ERADICATION OF DISSEMINATED MURINE LEUKEMIA BY CHEMOIMMUNOTHERAPY WITH CYCLOPHOSPHAMIDE AND ADOPTIVELY TRANSFERRED IMMUNE SYNGENEIC Lyt-1$^+$2$^-$ LYMPHOCYTES*

By PHILIP D. GREENBERG, MARTIN A. CHEEVER, and ALEXANDER FEFER

From the Division of Oncology, Department of Medicine, University of Washington School of Medicine, and Fred Hutchinson Cancer Research Center, Seattle, Washington 98195

T lymphocytes immune to tumor-associated antigens (TAA) on syngeneic tumor cells can readily be shown to lyse tumor cells in vitro and inhibit the growth of transplanted tumors if administered shortly before, with, or shortly after the inoculation of tumor. However, after a tumor has become established in the host, therapy with adoptively transferred lymphocytes alone has generally been ineffective (1). The limited ability of adoptive immunotherapy to eradicate established tumors presumably reflects consequences of a growing tumor, such as a large tumor burden and induction of suppressor cells or suppressor factors in the host (2, 3). Thus, immunotherapeutic approaches that include modifying this underlying host-tumor relationship have been more successful. For example, in therapy of established locally growing rat and murine fibrosarcomas, the efficacy of immune T cells is enhanced if, before inoculation of the tumors, the host has been irradiated or rendered T cell deficient (3, 4). Several immunotherapy models have been developed in our laboratory in which mice with established disseminated syngeneic leukemias can be effectively treated with a combination of noncurative, nonlethal chemotherapy with cyclophosphamide (CY),[1] followed by adoptively transferred syngeneic immune cells (5–8). In these models, the CY has a direct tumoricidal effect (6), as well as potentially facilitating effects on host tumor immunity, and therapy with immune cells without CY has no apparent in vivo anti-tumor effect (5). In vivo efficacy in such adoptive chemoimmunotherapy (ACIT) requires immune T cells capable of proliferating in the host after adoptive transfer (5, 7), and studies of ACIT of two non-cross-reactive tumors of C57BL/6 origin have confirmed that the lymphocytes must be specifically immune to the relevant tumor (8).

The cellular mechanisms by which adoptively transferred T cells mediate tumor destruction in vivo might be further elucidated by determination of the functional T cell subsets operative in immunotherapy. Although the end results might reflect direct

* Supported by grant CA10777 and contract N01-CB-84247 from the National Cancer Institute, National Institutes of Health. Dr. Greenberg is an American Cancer Society Junior Faculty Fellow. Dr. Fefer is an American Cancer Society Professor of Clinical Oncology.

[1] *Abbreviations used in this paper:* ACIT, adoptive chemoimmunotherapy; C, complement; CRA, chromium-release assay; CTL, cytotoxic T lymphocyte; CY, cyclophosphamide; DTH, delayed-type hypersensitivity; FBL, Friend virus-induced leukemia of C57BL/6 origin; IL-2, interleukin 2; MBL, Moloney virus-induced leukemia of C57BL/6 origin; MLC, mixed leukocyte culture; MLTC, mixed leukocyte-tumor culture.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/81/09/0952/12 $1.00
Volume 154 September 1981 952–963

tumor lysis by infused cytotoxic donor cells, comparisons of the ability of immune lymphocytes to mediate in vitro tumor lysis and in vivo immunotherapy have generally revealed a poor correlation between these two effector functions (4, 9, 10). Expression of Lyt antigens has been shown to be useful for separating functional T cell subsets (reviewed in 11, 12). By negative selection using cytotoxic depletion with antibody and complement, T cells can be operationally separated into Lyt-1^+2^- cells containing the amplifier, helper, and delayed type hypersensitivity (DTH) effector cells, Lyt-1^-2^+ cells containing cytotoxic and suppressor cells, and an Lyt-1^+2^+ compartment containing precursors for the other T cell subsets, as well as a portion of cytotoxic and suppressor effector cells (11, 12). In particular, cytotoxic T lymphocytes (CTL) to syngeneic tumors appear to reside in both the Lyt-1^-2^+ and Lyt-1^+2^+ compartments (13–17). Therefore, in the present studies, the efficacy of T cell subpopulations, as distinguished by expression of Lyt antigens, was analyzed in ACIT of established murine leukemia and in in vitro assays for the generation and expression of cytotoxicity. The results demonstrate that the phenotype of the predominant T cell subset required for efficacy in ACIT is Lyt-1^+2^-, which is distinct from the T cell phenotypes mediating in vitro tumor lysis.

