Cancer Immunol Immunother (1982) 13:5–13

Cancer Immunology and Immunotherapy

Adoptive Chemoimmunotherapy of a Syngeneic Murine Lymphoma with Long-Term Lymphoid Cell Lines Expanded in T Cell Growth Factor

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Summary. *Recently techniques have been developed for the long-term growth of cytotoxic T-lymphoid cells in vitro with T cell growth factor (TCGF). We have investigated the use of these in vitro-expanded T cells for the immunotherapy of a disseminated syngeneic murine FBL-3 lymphoma. In this model, mice with disseminated tumor were treated on day 5 with 180 mg cytoxan/kg and then 5 h later were given lymphoid cells IP. In vivo-immunized lymphocytes resulted in significantly improved survival in three of three experiments, curing 52% of 38 animals, compared with treatment with cytoxan alone (0 of 31 cured) or cytoxan plus unimmunized cells (0 of 40 cured) ($P < 0.0005$). In vivo-immunized lymphocytes were re-exposed to FBL-3 tumor in vitro for 5 days in complete medium (CM) or lectin-free TCGF (LF-TCGF). Both groups showed significantly improved survival in six of six experiments. Cytoxan cured 17% of 66 animals, while cytoxan plus normal lymphocytes after IVS cured 6% of 47 animals. In vivo-immunized cells resensitized in vitro to FBL-3 in CM or LF-TCGF cured 82% of 50 animals ($P < 0.001$) and 72% of 61 animals ($P < 0.001$), respectively. Cells from in vivo- and in vitro-sensitized lymphocytes exhibited no cytotoxicity in our in vitro ^{51}Cr-release assay; expansion of these cells resulted in significant specific lysis of fresh FBL-3 targets. Adoptive transfer of immune lymphocytes resensitized to FBL-3 tumor in vitro and expanded in LF-TCGF conferred a significant survival benefit ($P < 0.001$, curing 7 of 27 animals) compared with all controls. These expanded cells were then continuously grown in LF-TCGF for 2½ months. Again, in vivo-immunized lymphocytes resensitized to FBL-3 tumor and expanded in LF-TCGF for 2½ months cured 56% of the animals with disseminated tumor, significantly prolonging survival over that recorded in any control group ($P < 0.0002$). Irradiation of these same cells totally abolished their efficacy. Clones were generated from IVS and continuously grown in LF-TCGF. Two of these clones were very cytotoxic for fresh FBL-3 (> 4,000 lytic units/10^6 cells). When adoptively transferred to mice in this chemoimmunotherapy model these cytotoxic clones significantly enhanced survival over that recorded following treatment with cytoxan alone ($P < 0.00001$), though prolongation of survival was small. Implications of these results for application of these techniques to other less antigenic tumors and human cancers are discussed.*

Introduction

The possibility of treating malignant neoplasms by the use of passive immunotherapy has been the subject of much research [30]. This approach depends upon obtaining immunologically reactive cells or serum capable of eliminating tumor in vivo. Reports of specific adoptive immunotherapy of murine tumors have described the use of in vivo-immunized cells [4, 6, 11–13, 17, 24, 34, 36, 38, 42, 45, 48, 49], in vitro-sensitized cells [1, 5, 7–9, 21, 29, 43, 44, 46], or cells with enhanced immune reactivity due to the addition of a variety of soluble supernatants [10, 25, 28, 37]. Though some immunotherapy protocols have utilized a Winn assay [1, 4, 7, 8, 29, 34, 36, 45, 46, 49] (the admixture of tumor cells and immune cells in vitro prior to in vivo adoptive transfer), the systemic treatment of an established tumor nodule or disseminated lethal tumor [4, 6–10, 13, 21, 24, 25, 38, 43, 45] is a far better test of the therapeutic potential of immune cells in vivo.

It has been demonstrated [32, 40] that immunologically reactive T cells can be expanded in vitro by means of T cell growth factor (TCGF) and that this lymphokine is capable of actively enhancing the cytotoxicity of cells sensitized in its presence [2, 41]. These findings have opened new possibilities for the adoptive immunotherapy of tumors with expanded, specifically reactive syngeneic or autologous lymphoid cells. We [35] have previously demonstrated that adoptive transfer of alloreactive cells expanded in TCGF is capable of accelerating the rejection of allogeneic skin grafts. It was of interest to test the effect of anti-tumor reactive cells on tumor growth in vivo.

Recently (T. J. Eberlein et al. 1982) we have described techniques for the generation of highly cytotoxic lymphoid cell lines and cloned cell lines directed against the syngeneic FBL-3 lymphoma in vitro. This was achieved with in vitro expansion of appropriately in vitro-sensitized cells in lectin-free T cell growth factor (LF-TCGF).

In this paper we have demonstrated that the adoptive transfer of these cells can cure mice with disseminated lethal syngeneic lymphoma. Modifications of these techniques may also be applicable to the therapy of human cancers.

Materials and Methods

Animals. Female C57BL/6 mice were obtained from Jackson Laboratory, Bar Harbor, Maine, and used in experiments when they were at least 12 weeks old.

Tumors. FBL-3 is a transplanted ascitic Friend virus-induced lymphoma/leukemia of C57BL/6 origin (supplied to us by Dr C. C. Ting, NCI, NIH). This tumor bears tumor-specific and/or viral antigens, which cross react with other tumors induced by the Friend, Maloney, and Rauscher viruses [19].

