THERAPY OF DISSEMINATED MURINE LEUKEMIA WITH CYCLOPHOSPHAMIDE AND IMMUNE Lyt-1$^+$,2$^-$ T CELLS

Tumor Eradication Does Not Require Participation of Cytotoxic T Cells

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Animal models have been developed (1–4) in which established and disseminated tumors can be eradicated by the adoptive transfer of syngeneic T cells specifically immune to tumor-associated antigens. These models have served as prototypes for understanding how the immune system of a tumor-bearing host might be manipulated to promote in vivo lysis of advanced tumors. Many factors necessary for successful immunotherapy have now been elucidated, including the need for immune effector T cells capable of persisting in the host (5, 6), the long time period required for elimination of all tumor cells by immune cells in vivo (5), and the importance of ablating suppressor cells, induced in the host by the growing tumor, which interfere with the expression of transferred immunity (3, 7). Although specific T cell immunity is necessary for tumor eradication, it has been difficult to determine which immunologic effector mechanism(s) must be operative in vivo for these transferred T cells to be effective.

Cytolytic T cells (CTL)[1] can directly and immediately kill tumor cells in vitro. Adoptively transferred CTL have been shown to prevent the outgrowth of recently transplanted tumors, and to contribute to tumor elimination in some therapy settings (4, 8, 9); the rejection of a murine sarcoma following transfer of immune T cells has been correlated with the in vivo generation of CTL (10). Thus, CTL may be important for the elimination of established disseminated tumors. However, studies using purified functional subsets of immune T cells have suggested that transfer of the noncytolytic helper T cell subset, which presumably can induce a tumoricidal delayed-type hypersensitivity (DTH) response in vivo, is necessary and sufficient for tumor eradication (11, 12). This conclusion was supported by studies (13) suggesting that a similar DTH phenom-

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[1] *Abbreviations used in this paper:* ACIT, adoptive chemoimmunotherapy; ATXBM, adult, thymectomized, irradiated, bone marrow-reconstituted mouse; CTL, cytotoxic T lymphocyte; CY, cyclophosphamide; DTH, delayed-type hypersensitivity; IFN, interferon; IL-2, interleukin 2; mAb, monoclonal antibody; MHC, major histocompatibility complex.

enon is responsible for rejection of allografts. Since, in these settings, the transferred helper cells could also be amplifying CTL responses in vivo (11, 14), it still remains unclear whether mechanisms other than direct tumor lysis by CTL in vivo are important in adoptive tumor therapy. Although this issue might theoretically be resolved most directly by the in vivo infusion of T cell clones with specific and restricted function, the results of therapy of syngeneic tumors with such clones have, to date, been disappointing (15–17). The therapeutic failures with T cell clones might reflect problems of T cell survival and homing in vivo (18, 19), rather than reflecting inadequate or inappropriate effector function.

Our studies have been designed to determine whether noncytolytic Lyt-1^+,2^- cells can effect eradication of established tumors without inducing CTL. We have utilized adoptive chemoimmunotherapy (ACIT) models in which mice bearing disseminated tumors can be cured by combined treatment with cyclophosphamide (CY) (which has both direct tumoricidal activity and can ablate tumor-induced suppressor cells in the host [5, 7]), followed by transfer of specifically immune T cells (1, 20). Previous studies of ACIT using immunologically intact mice as hosts have demonstrated that the adoptive transfer of the immune Lyt-1^+,2^- T cell subset can achieve therapeutic results nearly as good as those obtained with unfractionated T cells (11). The transfer of purified Lyt-1^+,2^- T cells ruled out participation of donor CTL in tumor eradication, but a potentially significant contribution by host-derived CTL could not be excluded. To eliminate the possibility of such a host contribution, we infused purified Lyt-1^+,2^- T cells into T-deficient ATXBM (adult, thymectomized, irradiated, and T-depleted bone-marrow reconstituted) B6 mice bearing disseminated FBL-3 leukemia. However, even such T-deficient hosts contain T cell precursors with the potential to differentiate under certain conditions (14, 21). Therefore, donor cells were obtained from congenic B6/Thy-1.1 mice, which permitted both the phenotypic distinction and the functional assessment of the tumor reactivity of transferred donor T cells (Thy-1.1^+) from any residual or newly generated host T cells (Thy-1.2^+). The results demonstrate that immune Lyt-1^+,2^- T cells, which are not directly cytolytic to tumor cells, can recognize and mediate rejection of a disseminated tumor without the participation of CTL.