Materials and Methods

Mice. 12–16-week-old (BALB/c × C57BL/6)F_1 mice, denoted CBF_1, were obtained from The Jackson Laboratory, Bar Harbor, Maine. B10.G ($H\text{-}2^q$) and AKR/J ($H\text{-}2^k$) mice were provided by our breeding facility at the Fred Hutchinson Cancer Research Center.

Tumor. FBL-3 is a Friend virus-induced leukemia of C57BL/6 origin maintained by serial intraperitoneal transplantation (>300 generations) in adult syngeneic mice. CBF_1 mice were sensitized to FBL-3 in vivo by inoculation of two doses of 2×10^7 irradiated FBL-3 intraperitoneally at 2-wk intervals, and the immune spleen cells were obtained 2–4 wk later.

ACIT. This assay, previously described in detail (5), consists of treating mice that have advanced disseminated FBL-3 leukemia with a combination of nonlethal noncurative chemotherapy and adoptively transferred immune syngeneic cells. CBF_1 mice were inoculated with 5×10^6 FBL on day 0, and treated on day 5, after tumor dissemination had occurred (6), with 180 mg/kg CY injected intraperitoneally, followed in 5 h by adoptively transferred CBF_1 cells. Therapy on day 5 with immune cells alone, without prior CY, has no demonstrable anti-tumor effect (5). Studies with non-cross-reactive tumors of C57BL/6 origin have demonstrated that therapeutic efficacy in ACIT requires the transfer of specifically immune lymphocytes (8), and the degree of prolongation of survival and percentage of cures is proportional to the number of immune cells transferred (8, 9). Survival curves in the present studies were constructed from the cumulative results of six experiments with mice treated with CY and a dose of 5×10^6 immune CBF_1 cells. This cell dose was selected for presentation because CY plus 5×10^6 unfractionated immune cells significantly prolonged survival but did not cure all mice, and thus was the optimal dose for comparisons of the efficacy of subpopulations. In groups of mice not shown, which were treated concurrently with smaller numbers of fractionated or unfractionated immune cells, survival was proportionately shorter and the percentage of cures was smaller for all groups, consistent with the dose-response relationship of the number of immune cells to the outcome of therapy reported in these ACIT models (8, 9).

Cell Depletion with Antibody and Complement (C). Spleen cells were incubated at 4°C for 30 min with monoclonal IgM antibody to Thy-1.2, a kind gift of Dr. E. Clark of the University of Washington, Seattle, at a dilution of $1{:}10^4/10^6$ cells, arsanilate-conjugated monoclonal IgG α-Lyt-1 antibody at 0.6 $\mu g/10^6$ cells, or arsanilate-conjugated monoclonal IgG α-Lyt-2 antibody at 0.3 $\mu g/10^6$ cells (Becton, Dickinson & Co., Oxnard, Calif.). Cells labeled with arsanilate-conjugated antibody were washed and incubated for an additional 30 min at 4°C with rabbit α-arsanilate antibody at 12 $\mu g/10^6$ cells. The cells were washed and incubated for 45 min at 37°C with agarose-absorbed selected rabbit C at a final dilution of 1:36. At these dilutions, the

antibodies were not directly toxic to cells, and with the addition of C maximally lysed thymocytes and spleen T cells.

In Vitro Sensitization. Mixed leukocyte cultures (MLC) and mixed leukocyte-tumor cultures (MLTC) were established as described previously (9). Briefly, 60×10^6 CBF_1 responder spleen cells were cultured for 5 d in flasks containing 20 ml of supplemented 5% fetal calf serum with 15×10^6 irradiated stimulator allogeneic spleen cells or 3×10^6 irradiated FBL-3 tumor cells.

The potential role of Lyt-1$^+$ T cells as helper-amplifier cells in the generation of in vitro cytotoxic responses was examined by depleting responder cells of Lyt-1$^+$ cells before in vitro culture and reconstituting the amplifier function of Lyt-1$^+$ cells by the addition of supernates containing interleukin 2 (IL-2). The supernates, induced by concanavalin A stimulation of rat spleen cells, were kindly provided by Dr. S. Gillis and Dr. C. Henney of the Fred Hutchinson Cancer Research Center, and had previously been purified on DEAE to remove concanavalin A, titered by microassay to quantify IL-2 activity, and demonstrated to reconstitute the amplifier activity of helper T cells for in vitro cytotoxic responses (18, 19). Therefore, CBF_1 spleen cells were depleted of Lyt-1$^+$ cells by treatment with α-Lyt-1 antibody and C before in vitro sensitization. At the initiation of 5-d sensitization culture, IL-2-containing supernates were added to selected groups at a final dilution of 1:10, a concentration sufficient to maximally support generation of CTL. After 5 d of culture in this IL-2-supplemented medium, cells were harvested and washed, and the generation of cytotoxicity was assessed.