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0340-7004/82/0013/0005/$ 01.80

MCA-103 is a fibrosarcoma induced in our laboratory in a C57BL/6 mouse by the injection of 0.1 ml 1% 3-methylcholanthrene in sesame oil into the hind limb as previously described [26]. It was maintained by serial intramuscular passage and was used for these experiments during the fourth to seventh transplant generations.

In vivo Immunization. C57BL/6 female mice aged 8–12 weeks were given 2-weekly IP injections of 2×10^7 irradiated (10,000 R) FBL-3 tumor cells in sterile Hank's balanced salt solution (HBSS). After 6 weeks, spleen cells were harvested and used for in vitro and in vivo experiments.

Spleen Cell Suspension. Spleens were removed asceptically, pooled, teased apart with two sterile forceps, and passed through a single layer of 100 mesh nylon in HBSS. These cells were centrifuged at 500 *g* for 5 min and the resulting pellet was resuspended in buffered ammonium chloride solution (ACK) for 1 min at room temperature to lyse the red blood cells. After three washes in HBSS the cells were resuspended in complete medium (CM). CM was composed of RPMI 1640 (Grand Island Biological Co., Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Grand Island Biological Co., USA), 0.03% fresh glutamine (NIH Media Unit), 1 μ*M* sodium pyruvate (Microbiological Associates, Walkersville, MD, USA), 0.1 m*M* nonessential amino acids (Microbiological Associates), 5×10^{-5} M2-mercaptoethanol, 100 units penicillin/ml, and 100 μg streptomycin/ml.

Production of LF-TCGF. Optimal conditions for the production of LF-TCGF have been previously described [41]. Briefly, splenocytes from ex-breeder DBA/2 or BALB/c mice were incubated with 10 μg Con A/ml (Miles Laboratories, Elkhart, IN) for 2 h. The cells were then washed three times with HBSS and the pellet resuspended in CM without Con A for an additional 24 h. Supernatants from these cultures were harvested, centrifuged, and passed through 0.45-μm filters (Millipore Corp., Bedford, MA, USA). LF-TCGF supernatants prepared in this manner were greater than 95% Con A-free as determined by absence of mitogenic activity on fresh lymphocytes and by measurement of removal of radiolabeled Con A [41].

LF-TCGF was also made from the EL-4 tumor cell line and is referred to as LF-TCGF-EL4. This factor was only used in the latter stages of the in vivo experiments with cloned lymphoid cells. Briefly, the EL-4 tumor lines (given to us by J. Farrer, NIH) were maintained in RPMI and 5% heat-inactivated FCS and 0.1% gentamicin. The cells were initially placed at 10^5 cells/ml. They expand approximately 5 to 10-fold in 5–7 days. They are grown in 750-ml tissue culture flasks (Falcon # 3028), standing up, with 200 ml tissue culture suspension per bottle.

To generate LF-TCGF-EL-4, EL-4 cells were harvested, centrifuged at 1500 *g* for 5 min, and washed twice in HBSS. Viable EL4 cells (10^6/ml) were incubated in RPMI 1640, with 10 ng phorbol myristic acid/ml and 0.1% gentamicin. This suspension was placed at 37°, 5% CO_2 for 48 h in 750-ml tissue culture flasks, upright, with 200 ml per bottle. After 48 h, the suspension was centrifuged at 2000 *g* for 10 min and the pellet discarded. The supernatant was recentrifuged at 6000 *g* for 30 min and the second pellet discarded. The second supernatant was filtered through 0.45 μm filters (Millipore Corp.) and stored at 4° C until used.

In vitro Sensitizations (IVS). Stimulator cells were irradiated in a gamma irradiator with 10,000 R (FBL-3) or 2,000 R (normal C57BL/6 lymphocytes). Viable responder cells (6×10^7) and irradiated stimulator cells (1×10^6) were added to upright flasks (Costar # 3013) in 20 ml total volume of CM (for generation of clones; see below) or 10 ml CM and 10 ml LF-TCGF (for generation of the whole population for expansion). Cells were harvested for expansion or in vitro testing on day 5. If used for in vivo testing, these cells were harvested, washed three times in HBSS, and resuspended to a concentration of 4×10^7 viable cells/ml.

Secondary IVS were performed by taking 6×10^6 viable cells recovered from a primary IVS on day 10 and adding 3×10^5 fresh irradiated tumor cells (or normal lymphocytes) to 24-well flat-bottom plates (Costar) in 2 ml fresh CM. Tertiary IVS were performed in the same fashion on day 20 with fresh CM and stimulators under the same culture conditions as for 2° IVS.

Expansion of IVS Lymphoid Cells. Cells were harvested from LF-TCGF IVS flasks, centrifuged, and resuspended in fresh CM. Cell viability was assessed by trypan blue dye exclusion. These sensitized cells were adjusted to a concentration of 10^5 viable cells/ml in CM and then diluted in an equal volume of LF-TCGF (final concentration of 5×10^4 viable cells/ml). Of this final cell suspension, 100 ml was placed in 750-ml tissue culture flasks (Falcon # 3028) lying flat. The cells expanded 7- to 10-fold in 6 days. When used for adoptive immunotherapy, cells were washed three times in HBSS and resuspended to a concentration of 4×10^7 viable cells/ml. Of this solution, 0.5 cm^3 was adoptively transferred.