Materials and Methods

Mice. C57BL/6 (H-2^b, Thy-1.2; denoted B6) mice, breeding pairs of congenic B6.PL(74NS) (H-2^b, Thy-1.1; denoted B6/Thy-1.1), and BALB/c (H-2^d) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. To prepare ATXBM hosts, 5-wk-old B6 mice were thymectomized, lethally irradiated (950 rad) 3 wk later, and injected intravenously with B6 bone marrow cells that had been depleted of T cells by treatment with monoclonal IgM anti-Thy-1.2 plus selected rabbit complement. These mice, used 4 wk later, were killed at the end of the experiments to confirm the absence of Thy-1.2^+ T cells.

Cell Depletion and Fluorescence Analysis With Monoclonal Antibodies (mAb). T cells were depleted with either IgM anti-Thy-1.2 or IgG anti-Lyt-2 mAb plus selected rabbit complement. T cell phenotype was assessed on a fluorescence-activated cell sorter and on a fluorescence microscope, with cytocentrifuged preparations of cells double-labeled with combinations of fluorescein-conjugated anti-Thy-1.2, anti-Lyt-1, or anti-Lyt-2 mAb and biotin-conjugated anti-Thy-1.1, anti-Lyt-1 or anti-Lyt-2 (Becton Dickinson Monoclonal

Center Inc., Mountain View, CA) plus avidin-phycoerythrin kindly provided by Dr. Jeff Ledbetter of Genetic Systems Corporation, Seattle, WA.

Assessment of Class I and Class II MHC antigen expression was performed with commercially obtained monoclonal IgG anti-K^bD^b (Australian Monoclonal Development Pty. Ltd, New South Wales, Australia), and antibodies derived from hybridomas obtained from the American Type Culture Collection, Rockville, MD, including 25-9-3S, producing an IgM anti-Ia^b; 34-5-8S, producing an IgG anti-D^d; and MKD6, producing an IgG anti-Ia^d. Following labelling with these antibodies, cells were prepared for analysis on the cell sorter with the use of fluorescein-conjugated goat anti–mouse IgG or rat anti–mouse IgM (Becton Dickinson Monoclonal Center Inc., Mountain View, CA).

Tumor. FBL-3 is a Friend virus–induced erythroleukemia of B6 origin that has been subcloned in vitro, and maintained by serial intraperitoneal transplantation in syngeneic mice. Donor B6/Thy-1.1 mice were sensitized to FBL-3 in vivo by inoculation of three doses of 2×10^7 irradiated FBL-3 intraperitoneally at 2-wk intervals, and the immune cells were obtained 2–4 wk later. This regimen has been shown (1, 20) to induce antigen-specific major histocompatibility complex (MHC)-restricted T cell immunity to FBL-3 tumor in B6 mice (1, 20). In vitro sensitization was performed in mixed lymphocyte tumor culture as described previously (11, 20). Briefly, 6×10^6 responder spleen cells were cultured for 5 d in 12-well tissue culture plates (Costar, Cambridge, MA) containing 30 ml of supplemented RPMI 1640 medium, 5% fetal calf serum, and either 1.5×10^6 irradiated allogeneic stimulator spleen cells, or 0.3×10^6 irradiated FBL-3 tumor cells.

For assessment of FBL expression of MHC antigens and target susceptibility following exposure to immune interferon (IFN), FBL tumor cells were incubated for 24 h with murine IFN-γ generated by recombinant DNA technology and kindly provided by Dr. Mike Shepard (Genentech Corporation, South San Francisco, CA), at concentrations of 2 and 10 U/ml. The enhancement by IFN-γ of the expression of MHC antigens has been shown (22, 23) with other tumor cell lines to be maximal following exposure to 1–2 U IFN-γ for 24 h.

Adoptive Chemoimmunotherapy. This assay, previously described in detail (1, 5, 6, 11), consists of treating mice suffering advanced disseminated FBL-3 leukemia with a combination of chemotherapy and adoptively transferred immune cells. ATXBM B6 hosts were inoculated on day 0 with 5×10^6 FBL-3, and treated on day 5, after tumor dissemination had occurred (5), with 180 mg/kg CY i.p., followed in 5 h by adoptively transferred B6/Thy-1.1 donor cells. Studies with noncrossreactive tumors of B6 origin have demonstrated that the therapeutic efficacy of ACIT requires the transfer of specifically immune T cells (1, 20); nonimmune spleen cells have no therapeutic activity (11), and the magnitude of survival prolongation and rate of cure are proportional to the number of immune cells transferred (1, 24).

