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SPECIFICITY OF ADOPTIVE CHEMOIMMUNOTHERAPY OF ESTABLISHED SYNGENEIC TUMORS[1]

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To examine the specificity of adoptive chemoimmunotherapy (ACIT) of established syngeneic tumors, two noncross-reactive C57BL/6 tumors were studied: a Friend virus-induced tumor (FBL-3) and a chemically induced virus-negative tumor EL-4(G-). *In vitro* studies confirmed that these tumors are antigenically distinct by demonstrating that the cytotoxic responses of spleen cells from mice immunized *in vivo* and reexposed to tumor *in vitro* are immunologically specific.

Studies of ACIT with cells from mice immunized *in vivo* demonstrated similar specificity. Mice receiving 5 × 10^6 FBL-3 on day 0 all died by day 13. Treatment on day 5 with cyclophosphamide (CY), 180 mg/kg, prolonged the median survival time (MST) to day 23. Treatment on day 5 with CY plus 2 × 10^7 normal nonimmune C57BL/6 cells or CY plus cells sensitized to EL-4(G-) had no additional effect on survival whereas 2 × 10^7 C57BL/6 cells sensitized to FBL-3 *in vivo* prolonged MST to day 64 and cured 13 of 32 mice. Similarly, mice given 2 × 10^5 EL-4(G-) on day 0 all died by day 16, and CY on day 5 prolonged the MST to day 22. As an adjunct to CY, 2 × 10^7 normal cells or cells sensitized to FBL-3 had a modest effect, prolonging the MST to days 37 and 36, respectively. However, treatment with CY plus 2 × 10^7 cells immune to EL-4(G-) cured 22 of 32 mice. The results demonstrate the immunologic specificity of ACIT of syngeneic tumors treated with immune syngeneic cells.

Immune cells adoptively transferred to nonimmune mice with or shortly before tumor cells can specifically prevent the out-growth of transplanted tumors (1). However, if used as the sole means of therapy, similar cells have only rarely been effective when administered *after* tumor challenge (2–7). Once syngeneic tumors have become clinically established, successful adoptive immunotherapy has usually required either treatment with a combination of chemotherapy plus adoptively transferred immune cells (8–14) or surgical removal of the primary tumor followed by an infusion of immune cells to eradicate residual micro-metastatic tumor (5, 15).

Immunotherapy of established tumors has been accomplished with both *in vivo* and *in vitro* sensitized cells. The therapeutic effect of the lymphoid cells has been assumed to result from specific immunization, since cells immune to the tumor-associated surface antigens (5, 6, 8–14) or to cross-reacting antigens were effective, (10, 12) whereas nonimmune cells (5, 6, 8–13) or cells immune to irrelevant alloantigens or tumor antigens were ineffective (5, 6, 9, 11, 13).

However, these previous reports have not shown that the putative specifically immune cells are ineffective against an antigenically distinct noncross-reacting tumor, which can be similarly eradicated by appropriately directed immunotherapy. Since rigorous proof of immunologic specificity requires such reciprocal controls (16), and moreover, since it has been recently established that normal nonimmune cells may be effective in adoptive chemoimmunotherapy (ACIT) (17), the question of the specificity of ACIT was reexamined.

The current study, performed with FBL-3 and EL-4(G-) in C57BL/6 mice, confirmed that these are antigenically distinct tumors (18–20), since spleen cells from mice immunized *in vivo* with FBL-3 or EL-4(G-) and reexposed to the sensitizing tumor *in vitro* displayed enhanced *in vitro* cytotoxicity specific for the sensitizing tumor. Moreover, *in vivo* immunization induced cells that were therapeutically effective only in the adoptive chemoimmunotherapy of the sensitizing tumor.

MATERIALS AND METHODS

Mice. Eight to 12-week-old C57BL/6 mice were obtained from Simonsen Laboratory (Gilroy, Calif.).

Tumors. FBL-3 is a transplanted ascitic Friend virus-induced leukemia of C57BL/6 origin. It contains leukemogenic virus particles and possesses tumor-associated surface antigens that cross-react with other FMR tumors (21, 22).

EL-4(G-) is a subline of EL-4, which was kindly supplied by C. C. Ting. It is a dimethylbenzanthracene-induced lymphoma of C57BL origin maintained by i.p. transplantation in adult C57BL/6 mice, and is negative for Gross cell-surface antigens and mouse endogenous virus-associated surface antigen 1 (18).

In vivo sensitization. C57BL/6 mice were immunized by two weekly inoculations of 2 × 10^7 FBL-3 or EL-4(G-) irradiated with 10,000 R. Six weeks after the final inoculation, spleen cells were removed and teased to form single cell suspensions. Viable nucleated cells were enumerated by Trypan blue dye exclusion.