In experiments where cells were expanded in LF-TCGF for greater than 2 weeks, 10^5 irradiated FBL-3 tumor cells/ml (10,000 R) were added to each culture. Every 5–7 days the cells growing in TCGF were harvested, centrifuged at 1500 *g* for 5 min, and readjusted to 5×10^4 cells/ml in fresh medium containing LF-TCGF and irradiated tumor cells as described above. For the 10 days (two culture splits) prior to adoptive transfer, the cells were grown in media containing LF-TCGF but no tumor cells were added to the cultures.

Chromium-Release Cytotoxicity Assay. An 18-h chromium release assay under conditions previously described in detail was used [31]. Briefly, varying ratios of effector cells were plated in 96-well round-bottom plates (Linbro; Flow Laboratories) with 10^4 viable ^{51}Cr-labeled target cells/well. The plates were then centrifuged at 800 *g* for 5 min and incubated for 18 h. The plates were then centrifuged at 2000 *g* for 10 min and the supernatants harvested by the Titertek Collecting System (Flow Laboratories).

Fresh FBL-3 tumor was harvested for each assay, washed, and labeled with ^{51}Cr. For use as targets, MCA-103 tumor was minced with fine scissors, trypsinized for 7 min, passed through double-layer 100 mesh nylon, and then washed three times and labeled with ^{51}Cr. Spontaneous release was no greater than 50% in any experiment. The 18-h ^{51}Cr-release assays revealed substantially higher levels of specific tumor lysis than were seen in 4-h ^{51}Cr-release assays.

Cloning of IVS Lymphoid T Cells. Clones were generated by means of a limiting dilution technique as previously described in 96-well plates (Costar # 3596), cells from primary, secondary, or tertiary IVS being used [33].

Clones were screened for cytotoxicity by a replica plating technique as described elsewhere (T. J. Eberlein et al. 1982). In brief, of the contents of each well of a 96-well plate from limiting dilution cloning (Costar # 3596), 0.1 ml was transferred to a fresh 96-well plate containing ^{51}Cr-labeled target cells. An 18-h ^{51}Cr-release assay was then conducted as described above. Clones from the original plate that appeared 'interesting' and required further growth were then transferred to 2-ml flat-bottomed plates (Costar) in a 1 : 1 concentration of LF-TCGF and CM and expanded.

In vivo Assay of Adoptive Immunotherapy. An adoptive chemoimmunotherapy model developed by Fass and Fefer [11, 12, 14] was used. In brief, 12-week-old C57BL/6 female mice were inoculated IP on day 0 with 10^7 live FBL-3 tumor. By day 5, tumor cells can be found in blood and lymph nodes in addition to the peritoneal cavity. Mice die of this disseminated lymphoma if not treated.

On day 5, the control mice were removed randomly and all other animals were given 180 mg cyclophosphamide/kg IP (individual weighing of each animal). After 5 h a group of cytoxan-treated control mice were removed randomly and then the other mice received experimental or control cells by IP injection in 0.5 cm^3 sterile HBSS. Survival of mice was checked daily.

Statistical Methods. Survival of mice in these experiments was computed by the method of Kaplan and Meier [27]. For comparing survival curves to determine whether groups were statistically different we used the log-rank test described by Peto et al. [27]. An overall analysis of all groups in an experiment was done first. If this was statistically significant at the 0.05 level, pairwise comparisons of treatments were done. All P-values reported are two-sided.

Results

Treatment of Disseminated FBL-3 with in vivo-Immunized Lymphoid Cells

Adult female C57BL/6 mice were immunized in vivo with irradiated FBL-3 tumor cells (10,000 R), and 6 weeks later their spleens were harvested and tested both in vitro and in vivo. In each of three experiments, these immune lymphocytes did not exhibit in vitro cytotoxicity in an 18-h ^{51}Cr-release assay (data not shown). These same cells, when adoptively transferred in conjunction with cytoxan, conferred significant survival benefit to mice with disseminated FBL-3 tumor, compared with treatment with cytoxan alone or treatment with cytoxan plus normal lymphocytes. Figure 1 is the cumulative result of all three experiments performed; each experiment showed a similar result. In all experiments, 20 of 38 mice receiving cytoxan plus in vivo-immunized cells were cured of tumor, whereas 0 of 31 mice were cured when treated with cytoxan plus normal lymphocytes ($P < 0.005$). Cytoxan treatment alone cured 0 of 40 mice ($P < 0.0005$).

Adoptive Transfer of IVS Lymphoid Cells Before Expansion in LF-TCGF

In vivo-immunized lymphocytes were resensitized to irradiated FBL-3 tumor in CM or LF-TCGF. Simultaneously, nonimmune lymphocytes were co-cultured with normal C57BL/6 irradiated stimulators in LF-TCGF. On day 5 the cells were harvested, washed, and counted. 2×10^7 Viable cells were adoptively transferred 5 h after cytoxan was administered.