In Vitro Detection of Antitumor Reactivity. Cytolytic activity was determined, as previously described, by 4-h incubation of effector cells with 10^4 chromium-labelled FBL-3 syngeneic tumor targets or concanavalin A–induced BALB/c allogeneic spleen cell blast targets (11, 20). To assess interleukin 2 (IL-2) production in response to FBL-3, 5×10^6 responder spleen cells were cultured with 5×10^5 irradiated stimulator cells in 48-well plates and supernatants were removed after 48 h. These supernatants were diluted and added to 24-well plates containing CTLL-2 cells, an IL-2-dependent T cell line kindly provided by Dr. Steve Gillis (Immunex Corporation, Seattle, WA), and [^{3}H]thymidine uptake was measured 24 h later, as previously described (25).

Results

Eradication of Disseminated FBL-3 in ATXBM Hosts by Treatment with CY and Lyt-1$^+$,2$^-$ Immune T Cells. To determine whether noncytolytic T cells are sufficient to mediate complete eradication of disseminated antigenic leukemia, T cell–deficient host mice were injected with a lethal tumor dose, then treated with a purified subpopulation of immune Lyt-1$^+$,2$^-$ T cells. On day 0, ATXBM host B6 mice were inoculated with 5×10^6 FBL-3 cells. Some were then treated

on day 5, by which time the tumor had disseminated to peripheral blood and spleen (5). Untreated ATXBM hosts had a median survival time of 10 d, which could be prolonged to 24 d by treatment on day 5 with CY alone, but all mice in both groups died of progressive tumor growth (Fig. 1). Treatment of ATXBM mice with 10^7 immune cells alone on day 5 had no effect on survival, consistent with previous studies (5) in this model, showing that reduction of the tumor burden by treatment with CY before cell transfer is necessary to detect an antitumor effect. Tumor growth patterns similar to those demonstrated in these T-deficient hosts were observed in immunologically intact B6 hosts. Thus, as previously published (1, 11), untreated B6 hosts had a median survival time of 11 d, and treatment on day 5 with CY alone or CY plus 10^7 normal, nonimmune spleen cells prolonged median survival to 25 d (data not shown).

Treatment of ATXBM hosts on day 5 with CY plus 10^7 unfractionated spleen cells from B6/Thy-1.1 donors immune to FBL-3 cured 11 of 12 mice (Fig. 1). The results of therapy of these ATXBM B6 mice (lacking host T cells) with CY plus subpopulations of immune T cells paralleled results seen (11) in the treatment of tumor-bearing immunocompetent hosts. Thus, treatment with CY plus 10^7 cells functionally defined as Lyt-1^-,2^+ (i.e., depleted of helper activity with anti-Lyt-1 and complement) had only a minimal antitumor effect, whereas therapy with CY plus Lyt-1^+,2^- T cells (i.e., depleted of CTL and CTL precursors by anti-Lyt-2 and complement) cured all 12 mice. Mice were observed for 80–100 d to be certain that the tumor had been completely eliminated by ACIT, and were then killed for analysis of the T cell compartment.

Analysis of Splenic T Cells in ATXBM Mice Cured of Disseminated FBL-3 by ACIT. The results of therapy implied that eradication of disseminated leukemia

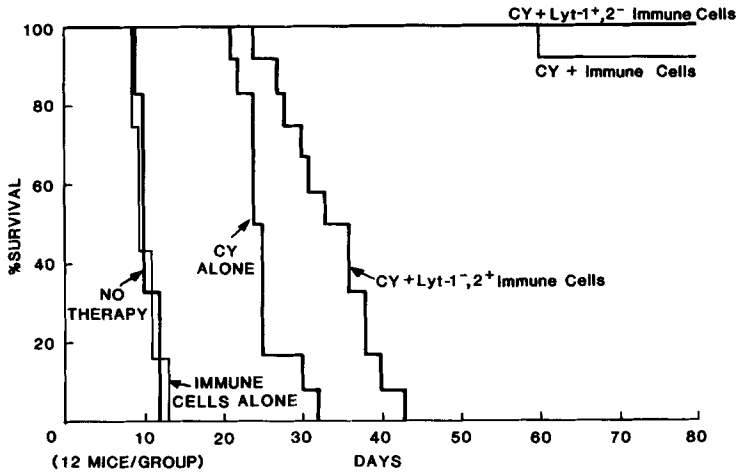

FIGURE 1. Therapy of ATXBM B6 hosts bearing disseminated FBL-3 leukemia. T-deficient ATXBM B6 hosts were inoculated with 5×10^6 FBL-3 cells intraperitoneally on day 0, then: left untreated (*NO THERAPY*); treated on day 5 with 10^7 immune spleen cells from congenic B6/Thy-1.1 donor mice previously sensitized to FBL-3 in vivo (*IMMUNE CELLS ALONE*); treated on day 5 with 180 mg/kg CY (*CY ALONE*); or treated on day 5 with CY plus 10^7 immune B6/Thy-1.1 spleen cells. The immune cells were either used unfractionated (*CY + Immune Cells*), or following cell depletion with anti-Lyt-2 and complement (*CY + Lyt-1^+,2^- Immune Cells*) or anti-Lyt-1 and complement (*CY + Lyt-1^-,2^+ Immune Cells*).

