e., 5×10^6 anti-CD3 expanded T cells were equivalent in efficacy to 10^6 noncultured immune T cells). Despite the fivefold decrease in efficacy on a per cell basis associated with one cycle of anti-CD3 expansion, an increase of greater than 50-fold in T cell number was achieved. Thus, there was an overall net increase of therapeutic efficacy on a per cell population basis after in vitro culture with anti-CD3.

Figure 1*B* depicts the relative in vivo efficacy of transferred spleen cells stimulated by anti-CD3 for one, two, or three cycles in vitro. Results show that there was a general decrease in efficacy of cultured T cells on a per cell basis between cycles 1 and 2 of in vitro anti-CD3 expansion. After the second round of anti-CD3 plus IL-2 expansion, a T cell dose of 2×10^7 cells/mouse did not result in any long-term survival and it required 5×10^7 T cells/mouse to achieve a detectable rate of long term cure. This represented a greater than 100-fold increase in the dose of T cells required per mouse for significant cure, as compared to the cure rate achieved by 5×10^5 noncultured tumor-immune splenic T cells (Fig. 1*A*). However, an approximately 2500-fold increase in total T cell number was obtained over the initiation of culture. Thus, as with one cycle, two cycles of expansion also produced a decrease in anti-tumor T cell efficacy on a per cell basis, but an increase in total efficacy on a cell population basis. Finally, splenic T cells cultured for three cycles with anti-CD3 and IL-2, were shown to be totally ineffective in vivo at a cell dose of 2×10^7 cells/mouse (Fig. 1*B*). No higher T cell doses were tested in vivo as it was demonstrated in vitro (Table III) that this cell population was no longer capable of becoming specifically cytolytic when restimulated with FBL.

DISCUSSION

Successful immunotherapy of cancer with adoptively transferred tumor-specific T cells requires large numbers of functional T cells. The limited therapeutic efficacy of a small number of tumor-immune T cells can be greatly augmented by in vitro growth with specific and/or nonspecific stimuli. However, conventional specific methods of in vitro T cell culture with the use of tumor material have been difficult to reproduce in various human systems and conventional nonspecific methods using IL-2 select for IL-2 dependent and often Ag nonspecific T cells. Therefore, we have studied an alternative means for T cell expansion in vitro with the use of a mAb to CD3. CD3 is thought to be involved in normal TCR signaling events leading to T cell activation. Triggering via this cluster of determinants by an antibody should be similar to Ag stimulation. mAb can be readily obtained in large quantities in purified forms and thus, the same reagent can be used in a variety of settings of T cell culture. Moreover, the stimulating activities of antibodies do not require Ag processing and presentation by MHC-compatible APC. In this paper, we show that anti-CD3 antibody can be used to stimulate and expand tumor-specific T cells in vitro from tumor-immune mouse spleens containing a heterogeneous population of tumor-reactive and tumor nonreactive T cells. Furthermore, the expanded cell population was shown to be functional in vitro and effective in the eradication of disseminated leukemia in vivo.

Reports have demonstrated that the use of anti-CD3 and high concentrations of IL-2 (1000 U/ml) could lead to activation of cytotoxic cells that were capable of nonspecifically lysing autologous and heterologous tumor targets (22–25). In our studies, we have purposely avoided the selection of Ag nonspecific lymphocyte populations, such as LAK cells or activated NK cells, by expansion of spleen cells with the use of anti-CD3 in the presence of low doses (20 U/ml) of IL-2. Low dose IL-2 alone neither induced significant expansion nor cytotoxic activity in the spleen cultures and when used in combination with anti-CD3 did not activate nonspecific cytolytic cells. Fewer than 5% of the expanded cells were of the NK-1.1^+ phenotype, and virtually no nonspecific cytolytic activity was detected.