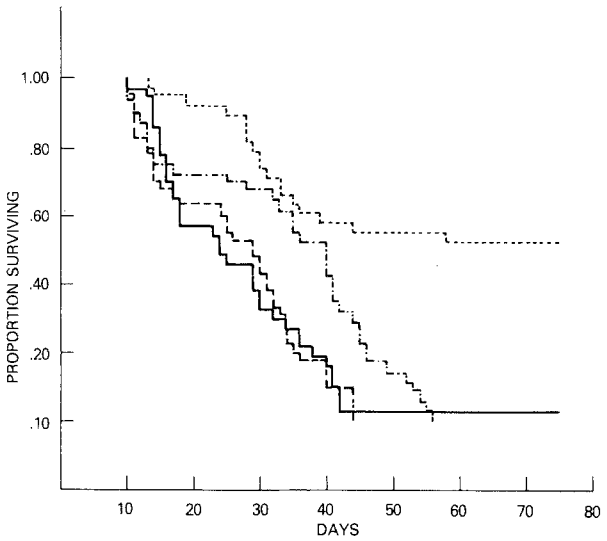

Fig. 1. Survival of mice with disseminated FBL-3 lymphoma treated with in vivo immunized lymphocytes. Treatment with cytoxan plus immune lymphocytes conferred significant survival benefit compared with treatment with cytoxan alone or cytoxan plus nonimmune lymphocytes. $N = 37$ (———) tumor only; 40 (– – –) cytoxan only; 31 (– · –) cytoxan + Nl; 38 (- - - -) cytoxan + I. Cy vs I $P < 0.0005$; NL vs I $P < 0.0005$

In Fig. 2, a composite of six identical experiments is shown. Immune lymphocytes resensitized to FBL-3 tumor in CM or in LF-TCGF both conferred significant survival benefit when adoptively transferred to mice with disseminated FBL-3 tumor. In the CM group, 41 of 50 animals were cured ($P < 0.001$). In the LF-TCGF group, 36 of 61 animals were cured ($P < 0.001$). Lymphocytes co-cultured with normal stimulators and injected after cytoxan therapy cured 7 of 47 animals, not a greatly different result than that obtained following treatment with cytoxan alone (4 of 66 animals cured). There seemed to be no difference between CM and LF-TCGF IVS groups ($P = 0.88$). To further compare the effects of IVS in CM and LF-TCGF, varying numbers of cells were used for in vivo therapy.

Table 1 shows one such titration experiment (2 others showed identical results), comparing IVS in CM and IVS in LF-TCGF. There was no significant difference in the in vivo effects of cells IVS in CM or LF-TCGF. The minimum effective dose that prolonged survival and cured mice appeared to be 5×10^6 cells.

In vitro Cytotoxicity Before and After Expansion in LF-TCGF

In vivo-sensitized cells were resensitized in vitro to irradiated FBL-3 tumor for 5 days as described above. Simultaneously, normal C57BL 6 lymphocytes were co-cultured with normal C57BL/6 irradiated stimulators. Neither of these groups showed significant lysis of fresh FBL-3 tumor in an 18-h ^{51}Cr-release assay in six of eight experiments. In the other two experiments low levels of lysis were seen only by the immune lymphocytes resensitized to FBL-3 tumor and only at the highest effector-to-target ratios (data not shown). In every experiment, however, after expansion in LF-TCGF, high degrees of lysis of fresh FBL-3 were seen only in immune lymphocytes previously co-cultured with FBL-3 tumor. None