required neither donor nor host CTL. To confirm that CTL were indeed not present, splenic T cells of cured mice were analyzed for both CTL phenotype and function. Thus, spleen cells, obtained from ATXBM B6 hosts 80–100 d after ACIT treatment for disseminated FBL-3 leukemia, were labelled with mAb conjugated for fluorescence analysis, and examined in vitro for immunologic reactivity. ATXBM B6 hosts cured by ACIT with either unfractionated or Lyt-1$^+$,2$^-$ T cells from B6/Thy-1.1 donors contained 8–11% donor Thy-1.1$^+$ T cells (Table I). Although these mice were usually devoid of any host T cells, a few mice contained 1–2% Thy-1.2$^+$ cells. By comparison, <1% background fluorescence with anti-Thy-1.2 was detected in normal B6/Thy-1.1 spleens, and 22–28% Thy-1.2$^+$ cells were detected in normal B6 spleens. ATXBM hosts that had received donor cells treated with anti-Lyt-2 and complement before transfer contained <1% Lyt-2$^+$ spleen cells, as compared to 2–4% in hosts receiving unfractionated donor cells, and 4–7% in the spleens of normal donors. Thus, most ATXBM hosts cured of FBL-3 leukemia by ACIT with Lyt-1$^+$,2$^-$ T cells contained no host-derived T cells and no Lyt-2$^+$ T cells.

Freshly obtained spleen cells from cured mice or mice immune to FBL-3 were not directly cytotoxic to either FBL-3 tumor or allogeneic spleen cell blasts. Therefore, cytolytic activity was assessed following 5-d cultures of spleen cells grown in vitro with irradiated FBL-3 tumor, or allogeneic spleen cell stimulators. These cultures were done under conditions known to generate secondary, but not primary responses to syngeneic FBL-3 tumor, and primary allogeneic responses (11, 20). Cells obtained from ATXBM hosts that had been treated with unfractionated B6/Thy-1.1 donor spleen cells immune to FBL-3 could be stimulated to produce both tumor-specific and allospecific CTL (Table I). By contrast,

TABLE I

Phenotype and Cytolytic Reactivity of Spleen Cells from ATXBM Hosts Cured by ACIT

Source of responder cells*	Phenotype of responder spleen cells‡				In vitro stimulator§	Specific lysis‖	
	Thy-1.2$^+$	Thy-1.1$^+$	Lyt-1$^+$	Lyt-2$^+$		FBL-3	BALB/c
	%					%	
B6/Thy-1.1$_{\text{anti-FBL-3}}$ $\xrightarrow{\text{anti-Lyt-2}}$ B6 (ATXBM)	0–2	8–10	8–11	<1	FBL-3	2	0
					BALB/c	0	0
B6/Thy-1.1$_{\text{anti-FBL-3}}$ → B6 (ATXBM)	0–2	8–11	8–12	2–4	FBL-3	41	4
					BALB/c	2	33
B6/Thy-1.1$_{\text{anti-FBL-3}}$	<1	22–26	21–25	4–7	FBL-3	42	2
					BALB/c	0	25
B6/Thy-1.1	<1	21–26	20–25	4–6	FBL-3	1	1
					BALB/c	1	26

* Responder spleen cells were obtained on days 80–100 from the cured B6 (ATXBM) hosts (described in Fig. 1) that had been treated with either unfractionated spleen cells from immune B6/Thy-1.1 donors [B6/Thy-1.1$_{\text{anti-FBL-3}}$→ B6 (ATXBM)], or donor cells depleted of Lyt-2$^+$ T cells [B6/Thy-1.1$_{\text{anti-FBL-3}}$ $\xrightarrow{\text{anti-Lyt-2}}$ B6 (ATXBM)]. Or, responder cells were obtained from normal B6/Thy-1.1 donors or donors sensitized to FBL-3 in vivo (B6/Thy-1.1$_{\text{anti-FBL-3}}$).

‡ Spleen cells were labelled with conjugated mAb. For analysis of T cell subsets, two-color fluorescence was performed with fluoresceinated anti-Lyt-1 or anti-Lyt-2 and phycoerythrin-avidin with biotinylated anti-Thy-1.1 Data is presented as the range of percent positive cells from six spleens.