In vitro sensitization. Culture conditions for mixed lymphocyte-tumor cultures have been previously described (23). Cultures of 60 × 10^6 responder spleen cells and 3 × 10^6 stimulator cells were established in 20 ml of RPMI supplemented with fetal calf serum, 2-mercaptoethanol, and antibotics. Responder

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cells were obtained from normal nonimmune mice (C57BL/6_{normal})[4] or mice previously immunized with FBL-3 (C57BL/$6_{\alpha FBL}$) or EL-4(G-) (C57BL/$6_{\alpha EL-4}$) and cultured for 5 days at 37°C in a 5% CO_2 humidified atmosphere with irradiated FBL-3, EL-4(G-), or C57BL/6_{normal}, denoted $(FBL)_x$, $(EL-4)_x$, or $(C57BL/6)_x$, respectively.

In vitro cytotoxicity. Cytolytic activity was measured in a 4-hr chromium release assay as previously detailed (23). Targets for cytotoxicity were FBL-3 and EL-4(G-) that had been passaged *in vitro* 2 to 4 weeks before testing. Cytotoxicity was determined by the following formula:

$$\% \text{ Specific lysis} = \frac{\text{Test counts} - \text{spontaneous release counts}}{\text{Maximal releasable counts} - \text{spontaneous releasable counts}} \times 100$$

Maximal releasable counts were determined by freezing and thawing the target cells four times. Spontaneous release was approximately 10 to 15% of maximal release. The means of triplicate samples were determined.

Treatment of cells with anti-Thy 1.2 serum and Complement (C). Monoclonal IgM antibody to Thy 1.2 antigen was provided as a gift by Dr. E. Clark. The antibody was produced by hybridization of spleen cells from AKR mice immunized to CBA thymocytes with a BALB/c plasmacytoma. The specificity for Thy 1.2 antigen was determined by tests with Thy-1 congenic mice and studies of the tissue distribution of lymphoid sensitivity (24). The 50% cytotoxicity titer was greater than 1/500,000. Rabbit C was absorbed with agarose and screened for toxicity before use. Lymphocytes (1×10^7/ml) were incubated for 60 min at 37°C with equal volumes of antisera and C at respective final dilutions of 1:30,000 and 1:15.

In vivo adoptive chemoimmunotherapy (ACIT). The assay for treating advanced disseminated FBL-3 with a combination of nonlethal, noncurative chemotherapy and adoptively transferred immune cells has previously been described (11, 12). C57BL/6 mice inoculated with 5×10^6 FBL-3 on day 0 were treated on day 5 with cyclophosphamide (CY) (180 mg/kg) and 6 hr later with adoptively transferred cells i.p. Mice developed disseminated leukemia by day 5. Studies have shown that CY prolongs the median survival to approximately 4 weeks but cures no mice, that treatment with CY plus nonimmune cells or cells immune to unrelated antigens is no more effective than CY alone, and that treatment with CY plus cells from mice immunized with FBL-3 prolongs survival and cures mice depending on the dose of cells administered (11, 12).

An assay for treating established EL-4(G-) was constructed similarly for the present study. C57BL/6 mice inoculated with 2×10^5 EL-4(G-) on day 0 were treated on day 5 with CY (180 mg/kg) and 6 hr later with adoptively transferred cells i.p.

Statistical analysis. Survival curves were derived from cumulative experiments, with each data point representing the percentage of mice surviving on that day. The significance of the observed differences in survivals between groups was determined by a rank test designed for the analysis of multiple samples with censored observation (25). Two sample differences were confirmed by using the generalized Wilcoxon text (26).

[4] Abbreviations used in this paper: ACIT, adoptive chemoimmunotherapy; CY, cyclophosphamide; C57BL/6_{normal}, normal nonimmune C57BL/6 spleen cells; C57BL/$6_{\alpha EL-4}$, spleen cells from mice immunized *in vivo* with EL-4(G-); C57BL/$6_{\alpha FBL}$, spleen cells from mice immunized *in vivo* with FBL-3; $(C57BL/6)_x$, irradiated C57BL/6 spleen cells; $(EL-4)_x$; irradiated EL-4(G-); $(FBL)_x$, irradiated FBL-3; MST, median survival time.