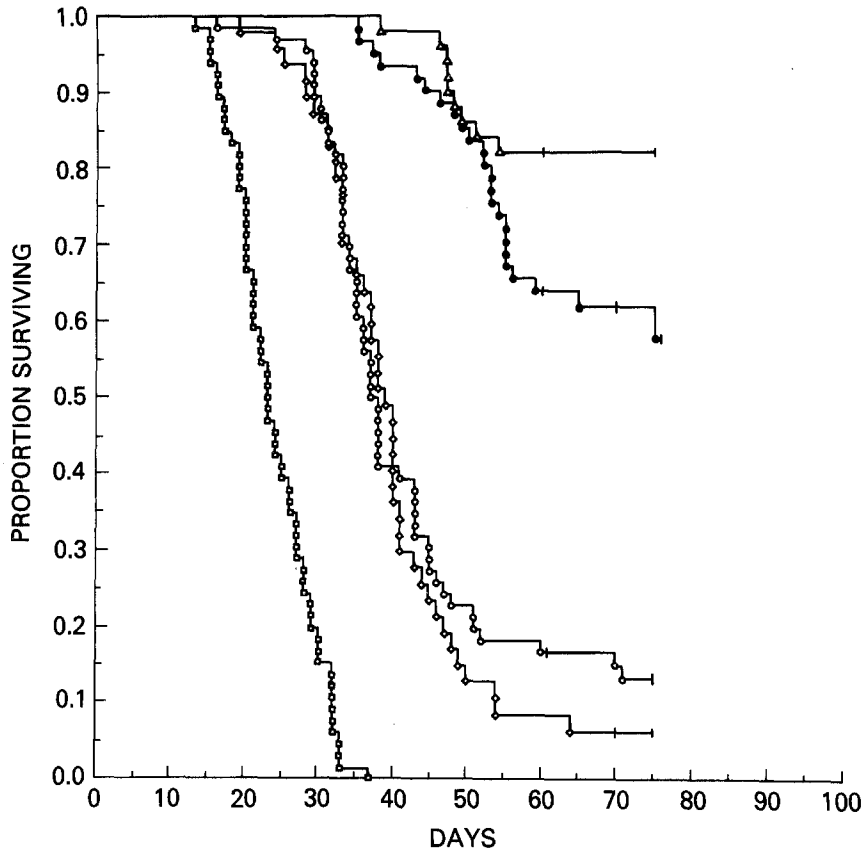

Fig. 2. Survival of mice with disseminated FBL-3 lymphoma treated with IVS lymphocytes. Treatment with cytoxan plus immune lymphocytes co-cultured with FBL-3 tumor conferred significant survival benefit compared with treatment with cytoxan only or cytoxan plus nonimmune lymphocytes co-cultured with normal lymphocytes. This significant survival benefit occurred whether the IVS was performed in CM or LF-TCGF. N = 66 (■—■) no treatment (T) · 66 (○—○) cytoxan (Cy); 50 (△—△) Iα FBL-3 CM (CM); 61 (●—●) Iα FBL-3 LF (LF); 47 (◇—◇) Nl αB6 LF (Nl). Cy vs CM $P < 0.001$; Cy vs LF $P < 0.001$; NL vs CM $P < 0.001$; NL vs LF $P < 0.001$. CM vs LF $P = 0.88$

Table 1. Comparison of treatment with IVS cells in CM or LF-TCGF

Treatment	IVS in	
	CM	LF-TCGF
	(% surviving at 75 days)	
None	[a]	[a]
Cytoxan alone	[a]	[a]
Cytoxan + 2×10^7 IVS cells	57	63
Cytoxan + 10^7 IVS cells	57	50
Cytoxan + 5×10^6 IVS cells	29	29
Cytoxan + 10^6 IVS cells	0	0

[a] These mice did not receive adoptively transferred cells

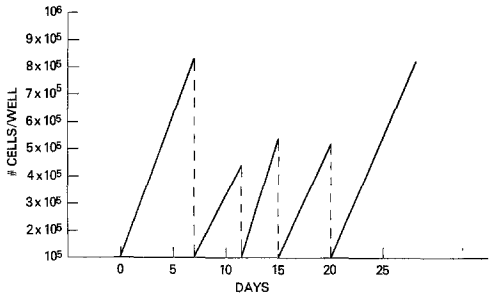

Fig. 3. Growth of cells expanding in LF-TCGF. There was a doubling time of approximately 24 h during this 28-day period. These cells expanded over 8,000-fold

Table 2. Lysis of FBL-3 tumor by lymphoid cells in TCGF

Day of expansion	I α FBL-3[a] (lytic units/10^6 cells)[c]		NL α NL[b] (lytic units/10^6 cells)[c]	
	Vs. fresh FBL-3	Vs. fresh 103	Vs. fresh FBL-3	Vs. fresh 103
7	714	n.d.	< 1	n.d.
18	3,225	< 2	17.4	< 2
28	1,724	< 2	< 1	< 1

[a] I α FBL-3, in vivo immunized lymphocytes resensitized to FBL-3 tumor in vitro prior to expansion in LF-TCGF

[b] NL α NL, normal C57BL/6 lymphocytes resensitized to normal irradiated stimulators in vitro prior to expansion in LF-TCGF

[c] A lytic unit is defined as the number of effector cells that causes 50% lysis of 10^4 ^{51}Cr-labeled target cells

of the other combinations of IVS (normal lymphocytes sensitized to FBL-3 tumor, immune lymphocytes co-cultured with normal C57BL/6 stimulators, or normal lymphocytes resensitized to normal C57BL/6 stimulators) had any significant lysis of fresh FBL-3 tumor after expansion in LF-TCGF. This lysis was specific for FBL-3. No lysis of fresh syngeneic MCA-103 target cells was demonstrated in any experiment, though experiments with allosensitized cells demonstrated that the MCA-103 tumor target cells were as susceptible to lysis as FBL-3 target cells.

Figure 3 shows the growth pattern of the immune lymphocytes resensitized to FBL-3 tumor and expanded in LF-TCGF for 28 days. The doubling time is approximately 24 h. Table 2 shows the levels of lysis seen over the same 28 days of continuous growth in LF-TCGF. High levels of specific lysis were unique to the IVS immune lymphocytes