§ Spleen cells were cultured for 5 d with irradiated syngeneic FBL-3 tumor cells or allogeneic BALB/c spleen cell stimulators.

‖ Cytolytic activity was tested in a 4-h chromium-release assay with labelled FBL-3 or BALB/c spleen blast targets at an effector/target ratio of 20:1. The results represent the means of triplicate wells from six experimental groups.

cultures of cells from ATXBM hosts that had been cured by therapy with Lyt-2^+-depleted donor cells failed to lyse either target. Control cultures of spleen cells from normal mice primed in vivo to FBL-3 contained CTL reactive to FBL-3, whereas no CTL reactive with FBL-3 tumor were generated in spleens from normal unprimed mice. Thus, ATXBM hosts cured by therapy with CY plus Lyt-1^+,2^- donor T cells contained no Lyt-2^+ cells, by phenotypic analysis, and no tumor-reactive or alloreactive CTL, by functional analysis.

The ability of the persistent donor noncytolytic T cells to respond to FBL-3 tumor was assessed by measuring production of the lymphokine, IL-2, following in vitro stimulation (Table II). Spleen cells from ATXBM hosts treated with either Lyt-2-depleted, or unfractionated donor cells immune to FBL-3 produced IL-2 in response to stimulation by FBL-3. The levels of IL-2 detected after stimulation of the cells, which were obtained from cured mice, was higher than that detected from cells obtained directly from primed donor mice, possibly reflecting further expansion of the antigen-reactive cell population following transfer into a tumor-bearing animal (26). Unprimed cells did not generate measurable amounts of IL-2 in response to FBL-3 (data not shown), consistent with the difficulty in generating a primary in vitro cytotoxic response to FBL-3 (Table I). Thus, ATXBM B6 host mice, cured of disseminated FBL-3 by ACIT with immune Lyt-1^+,2^- donor cells, contained no phenotypic or functional CTL against FBL-3, but did contain donor cells capable of secreting lymphokines in response to the tumor.

Analysis of MHC Antigen Expression on FBL-3 Tumor Cells Following Exposure to IFN-γ. Although Lyt-1^+,2^- T cells generally recognize antigens in the context of class II MHC determinants expressed on antigen-presenting cells and perform noncytolytic functions, some Lyt-1^+,2^- T cells can act as CTL restricted to targets expressing class II antigens. FBL-3 is an erythroleukemia that expresses only

TABLE II

Tumor-induced Lymphokine Production by Spleen Cells from ATXBM Hosts Cured by ACIT

IL-2 generation culture*		Supernatant-induced proliferation of CTLL-2 at dilutions of:‡		
Source of responder cells	Stimulator	1:2	1:8	1:32
B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$ $\xrightarrow{anti\text{-}Lyt\text{-}2}$ B6 (ATXBM)	FBL-3	9,506	5,986	3,972
	B6	610	1,430	2,124
		Δ8,896	Δ4,556	Δ1,848
B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$ → B6 (ATXBM)	FBL-3	14,610	8,358	4,265
	B6	2,199	2,338	2,731
		Δ12,411	Δ6,020	Δ1,534
B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$	FBL-3	3,627	1,729	1,544
	B6	1,859	1,544	2,150
		Δ1,768	Δ175	Δ −606

* Responder spleen cells, as designated in Table I, were incubated for 48 h with irradiated syngeneic stimulators, FBL-3 cells, or spleen cells, and the culture supernatant was harvested.

‡ An IL-2-dependent T cell line (CTLL-2) was incubated for 24 h with culture supernatants at the denoted dilutions, and incorporation of [^{3}H]thymidine was measured. The results represent the means of triplicate wells from three experiments.

class I and no class II antigens (27). However, IFN-γ, a lymphokine known to be secreted by immune Lyt-1$^+$,2$^-$ T cells in response to stimulation by antigen (28), has been shown to induce and increase the expression of class II antigens on tumor cells (22, 23). Thus, CTL activity might still be necessary in tumor therapy if the Lyt-1$^+$,2$^-$ T cells responding to FBL-3 antigens induce Ia antigen expression on FBL-3 as a consequence of the secretion of IFN-γ, and then function as class II–restricted CTL. Therefore, the expression of Ia^b on FBL-3 tumor cells following exposure to IFN-γ was assessed.

The sensitivity and specificity of labelling with mAb against H-2^b antigens was demonstrated with B6 and BALB/c spleen cells. B6 spleen cells reacted with an mAb that recognizes a determinant on class I K^b and D^b antigens (Fig. 2*B*); the non–T cell subpopulation reacted with an mAb against Ia^b (Fig. 2*A*). Studies with H-2^d BALB/c spleen cells and antibodies against Ia^d and D^d confirmed the haplotype specificity of these previously well-characterized mAb (data not shown).