In Vitro Cytotoxicity. Responder cells were harvested and tested for cytolytic activity by 4-h incubation with 10^4 chromium-labeled target cells in microtiter plates. Cytotoxicity is expressed as: percent specific lysis = 100 (A − B)/(C − B), with A = test sample counts per minute, B = spontaneously released counts per minute, and C = total counts per minute. Target cells were either FBL tumor cells or blast cells induced by 48 h incubation of normal spleen cells with concanavalin A at 2 μg/ml.

Results

Analysis of the Lyt phenotype(s) of the T cells responsible for efficacy in ACIT requires the transfer of large numbers of purified T cells. The availability of high titer monoclonal antibodies to Lyt determinants has recently made such analysis feasible. Before using these monoclonal reagents to separate Lyt subpopulations for immunotherapy, we examined whether these antibodies and selected absorbed rabbit C would appropriately delete CTL to alloantigens and syngeneic tumors, which have been well characterized phenotypically by others using conventional alloantisera to Lyt antigens. Therefore, CBF_1 spleen cells were sensitized to allogeneic spleen cells or syngeneic tumor cells in MLC, and cytotoxicity was examined after 5 d culture (Table I). Treatment of alloreactive effector cells immediately before testing in a 4-h chromium release assay (CRA) with α-Thy-1.2 and C eliminated all cytotoxicity. The majority of cytotoxic reactivity was similarly eliminated by treatment of effector cells with α-Lyt-2 and C, whereas depletion of Lyt-1$^+$ cells had only a minimal effect on cytotoxicity. Thus, as defined functionally, alloreactive CTL were predominantly of the Lyt-1$^-$2$^+$ phenotype.

CTL that are specific for syngeneic tumor can be readily generated by in vitro sensitization of in vivo-primed spleen cells (8, 9). For this analysis, tumor-primed cells were obtained from spleens of CBF_1 mice that had previously been inoculated twice with irradiated FBL, because these spleen cells subsequently served as the source of donor immune cells in adoptive immunotherapy. CBF_1 cells from nonimmune mice did not become significantly cytotoxic after primary in vitro sensitization to FBL and noncultured spleen cells from mice immunized to FBL in vivo were not directly cytotoxic to tumor (Table I). However, cytotoxic reactivity was readily detectable after 5 d in vitro sensitization of CBF_1 cells previously sensitized in vivo to FBL.

TABLE I
Phenotypes of CBF_1 ($H\text{-}2^{dxb}$) Cytotoxic Cells

Cytotoxicity generation culture*		Treatment of effector cells‡	Percent specific lysis§	
Responder	Stimulator		B10.G ($H\text{-}2^a$)	FBL ($H\text{-}2^b$)
Allogeneic response				
CBF	—	None	0 ± 0.1	0 ± 0.6
CBF	B10.G	None	33 ± 2.0	2 ± 1.5
CBF	B10.G	C only	31 ± 2.4	
CBF	B10.G	α-Thy-1.2 + C	0 ± 0.6	
CBF	B10.G	α-Lyt-1 + C	25 ± 1.0	
CBF	B10.G	α-Lyt-2 + C	9 ± 1.5	
Syngeneic anti-tumor response				
CBF	—	None		2 ± 0.6
CBF	FBL	None		5 ± 1.9
$CBF_{\alpha FBL}$	Not cultured	None		0 ± 0.3
$CBF_{\alpha FBL}$	—	None	0 ± 0.1	3 ± 2.1
$CBF_{\alpha FBL}$	FBL	None	1 ± 0.6	53 ± 6.5
$CBF_{\alpha FBL}$	FBL	C only		50 ± 5.9
$CBF_{\alpha FBL}$	FBL	α-Thy-1.2 + C		5 ± 1.8
$CBF_{\alpha FBL}$	FBL	α-Lyt-1 + C		27 ± 2.7
$CBF_{\alpha FBL}$	FBL	α-Lyt-2 + C		11 ± 3.5

* 60×10^6 responder cells derived from normal unprimed CBF_1 mice (CBF) or mice primed and boosted in vivo with irradiated FBL ($CBF_{\alpha FBL}$) were cultured with 15×10^6 irradiated allogeneic B10.G spleen cells or 3×10^6 irradiated FBL tumor cells.