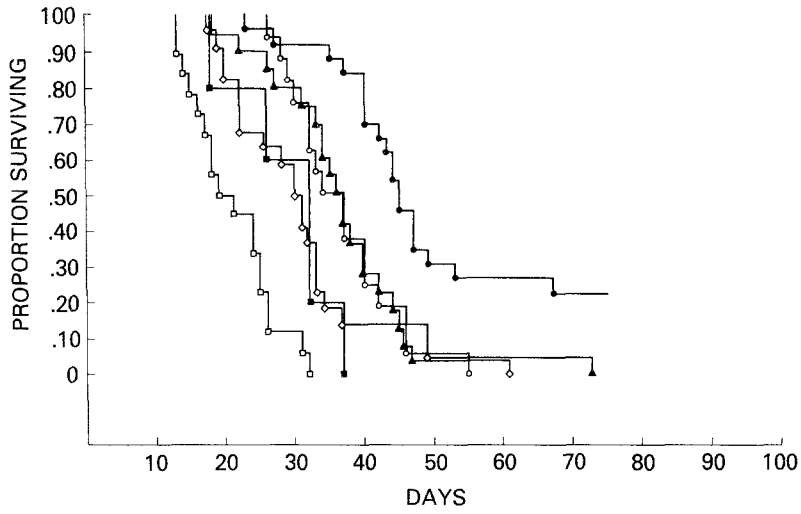

Fig. 4. Survival of mice with disseminated FBL-3 lymphoma treated with IVS lymphocytes expanded 7- to 10-fold in LF-TCGF for 6 days. Treatment with immune lymphocytes resensitized to FBL-3 tumor and then expanded in LF-TCGF conferred significant survival benefit compared with cytoxan treatment alone, cytoxan plus immune lymphocytes sensitized to normal C57BL/6 stimulators prior to expansion in LF-TCGF, or cytoxan plus nonimmune lymphocytes sensitized to normal stimulators or FBL-3 tumor and then expanded in LF-TCGF. Total = 18 (■—■) no treatment; 16 (○—○) cytoxan; 27 (●—●) cytoxan + Iα FBL-3 lymphocytes; 21 (▲—▲) cytoxan + Iα B6 lymphocytes; 5 (■—■) cytoxan + NL α FBL-3 lymphocytes; 22 (◇—◇) cytoxan + NL α B6 lymphocytes. Cy vs I α FBL-3 $P < 0.001$. I α B6 vs I α FBL-3 $P < 0.001$. NL α FBL-3 vs I α FBL-3 $P < 0.001$; NL α B6 vs I α FBL-3 $P < 0.001$

resensitized to FBL-3 tumor and then expanded in LF-TCGF. Normal C57BL/6 lymphocytes co-cultured with normal C57BL/6 stimulators showed little lysis of fresh FBL-3 or fresh MCA-103.

Adoptive Transfer of Lymphoid Cells Expanded in LF-TCGF

Cells from the IVS were washed, counted, and placed in LF-TCGF at 5×10^4 cells/ml. After 6 days there was a 7- to 10-fold expansion in cell number. The cells were harvested, washed three times, and resuspended in HBSS. Viable cells were injected in vivo (2×10^7 in 0.5 cm^3 IP).

Figure 4 shows the in vivo results of the first two experiments with the adoptive transfer of expanded cells. The animals receiving no treatment all died by day 32. Cytoxan prolonged the mean survival time but no animals were cured. Treatment of mice with cytoxan plus immune lymphocytes sensitized to normal C57BL/6 stimulators and then expanded in LF-TCGF resulted in no increase in survival compared with cytoxan alone. Similarly, treatment of mice with cytoxan plus normal nonimmune lymphocytes sensitized either to FBL-3 tumor or to normal lymphocyte stimulators resulted in no increase in survival compared with treatment with cytoxan. However, treatment of mice with cytoxan plus immune lymphocytes resensitized to FBL-3 tumor in vitro and expanded in LF-TCGF led to cures in 7 of 27 animals, and prolonged survival over that in all control groups ($P < 0.001$).

Adoptive Immunotherapy with Cells Expanded in LF-TCGF Continuously for 2½ Months

The cells in Fig. 5 were expanded in LF-TCGF for 2½ months, as described in *Materials and Methods*. The growth curve is presented in Fig. 3. Again, the animals receiving no treatment all died by day 33. Cytoxan prolonged survival until day 41 but all animals died of tumor. Treatment with cytoxan plus normal nonimmune lymphocytes resensitized to normal C57BL/6 stimulators and expanded in LF-TCGF for 2½ months did not prolong survival compared with cytoxan controls. Cytoxan plus immune lymphocytes resensitized to FBL-3 tumor and expanded continuously in LF-TCGF for 2½ months showed marked prolongation of survival and cured five of nine animals ($P = 0.0002$). These cells had almost 2000 lytic units (LU)/10^6 cells when tested in a ^{51}Cr-release assay just prior to adoptive transfer (data not shown).

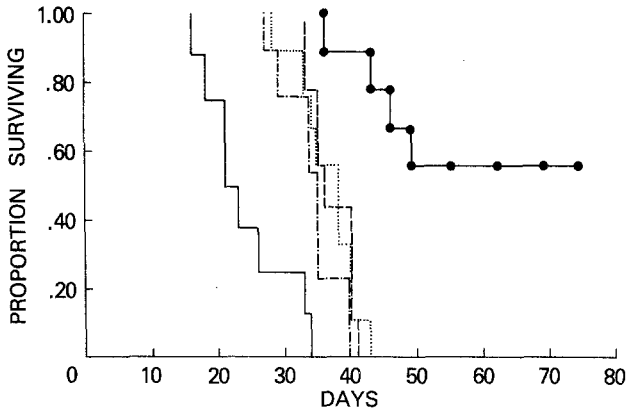

Fig. 5. Survival of mice with disseminated FBL-3 lymphoma treated with immune lymphocytes resensitized to FBL-3 tumor and continuously grown for 2½ months in LF-TCGF. Significant survival benefit was conferred by this treatment compared with treatment with cytoxan alone or cytoxan plus normal lymphocytes resensitized to normal lymphocytes and expanded in LF-TCGF for the same length of time. Irradiation (2,000 R) of the transferred cells abolished their ability to prolong survival. $N = 8$ (———) no treatment; 9 (– – –) Cytoxan (Cy); 9 (· · · · · ·) Cy + B6 α B6 (NL); 9 (– · –) Cy + I α FBL-3 (2,000 R) (XRAY); (■———■) Cy + I α FBL-3 (Exp) (I). Cy vs I $P < 0.0002$; B6 vs I $P < 0.0003$. X-ray vs I $P < 0.0002$

When the same cells that cured 56% of the treated animals received 2,000 R just prior to adoptive transfer, the survival benefit conferred by these cells was abolished. No animals were cured and the survival time was no better than the cytoxan alone treatment group. Thus it appeared that a cell capable of dividing in vivo was necessary to mediate an increase in survival.