FBL-3 tumor cells were labelled either directly, or after incubation for 24 h with 2 U/ml recombinant murine IFN-γ. This dose was selected because it had been shown (22, 23) to induce maximal expression of Ia antigens and enhance expression of K and D antigens in other tumor cell lines. FBL-3 cells reacted with the antibody against K^b and D^b, and the level of antigen expression was increased if the tumor had been cultured with IFN-γ (Fig. 2*D*). However, FBL-3 cells did not react with antibody against Ia^b, either before or following the incubation with IFN-γ (Fig. 2*C*). Thus, exposure of FBL-3 to IFN-γ resulted in enhanced expression of class I antigens, but failed to induce expression of the class II Ia^b determinants.

Analysis of Splenic T Cells in ATXBM Mice During the Period of Tumor Rejection by ACIT. The lack of class II antigen expression by FBL-3, and the absence of

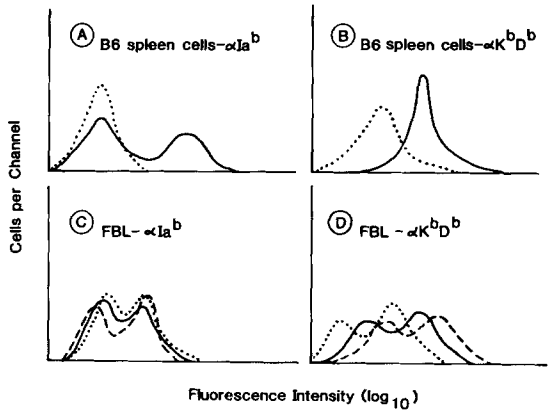

FIGURE 2. MHC antigen expression on B6 spleen cells and FBL tumor cells. Cells were incubated with either mAb against Ia^b (αIa^b) or K^{b}D^b (αK^bD^b), followed by labelling with a fluorescein-conjugated second step reagent reactive with the mAb, then analyzed on a fluorescence-activated cell sorter. To assess the influence of IFN-γ on MHC antigen expression by FBL-3, tumor cells were cultured for 24 h before labelling, either in medium alone, or in the presence of 2 U/ml recombinant IFN-γ. Unlabelled cells are represented by the dotted lines (· · · · ·), labelled cells by the solid lines (——) and IFN-γ-treated and labelled cells by the dashed lines (-----).

Lyt-2^+ CTL in ATXBM hosts that had been cured of FBL-3 by ACIT with Lyt-1^+,2^- T cells imply that T cells directly cytolytic to tumor are not necessary for tumor eradication. However, the functional studies performed in cured mice would have missed any short-lived T cells having direct cytolytic activity and that might have made an important contribution during the 30-d period following ACIT that is necessary for complete tumor elimination (5). Therefore, ATXBM B6 host mice were ACIT-treated on day 5, and T cell function was assessed on days 16, 26, and 30. Donor cells depleted of Lyt-2^+ T cells contained no CTL reactive to FBL-3 nor to allogeneic targets before transfer (Table III), and ATXBM hosts that received these cells contained no CTL during the period of tumor eradication by immune cells. By contrast, ATXBM B6 hosts treated with unfractionated donor cells contained both tumor and alloreactive CTL. The pattern of cytolytic reactivity against FBL-3 was qualitatively unchanged if the tumor target was preincubated with IFN-γ, consistent with the failure of this lymphokine to induce expression of class II molecules on FBL-3 (Fig. 2). Thus,

TABLE III

Cytolytic Reactivity of Spleen Cells from ATXBM Hosts During Period of Tumor Eradication by ACIT

Donor responder cells*	Day donor cells obtained from B6 (ATXBM) host	In vitro stimulator‡	Specific lysis§			
			FBL-3	FBL-3 + 2 U IFN-γ	FBL-3 + 10 U IFN-γ	BALB/c
			%			
B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$	—	FBL	18	17	20	0
		BALB/c	1	0	0	18
(B6/Thy1.1$_{anti\text{-}FBL\text{-}3}$)anti-Lyt-2	—	FBL	2	1	0	0
		BALB/c	0	1	0	0
B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$	16	FBL	NT	13	13	NT
(B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$)anti-Lyt-2	16	FBL	NT	2	1	NT
B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$	26	FBL	15	34	35	0
(B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$)anti-Lyt-2	26	FBL	0	1	3	0
B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$	30	FBL	31	30	32	0
		BALB/c	2	3	4	26
(B6/Thy-1.1$_{anti\text{-}FBL\text{-}3}$)anti-Lyt-2	30	FBL	4	1	1	0
		BALB/c	3	2	1	2

* Responder spleen cells were obtained either before adoptive transfer from B6/Thy-1.1 donors immune to FBL-3; or they were obtained on days 16, 26, or 30 from B6 (ATXBM) hosts bearing disseminated FBL-3 that had been treated on day 5 with CY and 10^7 adoptively transferred immune donor B6/Thy-1.1 spleen cells. The immune donor spleen cells were either used unfractionated or were depleted of Lyt-2^+ T cells before direct testing or adoptive transfer.