The T cells activated by anti-CD3 were expanded nonspecifically in vitro with low dose IL-2 for up to two cycles (3 wk in culture) from tumor-immune spleens and were shown to retain Ag-specific function when assayed in vitro, with no significant cytotoxicity against an antigenically distinct LAK-sensitive target, EL-4 (1). This suggests that the tumor-specific memory cytolytic T cell populations in the tumor-immune spleens had been preserved and expanded by our culture conditions with anti-CD3 plus IL-2, whereas LAK activity was not induced. However, there was a decrement in in vitro tumor-specific cytolytic activity on a per cell basis with each cycle of stimulation with anti-CD3 plus IL-2. Little or no tumor-specific cytolytic activity was detected after three cycles of anti-CD3 expansion in vitro (over 1 mo in culture) even at the highest E:T ratio tested (80:1). The reduction of anti-tumor activity in vitro correlated with therapeutic efficacy in in vivo adoptive chemoimmunotherapy experiments in which an increase in the dose of anti-CD3-expanded T cells was necessary for curing the same percentage of tumor-bearing mice after successive cycles of anti-CD3 stimulation. The loss of efficacy after expansion suggests that tumor-reactive T cells in the anti-CD3-expanded population might have been outgrown by T cells not reactive to the tumor. Alternatively, it is also possible that anti-CD3 stimulation affected the ability of the T cells to subsequently respond to Ag (i.e., by modulation of the TCR from the cell surface or direct inhibition of T cells to lyse tumor). However, the latter possibility does not appear to be likely because in preliminary experiments (not presented) FBL-specific T cell clones of either $CD4^+$ or $CD8^+$ phenotype could be grown long term in vitro (up to 60 days) with repetitive anti-CD3 plus IL-2 stimulation with no apparent loss of functional activity or specificity. In addition, tumor-immune spleen cells that had first undergone in vitro sensitization with tumor, and were therefore enriched for tumor-specific T cells, were much more efficacious on a per cell basis in adoptive chemoimmunotherapy after anti-CD3 expansion compared with cells that had only been exposed to anti-CD3 plus IL-2 in vitro. These results suggest that the use of anti-CD3 to expand tumor-specific T cells to large numbers for use in therapy may be optimal when supplemented by the use of tumor cells at the initiation of in vitro stimulation. This may be best applied to the expansion of cells such as tumor-infiltrating lympho-

cytes, which are presumed to have been partially enriched and primed by being at the site of tumor.

In order to explain the loss of function of tumor-specific T cells after anti-CD3 stimulation, it is noteworthy that the ratio of $CD4^+$ to $CD8^+$ cells in such spleen cultures changed in favor of the $CD8^+$ population with each additional cycle of stimulation. The generation of optimal $CD8^+$ cytolytic T cell responses to FBL from unfractionated spleen cell populations requires the help of $CD4^+$ T cells (19). FBL-specific $CD4^+$ T cells are not cytolytic but produce lymphokines after activation by MHC class II^+ APC such as macrophages that have processed FBL tumor Ag. Therefore, as the number of $CD4^+$ T cells diminished with each cycle of stimulation in culture, it is likely that the lymphokines available for supporting an optimal cytolytic response by the $CD8^+$ population became limiting, which led to a decline in T cell cytolytic function as seen in in vitro and in vivo experiments. This is further supported by the finding that FBL-specific $CD8^+$ cytolytic T cells possessed limited therapeutic efficacy against FBL in vivo in the absence of $CD4^+$ cells (26). It is not clear whether this selective expansion of the $CD8^+$ population is caused by a relative difference in growth rate between the two subsets after antibody stimulation or an absolute inability of some $CD4^+$ T cells to respond to the regimen employed. Similar experiments using established FBL-specific $CD4^+$ or $CD8^+$ T cell clones demonstrated that both types of cells grew in vitro to a similar degree in response to a similar range of antibody concentrations (data not shown).

Methods of cell culture that enhance the growth of $CD4^+$ T cells in the anti-CD3-stimulated culture might be able to better maintain anti-tumor function through successive cycles of expansion. It has been shown that the use of antibodies to both CD3 and CD4 together could lead to greater activation of $CD4^+$ T cells than with anti-CD3 alone (27). Such an approach is currently being tested in this system in an attempt to enhance the growth of $CD4^+$ T cells for promoting the subsequent expansion of $CD8^+$ T cells. Furthermore, because an antibody to the TCR/CD3 complex can be used to expand T cells, antibodies to the V region of the receptor may be used to induce the growth of more selective populations of T cells. Antibodies to $V\alpha$ and $V\beta$ gene products or to clonotypic structures of the TCR expressed by tumor-specific T cell clones could be similarly used to stimulate the corresponding T cell populations to expand, thereby achieving a greater degree of selectivity in culture than the use of anti-CD3 (28, 29).

In conclusion, we propose that stimulation with anti-CD3 plus low concentrations of IL-2 has been effective in expanding tumor-specific T cells that were present at a low frequency in spleens of tumor immune mice. The in vitro stimulation with anti-CD3 does not lead to a preferential activation of tumor-immune T cells. Rather, tumor-specific T cells appear to be selected against and expand more slowly than T cells not reactive to tumor. This apparent selection of tumor nonreactive T cells may in part be explained by the diminution of $CD4^+$ T cells after each cycle of stimulation which are known to be critical in promoting optimal $CD8^+$ T cell-mediated responses. Work is in progress to determine whether enhanced growth of $CD4^+$ T cells in anti-CD3-stimulated cultures can maintain the antitumor function through long-term expansion.

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