Adoptive Transfer of Cloned Lymphoid Cells Maintained in LF-TCGF for 2½ Months

Clones were generated by means of a limiting dilution technique (T. J. Eberlein et al. 1982). The proportion of wells with growth in the limiting dilution assay was 15%–30%. Based on the Poisson distribution there was about an 80% chance that growth in a single well arose from a single cell. Though we use the term clones to describe these cells we

Table 3. Lysis of FBL-3 tumor by cloned lymphoid cells expanded in TCGF

	Day of expansion	Cytotxic clones (lytic units/ 10^6 cells)		Noncytotoxic clone (lytic units/ 10^6 cells)	
		Vs. fresh FBL-3	Vs. fresh 103	Vs. fresh FBL-3	Vs. fresh 103
At 1 month	19	> 500[a]	< 1		
	17	> 500[a]	< 1	9′ < 1	< 1
	2	333	< 1		
At 2½ months	19	1,887	< 1		
	17	4,000	< 1	9′ < 1	< 1
	2	4,545	< 1		

[a] At the lowest ratio tested (0.2 : 1) greater than 80% of fresh FBL-3 were lysed

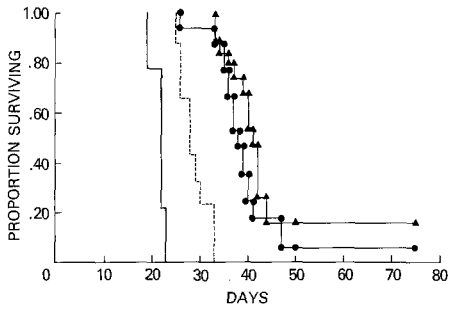

Fig. 6. Survival of mice with disseminated FBL-3 lymphoma treated with cytotoxic clones #2 and #17. Treatment with both of these clones prolonged survival beyond that obtained with treatment with cytoxan alone; however, there were few long-term cures. $N = 9$ (———) no treatment; 9 (– – –) cytoxan; 17 (●——●) clone 17; 19 (▲——▲) clone 2. Cy vs 17 $P < 0.00001$; Cy vs 2 $P < 0.00001$

realize that recloning is essential, and these studies are in progress.

Three 'cytotoxic' clones (Nos. 2, 17, and 19) and one 'noncytotoxic' clone (9′) were grown for 2½ months. Table 3 shows the specific cytotoxicity achieved with these cytotoxic clones. At 0.2 : 1 (the lowest effector-to-target ratio tested) clones 17 and 19 were 80% lytic for fresh FBL-3 at 1 month. This higher level of specific cytotoxicity was maintained for the entire 2½ months. Clone 9′, the noncytotoxic clone, showed no lysis of fresh FBL-3 or fresh MCA-103 at any time. Clones 2 and 17 were adoptively transferred to animals with disseminated FBL-3 lymphoma according to our standard adoptive immunotherapy model. Both clones were very lytic for fresh FBL-3 at the time of adoptive transfer (Table 3) and both clones resulted in prolonged survival compared with treatment with cytoxan alone ($P < 0.00001$) (Fig. 6).

Discussion

Many workers have utilized adoptive immunotherapy in the treatment of murine tumors [30]. Immune splenocytes from in vivo-immunized mice [4, 6, 11–13, 17, 24, 34, 36, 38, 42, 45, 48, 49] have prolonged survival and cured mice of tumors both in Winn assays [4, 34, 36, 45, 49] and in the treatment of established or disseminated tumors [4, 6, 24]. Other workers attempted to improve survival or eradicate tumor by boosting lymphoid cells in vitro to tumors or tumor antigens [1, 5, 7–9, 21, 29, 43, 44, 46] prior to use in Winn assays [1, 7, 8, 29, 46] or in the treatment of established neoplasms [7–9, 21, 43, 44]. However, the use of these cells has been limited because of the poor recovery of cells generally obtained following IVS.

Many workers have demonstrated the value of adoptive immunotherapy of immune lymphocytes or in vitro-sensitized cells in conjunction with chemotherapy for the treatment of a variety of mouse tumors [4, 7–9, 11–13, 45]. The mechanism of action of the chemotherapy has not been clearly identified, though several possibilities exist. Chemotherapeutic agents, such as cytoxan, may act by reducing tumor burden either by a direct cytotoxic effect on the tumor cell or possibly by making the tumor cell more susceptible to immunologic injury. Alternatively, some of the effect of cytoxan may be due to immunosuppression of the host that leads to prolonged survival of the adoptively transferred cells or by a decrease of the host's suppressor cells. In the FBL-3 tumor system we used in the present paper, Greenberg et al. [20] showed that the tumor regrows subclinically after the initial tumorcidal effect of the cytoxan and adoptively transferred cells, but before the total eradication of the tumor. Thus, while cytoxan appears to have an early cytoreductive effect, some immunologic events resulting from the adoptively transferred cells appear to result in total tumor eradication.