‡ After 5 d culture, the potential effector cells were harvested. Before testing for cytotoxicity, specific cell subpopulations were depleted by incubating these cells with the denoted monoclonal antibody and C.

§ Cytotoxicity of potential effector populations was measured in a 4-h CRA with labeled concanavalin A-induced B10.G spleen cell blast targets or FBL tumor cells at an effector:target ratio of 20:1. The results are the mean cytotoxicities ± SE of four experiments.

Treatment of the effector cells with either α-Thy-1.2 or α-Lyt-2 and C eliminated most cytotoxicity, whereas ~50% remained after depletion of $Lyt\text{-}1^+$ cells. Thus, as defined functionally, the CTL to syngeneic FBL tumor were most likely of two phenotypes, $Lyt\text{-}1^-2^+$ and $Lyt\text{-}1^+2^+$. Alternatively, the CTL could be only of the $Lyt\text{-}1^-2^+$ phenotype, but this would require that $Lyt\text{-}1^+2^+$ cytotoxic precursor cells differentiate to $Lyt\text{-}1^-2^+$ CTL during the 4-h assay, and would operationally still yield similar results in the therapy studies using cytotoxic depletion of subpopulations. Thus, these monoclonal antibodies characterize CTL with Lyt phenotypes similar to that expected with conventional alloantisera (11–17).

The efficacy of adoptive immunotherapy with these T lymphocyte subpopulations was examined. CBF_1 mice were inoculated intraperitoneally with 5×10^6 FBL-3 on day 0. On day 5, when the tumor has already disseminated to peripheral blood and spleen (6), mice were treated with ACIT, CY followed by spleen cells from CBF_1 mice that had been primed and boosted in vivo with irradiated FBL-3, a regimen shown to induce specific immunity to this tumor (8). The spleen cells were used either directly or after depletion of subpopulations by C and monoclonal antibody to Thy-1.2, Lyt-1, or Lyt-2 antigen. The cumulative results of six experiments with immunotherapy using 5×10^6 donor cells are shown in Fig. 1. Mice receiving no therapy died by day 19, and therapy with CY alone prolonged median survival time to day 26, but all mice died of progressive tumor. Therapy on day 5 without CY and with

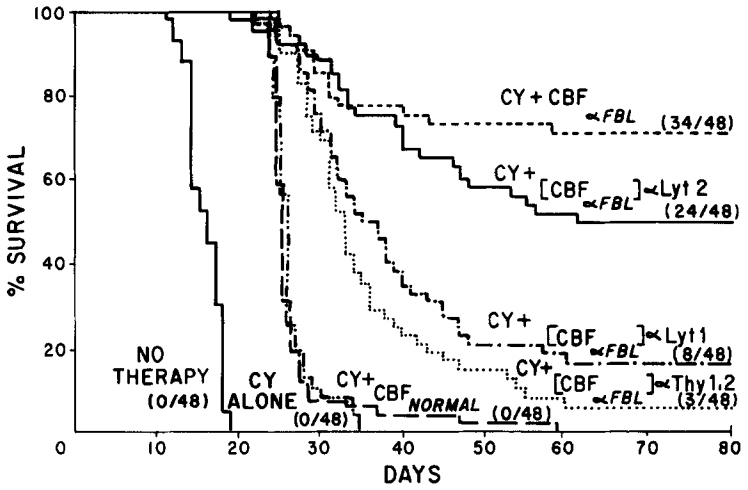

FIG. 1. In vivo efficacy of T cell subpopulations in ACIT of FBL. CBF_1 mice were inoculated with 5×10^6 FBL intraperitoneally on day 0, and left untreated (——, no therapy), treated on day 5 with cyclophosphamide (—··—, CY alone), or treated with CY plus 5×10^6 CBF_1 donor cells obtained either from normal nonimmune CBF_1 mice (– –, CBF_{normal}), or mice that had been primed and boosted in vivo with irradiated FBL. Donor immune spleen cells were used either unseparated (-----, $CBF_{\alpha FBL}$) or after depletion of subpopulations by in vitro incubation before adoptive transfer with C and antibody to Thy-1.2 (····, [$CBF_{\alpha FBL}$]α-Thy-1.2), Lyt-1 (—·—, [$CBF_{\alpha FBL}$]α-Lyt-1), or Lyt 2 (——, [$CBF_{\alpha FBL}$]α-Lyt-2). Fractions represent the number of mice surviving in the total cumulative group from six experiments.