RESULTS

Specificity of mixed lymphocyte-tumor responses. The ability of C57BL/6 spleen cells to recognize distinct antigenic determinants on our FBL-3 and EL-4(G-) lines was confirmed by examining the specificity of the cytotoxic responses generated after the reexposure of cells from mice immunized *in vivo* to tumor *in vitro* in mixed lymphocyte-tumor suspension cultures. (C57BL/6_{normal}, C57BL/$6_{\alpha FBL}$, or C57BL/$6_{\alpha EL-4}$ were cultured for 5 days with either irradiated FBL-3, EL-4(G-), or C57BL/6 spleen cells, denoted as $(FBL)_x$, $(EL-4)_x$, and $(C57BL/6)_x$, respectively, and then tested for *in vitro* cytotoxicity to FBL-3 and EL-4(G-) (Table I). The data represent the means of four experiments.

C57BL/6_{normal}, C57BL/$6_{\alpha FBL}$, and C57BL/$6_{\alpha EL-4}$ tested directly without culture were not cytotoxic to either target. After culture with tumor or spleen cells, C57BL/6_{normal} expressed low levels of cytotoxicity to both tumor targets. However, culture of C57BL/$6_{\alpha FBL}$ with $(FBL)_x$ resulted in significantly increased cytotoxicity against FBL-3 and not against EL-4(G-), whereas culture of C57BL/$6_{\alpha EL-4}$ with $(EL-4)_x$ resulted in significantly increased cytotoxicity to EL-4(G-) and not FBL-3. C57BL/$6_{\alpha FBL}$ cultured with $(EL-4)_x$ or C57BL/$6_{\alpha EL-4}$ cultured with $(FBL)_x$ became no more cytotoxic than cultures of normal nonimmune cells. Thus, the cytotoxic lymphocytes generated by the reexposure of cells from mice immunized *in vivo* to the sensitizing tumor recognized immunologic distinct antigens on these tumors, and the lack of a detectable specific cytotoxic response after *in vitro* exposure of *in vivo* immunized cells to the opposite tumor implies a lack of cross-immunization between these tumors.

The specific cytotoxicity generated during the 5-day mixed lymphocyte-tumor cultures was reduced by greater than 90% by further short-term incubation of effector cells with anti-Thy 1.2 plus C but not with C alone (data not shown). Thus, cell surface phenotype as well as specificity implicates the participation of thymus-derived lymphocytes in cytotoxic response generated after re-exposure to tumor *in vitro* of cells from *in vivo* immunized mice.

TABLE I

Specificity of mixed lymphocyte-tumor response[a]

Responder	Stimulator	% Specific Lysis			
		FBL-3		EL-4(G-)	
		50:1	12:1	50:1	12:1
C57BL/6_{normal}	Noncultured	0	0	0	0
C57BL/$6_{\alpha FBL}$	Noncultured	0	0	0	0
C57BL/$6_{\alpha EL-4}$	Noncultured	0	0	0	0
C57BL/6_{normal}	$(C57)_x$	10	0	2	1
C57BL/6_{normal}	$(FBL)_x$	14	5	4	2
C57BL/6_{normal}	$(EL-4)_x$	18	7	9	5
C57BL/$6_{\alpha FBL}$	$(C57)_x$	12	1	4	1
C57BL/$6_{\alpha FBL}$	$(FBL)_x$	50	30	4	1
C57BL/$6_{\alpha FBL}$	$(EL-4)_x$	10	5	10	4
C57BL/$6_{\alpha EL-4}$	$(C57)_x$	4	1	4	1
C57BL/$6_{\alpha EL-4}$	$(FBL)_x$	5	5	4	2
C57BL/$6_{\alpha EL-4}$	$(EL-4)_x$	2	1	37	36

[a] Spleen cells from normal nonimmune C57BL/6 mice (C57BL/6_{normal}), or from C57BL/6 mice that had been immunized *in vivo* with irradiated FBL-3 (C57BL/$6_{\alpha FBL}$) or with irradiated EL-4 (C57BL/$6_{\alpha EL-4}$) were cultured for 5 days with irradiated $(C57)_x$, $(FBL)_x$, or $(EL-4)_x$ and then tested in a 4-hr chromium release assay for cytotoxicity against FBL-3 and EL-4(G-) at effector to target ratios of 50:1 and 12:1. The data represent the means of four experiments.

The same pools of cells from the *in vivo* immunized mice utilized for these *in vitro* experiments were concurrently tested without culture for therapeutic efficacy against FBL-3 and EL-4(G-) in the four *in vivo* experiments presented below.

Specificity of ACIT. To examine the specificity of the adoptive therapy of established tumors, C57BL/6 mice inoculated i.p. with either FBL-3 or EL-4(G-) on day 0 were treated on day 5 with CY (180 mg/kg) plus C57BL/6_{normal}, C57BL/$6_{\alpha FBL}$, or C57BL/$6_{\alpha EL\text{-}4}$.