‡ Spleen cells were cultured for 5 d with irradiated syngeneic FBL-3 tumor or allogeneic BALB/c spleen cell stimulators. Due to low cell yields on days 16 and 26, responders were cultured with only tumor stimulators.

§ Cytolytic activity was tested in a 4-h chromium-release assay at an effector/target ratio of 20:1. Targets were prepared as previously described, or after 24 h incubation with recombinant INF-γ at 2 or 10 U/ml. Due to low cell yields on day 16, effectors were tested only against IFN-treated tumor targets.

mice being cured of disseminated FBL-3 by ACIT with Lyt-1$^+$,2$^-$ T cells contained no tumor-reactive CTL during the period of tumor elimination.

Discussion

These studies demonstrate that adoptively transferred noncytolytic Lyt-1$^+$,2$^-$ T cells that are immune to tumor-associated antigens can, in conjunction with CY, mediate rejection of a disseminated tumor in T-deficient ATXBM hosts, a setting in which neither donor nor host CTL should be available to participate in in vivo tumor lysis. To rule out the possibility that there had been incomplete cellular depletion, or that CTL precursors from ATXBM hosts had differentiated in vivo in the presence of antigen and lymphokines produced by helper T cells (14, 21), the treated animals were examined for CTL by phenotypic and functional analysis. The results showed that spleen cells from ATXBM hosts cured by therapy with CY and transferred Lyt-1$^+$,2$^-$ T cells contained neither Lyt-2$^+$ T cells nor CTL that could lyse FBL-3 tumor cells. Thus, the transferred T cells promoted tumor eradication either by inducing a DTH response, or by augmenting B cell antibody production. Although studies in which B cell responses have been suppressed or eliminated will be necessary to determine the extent to which antibody responses can contribute to tumor eradication, serotherapy has been ineffective in this model, and infusions of even very high titer mAb in similar models have produced only minimal effects in the therapy of advanced tumors (29, 30). Therefore, it is likely that a DTH cellular immune response, rather than a humoral response, is critical for tumor eradication following adoptive therapy.

The in vivo efficacy of the DTH effector mechanism in the rejection of viable nonmalignant cells has been demonstrated in studies with skin allografts. Adoptive transfer of immune, alloreactive Lyt-1$^+$,2$^-$ T cells can induce rejection of skin allografts in ATXBM hosts (13, 14). Initial studies correlated this graft rejection with the capacity of the noncytolytic Lyt-1$^+$,2$^-$ T cells to induce a DTH response to the alloantigens (13). Since subsequent studies (21) in ATXBM hosts suggested that the transferred alloreactive helper T cells might be capable of inducing differentiation of host prethymic CTL precursors in vivo, the potential importance of CTL in graft rejection in this model was reexamined (14). Analysis of the T cell compartments of ATXBM mice that had rejected allografts after transfer of Lyt-1$^+$,2$^-$ T cells revealed that many mice did, in fact, contain Lyt-2$^+$ CTL of host origin. However, several mice were identified that had rejected their grafts, but contained no CTL (14). Thus, it would appear that allograft rejection can be accomplished via a DTH mechanism, without the participation of CTL. We used a similar analysis to demonstrate the potential importance of DTH responses in the rejection of rapidly growing syngeneic tumors. In most, but not all mice cured of tumor, we found only donor-derived T cells, and no evidence that tumor-reactive CTL of host origin had been induced by the transferred helper cells. The absence of demonstrable CTL in our tumor therapy model, as compared to the more frequent detection of CTL in the allograft rejection model, may reflect the fact that the ATXBM hosts were treated with a lympholytic dose of CY before transfer of immune cells (31), and/or a lower

frequency of CTL precursors reactive to the syngeneic tumor as compared to allogeneic cells.