only normal CBF_1 cells or CBF_1 cells immune to FBL-3 had no effect on survival (curves not shown), and therapy on day 5 with CY plus normal cells yielded the same results as therapy with CY alone. However, therapy with CY plus cells immune to FBL prolonged survival and cured 34 of 48 mice. Depletion of T cells from the immune population by in vitro treatment with α-Thy-1.2 and C before adoptive transfer abrogated most of the therapeutic effect, with only 3 of 48 mice alive at day 80, thus confirming the T cell nature of the major effector cell for chemoimmunotherapy. Depletion of Lyt-1$^+$ cells, which permits persistence of only Lyt-1$^-$2$^+$ T cells, produced similar results with only 8 of 48 mice alive at day 80. In contrast, immune cells depleted of Lyt-2$^+$ cells, which permits persistence of only Lyt-1$^+$2$^-$ T cells, retained most of the therapeutic potential of the initial unseparated population, with 24 of 48 mice tumor free at day 80. Thus, the major therapeutic effect provided by adoptive transfer of immune spleen cells appeared to require the presence of Lyt-1$^+$2$^-$ T cells.

Although α-Lyt-2 antibody was more effective in removing CTL to syngeneic tumor than α-Lyt-1 antibody, ACIT was more negatively affected by depletion of Lyt-1$^+$ than Lyt-2$^+$ cells. Thus, other noncytotoxic functions of the Lyt-1$^+$ subpopulation must be important in immunotherapy. Lyt-1$^+$2$^-$ T cells, or their products, have been shown to promote differentiation and proliferation of CTL precursors (19–21). Because previous studies have demonstrated that adoptively transferred immune cells must proliferate in the host to be effective in ACIT (5), we examined whether Lyt-1$^+$ cells can provide an amplifier function in the generation of CTL to FBL tumor. Therefore, the generation of CTL in standard MLC and MLTC of unseparated CBF_1 spleen cells was compared with cultures of CBF_1 spleen cells depleted of Lyt-1$^+$ cells

before in vitro sensitization and to such Lyt-1^+ cell-depleted cultures to which a supernate containing IL-2, a T cell growth factor produced by Lyt-1^+2^- T cells (19, 20), was added at the initiation of culture as described in Materials and Methods. This factor has been shown to reconstitute the amplifier function provided by Lyt-1^+2^- cells in the generation of cytotoxic responses (20). Using this method, the generation of cytotoxic responses to alloantigens and syngeneic tumors was examined (Table II). Unseparated cells generated significant cytotoxicity, and depletion of Lyt-1^+ cells before in vitro sensitization prevented the generation of alloreactive cytotoxic cells. However, supplementation of Lyt-1^+-depleted cell cultures with IL-2 restored most of the cytotoxic reactivity detected in nondepleted cultures. These results suggest that IL-2 adequately reconstituted the amplifier function eliminated by depletion of the Lyt-1^+ population. The small diminution of cytotoxicity could reflect elimination of a minor fraction of CTL precursors because alloreactive CTL were shown to be predominantly of the Lyt-1^-2^+ phenotype (Table I). The generation of cytotoxicity

TABLE II

Amplification by IL-2 of CTL Responses in Populations Depleted of Lyt-1^+ Cells

Cytotoxicity generation culture*			Addition to culture‡	Percent specific lysis§	
Responder	Preculture treatment of responder cells	Stimulator		AKR (H-2^k)	FBL (H-2^b)
Allogeneic response					
CBF	None	—		0 ± 1.7	0 ± 0.5
CBF	None	AKR		25 ± 2.5	0 ± 0.5
CBF	α-Lyt-1 + C	—		0 ± 0.3	
CBF	α-Lyt-1 + C	AKR		1 ± 1.2	
CBF	α-Lyt-1 + C	—	IL-2	2 ± 0.4	0 ± 0.3
CBF	α-Lyt-1 + C	AKR	IL-2	20 ± 1.4	1 ± 0.6
Syngeneic anti-tumor response					
CBF	None	—		0 ± 1.7	0 ± 0.5
CBF	None	FBL		0 ± 1.1	0 ± 0.1
CBF	α-Lyt-1 + C	—			0 ± 0.3
CBF	α-Lyt-1 + C	FBL			0 ± 0.1
CBF	α-Lyt-1 + C	—	IL-2	2 ± 0.4	0 ± 0.3
CBF	α-Lyt-1 + C	FBL	IL-2	2 ± 0.9	0 ± 0.6
$CBF_{\alpha FBL}$	None	—		0 ± 0.5	0 ± 0.5
$CBF_{\alpha FBL}$	None	FBL		0 ± 0.7	27 ± 0.1
$CBF_{\alpha FBL}$	α-Lyt-1 + C	—			0 ± 0.6
$CBF_{\alpha FBL}$	α-Lyt-1 + C	FBL			2 ± 0.4
$CBF_{\alpha FBL}$	α-Lyt-1 + C	—	IL-2	1 ± 0.9	0 ± 0.3
$CBF_{\alpha FBL}$	α-Lyt-1 + C	FBL	IL-2	0 ± 0.9	9 ± 0.8