The cumulative results of four consecutive therapy experiments with FBL-3 are presented in Figure 1. Mice receiving 5×10^6 FBL-3 on day 0 and no treatment all died by day 13. Therapy with CY alone prolonged the median survival time (MST) to day 23, but all mice eventually died with tumor. As an adjunct to CY, therapy with 2×10^7 C57BL/6_{normal} or C57BL/$6_{\alpha EL\text{-}4}$ had no additional effect on survival, whereas C57BL/$6_{\alpha FBL}$ had a significant dose-dependent effect on survival. Thus, 5×10^6 C57BL/$6_{\alpha FBL}$ prolonged MST to day 31 ($p<0.01$) and 2×10^7 C57BL/$6_{\alpha FBL}$ prolonged the MST to day 64 and cured 41% of mice ($p<0.01$).

Established EL-4(G-) was treated concurrently with the same pool of cells used for treating FBL-3 (Fig. 2). Mice receiving 2×10^5 EL-4(G-) on day 0 and no therapy all died by day 16. Therapy with CY alone prolonged the MST to day 22 but cured no mice. C57BL/$6_{\alpha EL\text{-}4}$, which had no effect against FBL-3, demonstrated a significant dose-dependent therapeutic effect against EL-4(G-). Thus, as an adjunct to CY, 5×10^6 and 2×10^7 C57BL/$6_{\alpha EL\text{-}4}$ further prolonged the MST and cured 31% and 69% of mice, respectively. Cells from mice immune to FBL-3 did have a modest effect against EL-4(G-), CY plus 2×10^7 C57BL/$6_{\alpha FBL}$ prolonged the MST to day 36 and cured 12% of mice. However, this therapeutic effect was not the result of sensitization, since CY plus 2×10^7 C57BL/6_{normal} were equally effective, prolonging the MST to day 37 and curing 16% of mice. Thus, sensitization specifically enhanced the therapeutic efficacy of the adoptively transferred cells in ACIT.

DISCUSSION

The immunologic specificity of ACIT of FBL-3 has previously been based on the demonstration that lymphoid cells from donors previously sensitized to FBL-3 or to another tumor possessing cross-reacting antigens were therapeutically effective against FBL-3, whereas cells from nonimmune mice or mice sensitized to irrelevant antigens were ineffective (11, 12). However, it has not been shown that cells from mice sensitized to FBL-3 are ineffective against a noncross-reactive tumor that is susceptible to eradication by appropriately sensitized cells in ACIT. In the present study, EL-4(G-), a chemically induced leukemia, was utilized as the reciprocal specificity control for the therapy of FBL-3. This subline of EL-4 lacks mouse endogenous virus-associated surface antigen 1, an antigen related to endogenous murine C-type virus (18) and is antigenically distinct from tumors induced by Friend, Moloney, and Rauscher viruses by studies of transplantation resistance (20) and cold-target inhibition of specific cytolytic activity (18). Moreover, previous studies have shown that EL-4 is susceptible to therapy by a combination of CY plus syngeneic cells primarily sensitized *in vitro* (14).

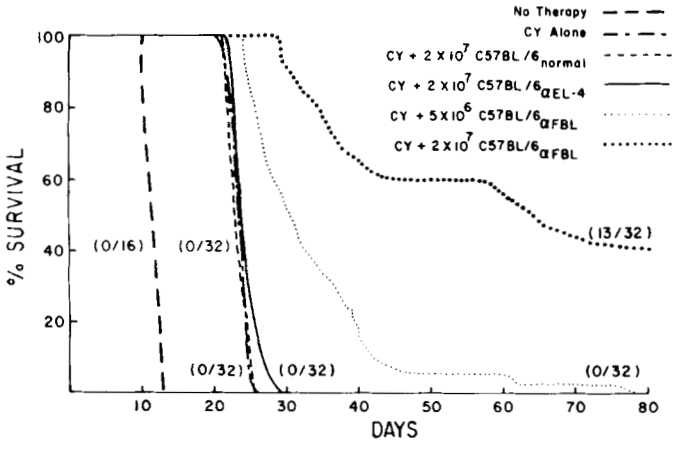

Figure 1. Adoptive chemoimmunotherapy of FBL-3. C57BL/6 mice inoculated i.p. with 5×10^6 FBL-3 on day 0 received either no therapy (— —), treatment on day 5 with cyclophosphamide (CY) at 180 mg/kg (—·—), or CY plus 2×10^7 normal nonimmune C57BL/6 spleen cells (-----), 2×10^7 cells from mice immunized with EL-4(G-) (——), 5×10^6 cells from mice immunized with FBL-3 (····) or 2×10^7 cells from mice immunized with FBL-3 (•••••••). Fractions represent number of mice surviving 80 days over total.