FBL-3 is an erythroleukemia that expresses class I MHC antigens but does not express the necessary class II restricting elements for direct recognition by the T cell receptor of Lyt-1^+,2^- T cells. Therefore, lysis of FBL-3 tumor by immune Lyt-1^+,2^- T cells must proceed via mechanisms other than direct cell-cell contact between lymphocytes and the tumor. Potentially, macrophages may process tumor antigens and then present them to Lyt-1^+,2^- cells in the context of class II MHC products. The Lyt-1^+,2^- cells may then secrete lymphokines, such as IFN-γ, macrophage-activating factor, and/or lymphotoxin, that promote tumor lysis via alternative effector pathways. Recent studies from our laboratory have demonstrated that specific stimulation of T cells immune to FBL-3 results in secretion of a macrophage-activating factor that renders previously nonlytic macrophages tumoricidal to FBL-3 in vitro (unpublished results). Similar macrophage-activating factors, such as IFN-γ, are produced by specific stimulation of helper T cell clones reactive to an antigenically related lymphoma, MBL (28).

Our studies do not suggest that CTL cannot function in the therapy of disseminated FBL-3 leukemia, but rather that CTL may not be necessary in this setting. Indeed, it seems likely that there should be conditions under which T cells directly cytolytic to tumor can have therapeutic activity. As examples, rejection of an established fibrosarcoma has been shown (10) to correlate with the in vivo generation of CTL reactive to the tumor, and the capacity to reject syngeneic solid tumors has been enhanced by the infusion of CTL along with nonlytic cells (4). The efficacy of CTL in therapy may depend on such factors as the nature of the tumor and the target antigens, the location of the tumor cells in the host, and whether the CTL must persist in the host and home to tumor sites. CTL may prove to be more effective when infused proximal to a solid tumor mass than if infused systemically in the treatment of a disseminated leukemia. However, regardless of the relative or potential contributions of CTL in some settings, our results emphasize the importance of effector mechanisms induced by noncytolytic T cells.

With improved means of selecting and growing reactive T cells, an understanding of the principles of successful adoptive transfer of immunity may assume increasing importance. This study demonstrates that, in conjunction with CY, transferred Lyt-1^+,2^- T cells can eradicate disseminated tumors in ATXBM hosts. As observed with therapy of disseminated FBL-3 in immunologically intact mice, immune cells did not have a significant effect unless the tumor-bearing hosts were treated with CY before cell transfer (11). CY can potentially both reduce the tumor burden by a direct tumoricidal effect (5), and ablate suppressor cells that may be induced in the host by the growing tumor and which inhibit the transfer of immunity (7). Since ATXBM hosts lack these suppressor T cells (3, 7), it is likely that the large burden of the FBL-3 tumor interferes with expression of transferred immunity. Such interference may not simply reflect infusion of an inadequate dose of lymphocytes, since much larger cell doses and multiple infusions of cells have also been ineffective (unpublished results). In addition to producing a large and rapidly increasing number of potential targets in vivo, the large tumor mass also may be secreting suppressor factors (32),

presenting excess antigen that directly inhibits the expression of antigen-specific reactivity by effector cells (33), and/or inducing suppressor cells in the transferred population, which subsequently suppress immunity. This last explanation is supported by evidence (34, 35) for the role of Lyt-1$^+$,2$^-$ T cells in the suppression of DTH and transferred tumor immunity. Moreover, such suppressor cells have been shown (35) to develop during, and then interfere with an ongoing antitumor response. Thus, full expression of transferred immunity may require a greater understanding of immunoregulatory pathways and appropriate perturbation of those pathways that develop after cell transfer.

Summary

The ability of noncytolytic Lyt-1$^+$,2$^-$ T cells immune to FBL-3 leukemia to effect eradication of disseminated FBL-3 was studied. Adult thymectomized, irradiated, and T-depleted bone marrow–reconstituted (ATXBM) B6 hosts were cured of disseminated FBL-3 by treatment with 180 mg/kg cyclophosphamide (CY) and adoptively transferred Lyt-1$^+$,2$^-$ T cells obtained from congenic B6/Thy-1.1 donors immune to FBL-3. Analysis of the T cell compartment of ATXBM hosts treated and rendered tumor-free by this therapy revealed that the only T cells present in the mice were donor-derived Lyt-1$^+$,2$^-$ T cells. In vitro stimulation of these T cells with FBL-3 tumor cells, which express class I but no class II major histocompatibility complex antigens, induced lymphokine secretion, but did not result in the generation of cytotoxic T lymphocytes (CTL). Thus, in a setting in which mice lack Lyt-2$^+$ T cells, and in which no CTL of either host or donor origin could be detected, immune Lyt-1$^+$,2$^-$ T cells, in conjunction with CY, mediated eradication of a disseminated leukemia. The results suggest that delayed-type hypersensitivity responses induced by immune T cells represent a potentially useful effector mechanism for in vivo elimination of disseminated tumor cells.

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