* Responder cells, derived from normal unprimed CBF_1 mice (CBF) or mice primed and boosted in vivo with irradiated FBL ($CBF_{\alpha FBL}$), were either used directly without pretreatment, or were depleted of Lyt-1^+ cells by incubation with α-Lyt-1 and C before in vitro sensitization. These cells were washed and then placed into culture with irradiated allogeneic AKR spleen cells or irradiated FBL at responder:stimulator ratios of 4:1 and 20:1, respectively.

‡ Partially purified supernates containing IL-2 were added to the designated responder-stimulator combinations at the initiation of 5-d culture at a final dilution of 1:10.

§ After 5 d culture, effector cells were harvested and washed. Cytotoxicity was measured in a 4-h CRA with labeled concanavalin A-induced AKR spleen cell blast targets or FBL tumor cells at an effector to target ratio of 20:1. The results are the mean cytotoxicities ± SE of triplicate determinations.

promoted by IL-2 was antigen specific in that it required the presence of alloantigen during sensitization and was specific for the allogeneic target.

The cytotoxic response to syngeneic tumor was similarly assessed (Table II). No significant cytotoxicity was generated by primary in vitro sensitization, including those cell cultures that had been depleted of Lyt-1$^+$ cells and supplemented with IL-2. This is consistent with a relative absence of Lyt-1$^-$2$^+$ CTL precursors to syngeneic tumor in unprimed populations. In vitro sensitization of CBF_1 cells previously immunized in vivo to FBL induced a strong cytotoxic response to the syngeneic tumor target. Depletion of Lyt-1$^+$ cells before in vitro culture prevented the generation of a significant cytotoxic response, and the addition of IL-2 to these cultures permitted only partial restoration of cytotoxicity. The cytotoxicity generated in the presence of IL-2 was specific for the tumor target, and required that the cells be previously immunized to FBL in vivo and cultured with FBL in vitro. The ability of IL-2 to restore only a fraction of the cytotoxicity to FBL generated in cultures that had not been depleted of Lyt-1$^+$ cells is consistent with the results in Table I, which demonstrated that unlike alloreactive CTL, a significant fraction of CTL and/or CTL precursors to syngeneic tumors are eliminated by α-Lyt-1 and C. However, despite this limitation, which resulted in the generation of only low levels of cytotoxicity, the results suggest that Lyt-1$^+$ cells, or a product derived from these cells, can augment the in vitro generation of cytotoxic responses to syngeneic tumor. Perhaps more important, depletion of Lyt-1$^+$ cells prevented the generation of significant cytotoxicity. Thus, Lyt-1$^+$ cells that were shown to be critical for efficacy in immunotherapy may similarly be required in vivo to permit sufficient expression and expansion of cytotoxic cells to mediate a significant anti-tumor effect.

Discussion

The results demonstrate that the predominant donor effector cell required for eradication of advanced disseminated leukemia by ACIT is an immune Lyt-1$^+$2$^-$ T cell that is not cytolytic in vitro. Immune cells depleted of Lyt-2$^+$ cells, such that the only donor T cells remaining were of the Lyt-1$^+$2$^-$ phenotype, were nearly as effective in ACIT as unselected cells, suggesting that other donor T cell subsets do not provide an essential contribution. Moreover, the potential therapeutic efficacy of immune cells was ablated nearly as completely by depletion of Lyt-1$^+$ cells as by depletion of all T cells. The small residual anti-tumor effect still evident after treatment of immune cells with α-Thy-1.2 and C probably reflected incomplete T cell removal because previous studies have demonstrated that removal of neither B cells nor macrophages diminished the in vivo efficacy of the effector population (7). Thus, although other subpopulations may also participate, donor Lyt-1$^+$2$^-$ T cells are necessary to mediate the major in vivo anti-tumor effect observed in ACIT after adoptive transfer.