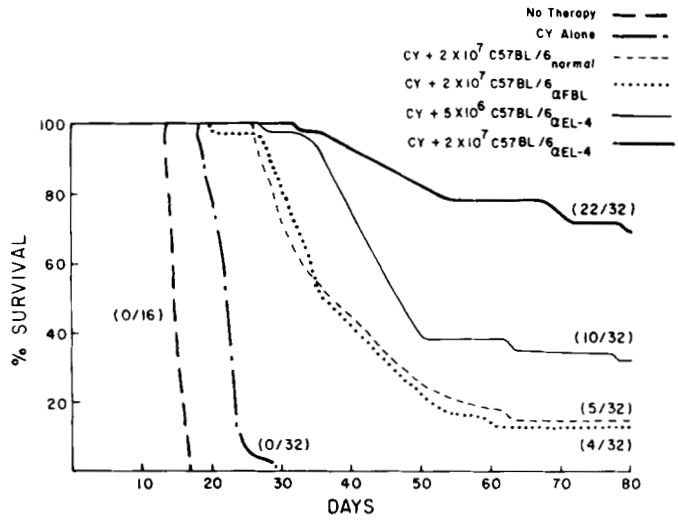

Figure 2. Adoptive chemoimmunotherapy of EL-4(G-). C57BL/6 mice inoculated with 2×10^5 EL-4(G-) on day 0 received either no therapy (— —), treatment on day 5 with cyclophosphamide (CY) at 180 mg/kg (—·—), or with CY plus 2×10^7 normal nonimmune C57BL/6 spleen cells (- - -), 2×10^7 cells from mice immunized with FBL-3 (····), 5×10^6 cells from mice immunized with EL-4(G-) (——), or 2×10^7 cells from mice immunized with EL-4(G-) (—). Fractions represent number of mice surviving 80 days over total.

The present study demonstrated that the cytotoxic cells generated after re-exposure of *in vivo* primed cells to the sensitizing tumor *in vitro* recognized antigenically distinct moieties on those tumors and also showed by inference that no cross-priming had occurred. This immunologic specificity of priming, response of re-exposure of primed cells to the sensitizing tumor, and tumor susceptibility to lysis allowed the subsequent testing of specificity of ACIT. In these therapy studies, cells from mice sensitized to EL-4(G-) were effective in therapy only against EL-4(G-) and not FBL-3, and conversely, cells from mice sensitized to FBL-3 were effective against FBL-3 and no more effective against EL-4(G-) than normal nonimmune cells. Thus, by employing reciprocal specificity controls in ACIT, these experiments demonstrated that the enhancement of therapeutic efficacy resulting from *in vivo* exposure to tumor is immunologically specific.

The efficacy of normal nonimmune cells against EL-4(G-) was unexpected, since normal cells as an adjunct to CY had previously been shown to be ineffective against EL-4 (14). However, in those studies a different subline of EL-4 was utilized, and therapy was administered on day 1 after tumor inoculation, whereas in our studies therapy was delayed to day

5 until the tumor was clearly established. Although we have not identified the effector cell in normal spleen cells, others have shown that normal nonimmune cells can prevent tumor growth *in vivo* through the mechanism of natural cell-mediated cytotoxicity (NK cells) (27–29). Moreover, we have recently demonstrated that cells with physical and surface characteristics similar to NK cells are an effective adjuvant to CY in the ACIT of a syngeneic Moloney virus-induced leukemia (LSTRA) in BALB/c mice (17).

Presumably the effector cells present in normal spleen account for some of the anti-tumor efficacy of spleen cells from mice immunized to EL-4(G-). The efficacy of these sensitized cells may reflect several effector cell subpopulations. The character of these different subpopulations is currently being investigated.

Although immunization specifically enhances the effect of donor cells in ACIT, the subsequent action of infused cells remains unclear. It cannot be assumed that cytotoxic cells, similar to those induced *in vitro* by re-exposure of primed cells to tumor, develop *in vivo* or are alone responsible for the anti-tumor effect demonstrated in ACIT. *In vivo* therapy is likely to involve a complicated series of interactions involving not only the generation of anti-tumor effector cells but also modulation of this response by both amplifier (30) and suppressor cells (31) as well as recruited cells (32). Additionally, the tumor-bearing host may contain cells that enhance or interfere with ACIT (33). The development of these two reciprocally specific models for tumor therapy may allow determination of which aspects of tumor eradication result from immunologically specific mechanisms or result from more generalized enhancement or suppression of immune reactivity.

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