The T cell subsets analyzed in ACIT were defined operationally by susceptibility to lysis by monoclonal antibody to Lyt antigens and C. Immunofluorescent analysis of Lyt antigen expression has suggested that such absolute distinctions of Lyt phenotypes are somewhat artificial (i.e., all T cells express some Lyt-1 antigen), and has implied that the source and titer of antibody and C can influence the separation of subsets (22, 23). Therefore, the effect of the reagents used in our studies on well-defined in vitro T cell functions was analyzed. Alloreactive CTL were predominantly of the Lyt-1$^-$2$^+$ phenotype, but were partially sensitive to α-Lyt-1. CTL to FBL tumor

were of both the Lyt-1^-2^+ and Lyt-1^+2^+ phenotypes. This representation of Lyt-1 antigen on a minor fraction of alloreactive CTL and on a larger fraction of CTL to syngeneic tumor is similar to reports using conventional α-Lyt alloantisera (13, 14, 23). More important, these studies confirm that the Lyt-1^+2^- cell active in ACIT is distinct from the major effector cell mediating in vitro tumor lysis.

The in vivo activity of T cell subsets against syngeneic tumors has been analyzed in only a few models. Tumor neutralization of a syngeneic sarcoma, in which tumor cells and immune cells were mixed before inoculation into the host, required Lyt-1^+2^+ effector cells (24), which were also required for in vitro cytolysis of this tumor in a microcytotoxicity assay (13). Prevention of outgrowth of a Moloney lymphoma, in which immune cells were inoculated before the tumor, required Lyt-1^-2^+ effector cells, which were also responsible for in vitro lysis of this tumor (25). Previous studies from our laboratory have suggested that the immunologic requirements for efficacy in ACIT of established FBL tumor differ from those for tumor neutralization (9), but do not readily explain why CTL should appear more important in neutralization and prevention of tumor than in therapy of established tumor. Also of note is a recent analysis of adoptive immunotherapy of an established vascular Moloney sarcoma in preirradiated rats that has identified an effector cell with some characteristics similar to those of the effector cell we identified for ACIT of disseminated leukemia. Although T cell subsets are less well characterized in the rat than in the mouse, the subset required for effective immunotherapy in vivo was not cytolytic to tumor in vitro (4).

The potential mechanisms by which Lyt-1^+2^- T cells can promote in vivo eradication of established FBL tumor must reflect one or several of the T effector functions characterized by this subset, helper cells for antibody responses, initiators of DTH responses, and amplifiers of CTL responses. A helper function to enhance antibody responses seems least likely, because immunotherapy of established tumors by infusion of antibody has generally been unsuccessful (reviewed in 1). Additionally, T cell-deficient "B" mice (i.e., thymectomized, lethally irradiated mice reconstituted with syngeneic T-depleted bone marrow) were not protected from lethal outgrowth of an inoculated Moloney-induced tumor of C57BL/6 origin (MBL) by previous adoptive transfer of Lyt-1^+2^- T cells immune to MBL lymphoma, which contained helper T cells for an antibody response to this virus-induced tumor (25).

A second mechanism by which Lyt-1^+2^- cells could influence tumor rejection is by initiation of a DTH response. Sensitization of A/J mice with a syngeneic fibrosarcoma induces T cells that can adoptively transfer a tumor-specific DTH response to a secondary host (26). However, the role of such T effector cells in the rejection of established tumors has not been demonstrated. Although regressing sarcomas contain inflammatory cells that could reflect an ongoing DTH response, these regressing tumors also contain directly cytolytic T cells as well as primed tumor-specific CTL precursors that can be expanded to produce increased numbers of CTL (21, 27). Moreover, inoculation of immune Lyt-1^+2^- T cells into "B" mice, a situation in which potential DTH effectors are present and CTL effectors are absent, did not prevent lethal outgrowth of a subsequent inoculum of MBL tumor (25). Thus, the importance of DTH effectors in ACIT of FBL, a tumor antigenically similar to MBL (28), remains uncertain.

The last alternative, that Lyt-1^+2^- cells provide an amplifier function in the host, is consistent with an ultimate in vivo role for tumor-specific CTL. Amplifier T cells

have been shown to augment generation of tumor-specific CTL in vitro and enhance neutralization of tumor in vivo (29). If the critical immune cell for ACIT of established tumor is an amplifier cell, one might predict that tumor eradication would be delayed for a period of time after adoptive transfer, during which time sufficient expansion of the pool of cytotoxic effector cells occurred. Bioassay experiments examining the time-course of tumor elimination in ACIT have demonstrated that there is in fact a period of tumor growth after ACIT before the tumor is eliminated (6). Furthermore, immunosuppression of the host 2 wk after ACIT with anti-thymocyte serum results in lethal tumor outgrowth (6). Similar delays in tumor eradication have been observed in adoptive immunotherapy of established fibrosarcomas in mice and rats; measurable tumor growth continued for 1–2 wk after transfer of immune cells before the onset of tumor rejection became apparent (3, 4).

Immune cells depleted of Lyt-1$^+$ cells were only minimally effective in ACIT, despite the presence of CTL and CTL precursors. This may reflect a predominantly quantitative phenomenon, in that an inadequate number of CTL may be present in the infusate to eradicate the tumor, and the CTL provided may be incapable of sufficient expansion in the absence of amplifier cells. In addition, Lyt-1$^-$2$^+$ CTL memory cells appear to have a short functional lifespan (16, 25, 30), and thus may be unable to mediate a prolonged in vivo anti-tumor effect. Therefore, continued generation of cytolytic effector cells in vivo might require the infusion of Lyt-1$^+$2$^-$ amplifier cells, which appear to have a long lifespan (16, 25, 30), and the presence of Lyt-1$^+$2$^+$ CTL precursors. The limited efficacy of the Lyt-1$^-$2$^+$ subpopulation in therapy might also reflect in part a relative inability of adoptively transferred cytotoxic spleen cells to traffic to sites of disseminated tumor in the host. However, although the homing patterns of adoptively transferred immune T cell subpopulations has not been well described, transferred Lyt-1$^-$2$^+$ cells have been shown to be capable of homing to and eliminating a small localized subcutaneous MBL tumor (25).

Immune cells depleted of Lyt-2$^+$ cells remained effective in ACIT. If the resulting Lyt-1$^+$2$^-$ cells operate in vivo by providing an amplifier function for CTL induction, the host must be capable of providing the necessary CTL precursors. The potential for a positive host contribution to the outcome of ACIT is very provocative. IL-2, which augmented the in vitro generation of CTL to FBL tumor, has been shown to induce in vitro differentiation and proliferation of CTL (19–21). Moreover, inoculation of IL-2 into nude mice can induce in vivo production of antigen-specific CTL (20, 31). Although normal mouse serum appears to contain an inhibitor of IL-2 activity, local injection of IL-2 into a footpad augmented in vivo generation of CTL to trinitrophenyl-modified cells in the draining lymph node (20). Thus, it is conceivable that under the proper conditions, the requirement for immune Lyt-1$^+$2$^-$ T cells in ACIT could be circumvented by the infusion of sufficient IL-2 to promote expansion of host precursor CTL. Such a therapeutic approach would have obvious practical implications.

Summary

The phenotype of T cells therapeutically effective in immunotherapy of advanced Friend virus-induced (FBL) leukemia in vivo and cytotoxic to FBL in vitro was determined. Mice bearing disseminated FBL leukemia were successfully treated by a combination of cyclophosphamide and adoptive transfer of syngeneic immune lym-

phocytes. Therapeutic efficacy was largely dependent on the presence of Lyt-1^+2^- T cells in the transferred cells, whereas cells cytotoxic to FBL tumor in vitro were derived from the Lyt-1^+2^+ and Lyt-1^-2^+ subsets. Thus, the predominate cell required to eradicate tumor in adoptive chemoimmunotherapy was not cytolytic to tumor in vitro.

Potentially, the Lyt-1^+2^- cell may operate in vivo as an amplifier cell rather than by a direct anti-tumor effect. Elimination of the Lyt-1^+ population with α-Lyt-1 and complement prevented the generation of significant cytotoxic responses during both primary in vitro sensitization to alloantigens and in vitro sensitization of tumor-primed cells. The capacity of the Lyt-1^+ cell-depleted population to generate cytotoxic responses was partially reconstituted by addition, at the initiation of culture, of interleukin 2, a T cell growth factor derived from Lyt-1^+2^- T cells. Thus the Lyt-1^+ population contained helper-amplifier T cells required for the in vitro generation of cytotoxic T lymphocytes (CTL).

Adoptive chemoimmunotherapy with immune cells depleted of Lyt-2^+ cells, which contain the CTL and CTL precursors, were nearly as effective in vivo as unseparated immune cells. If the remaining effector cells (i.e., Lyt-1^+2^- T cells) function in vivo predominantly as amplifier cells, then the tumor-bearing host must be capable of making a positive contribution to the outcome of therapy.

The authors thank Dr. C. Henney and Dr. S. Gillis for the generous gift of IL-2, and M. Bolton, S. Emery, and B. Keniston for expert techncal assistance, and J. Hoak for preparation of the manuscript.

Received for publication 11 May 1981 and in revised form 15 June 1981.

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