Because of the difficulty of obtaining in vivo-immunized cells in the human and the inefficient recovery of cells following in vitro sensitization, several workers have used soluble factors that enhance the development of cytotoxicity in vitro [10, 25, 28, 37]. Small and Trainin [37] showed that normal lymphocytes cultured with tumor and thymic humoral factor increased the cytotoxic potential of cells as evaluated by decreased growth of a syngeneic fibrosarcoma in an in vivo Winn assay. Similarly, Reyser et al. [28] described a supernatant product of a secondary allogeneic mixed lymphocyte culture that when added to primary mixed leukocyte tumor cell cultures increased the lytic activity against the syngeneic tumor target. These same authors showed the same effect when supernatants from Con-A-stimulated spleen cells were added to primary mixed leukocyte tumor cell cultures.

Since the description by Gillis and Smith [18] of long-term culture of tumor-specific cytotoxic T cells, much work has gone into attempts to grow and maintain cytotoxic T cells and clones with the aid of TCGF. We noted previously [41] a marked enhancement of the generation and expansion of cytotoxic cells attributable to LF-TCGF in allogeneic in vitro sensitization. Smith et al. [39] demonstrated that tumor-specific cytotoxic T lymphocytes cultured for prolonged periods in TCGF inhibited growth of a syngeneic tumor in a Winn assay.

More recently, others [10, 25] have treated established tumors with cells exposed to TCGF. Mills and Paetkau [25] used TCGF during in vitro sensitization of spleen cells from mice bearing the P815 syngeneic mastocytoma to generate cytotoxicity against the syngeneic tumor target cell. These cells were then shown to be effective in treating tumor in vivo. Cheever et al. [10] used TCGF to expand cells for 12 days after sequential in vivo immunization and re-exposure to FBL-3 tumor in vitro. They achieved specific cytotoxicity against the cultured tumor target in vitro and showed prolonged survival when these cells were used to treat mice with disseminated syngeneic tumor.

In the studies reported in this paper we have attempted to cure mice of disseminated syngeneic FBL-3 lymphoma by

using in vivo-immunized cells, in vitro-sensitized cells, whole lymphoid populations expanded in TCGF, and finally, cloned cytotoxic cells expanded in TCGF. Fresh spleen cells from mice immunized in vivo with irradiated FBL-3 tumor exhibited no in vitro cytotoxicity towards FBL-3 either before or after re-exposure to FBL-3 in vitro (data not shown). These same in vivo-immunized lymphocytes, when adoptively transferred to mice with disseminated lymphoma 5 h after a single cytoxan injection, cured 53% of the animals, and in each experiment (Fig. 1) showed significant benefit compared with treatment with cytoxan alone or treatment with cytoxan plus nonimmune cells. Similarly, when these in vivo-immunized lymphocytes were resensitized to FBL-3 tumor in vitro in either CM or LF-TCGF a similar survival benefit was conferred upon the mice thus treated (Fig. 2). IVS in CM cured 82% of the animals, and IVS in LF-TCGF cured 59%. Nonimmune lymphocytes resensitized to normal stimulators in LF-TCGF, when adoptively transferred after cytoxan therapy, cured only 15% of the animals ($P < 0.001$). There was no statistically significant difference in survival if IVS was performed in CM or LF-TCGF ($P = 0.88$). This increase in survival following IVS is similar to the findings of other workers [7, 8, 15].

We next took the cells from IVS and expanded them in LF-TCGF (Fig. 3 and Table 2). These cells exhibited specific cytotoxicity for fresh FBL-3 in an in vitro ^{51}Cr-release assay and conferred significant survival benefit when adoptively transferred to mice with disseminated FBL-3 lymphoma (Fig. 4), curing 7 of 27 animals. These results confirm those of Cheever et al. [10]. Lymphocytes from nonimmune mice co-cultured with FBL-3 or lymphocytes from immune mice sensitized to normal lymphocytes or normal lymphocytes resensitized to normal lymphocytes prior to expansion in LF-TCGF cured no animals and did not prolong survival over that obtained with cytoxan therapy alone (Fig. 4). Since there was a 7- to 10-fold expansion in the lymphoid population effective in this model, and 2×10^7 cells were adoptively transferred, it followed that no more than 2.9×10^6 cells from the original IVS cells could have been present at the time of adoptive transfer.

Table 1 shows that 5×10^6 IVS cells in either CM or LF-TCGF are capable of significantly prolonging survival in this model, but can cure only 29% of the animals; 10^6 adoptively transferred cells from IVS are incapable of prolonging survival beyond the effect of treatment with cytoxan alone. Thus while it appeared unlikely that IVS cells surviving during the expansion period were mediating this increase in survival, this was a possible explanation for our results and those of Cheever et al. [10], who saw a 3- to 8-fold expansion of cells in 12 days. We thus undertook to treat mice with cells expanded in LF-TCGF continuously for 2½ months with over 8,000-fold expansion in cell number (Fig. 5).

Figure 5 demonstrates that these cells were effective in vivo, curing 56% of the animals. Again, nonimmune lymphocytes co-cultured with normal stimulators and expanded in LF-TCGF for an equal length of time were no more effective than cytoxan alone. Also of importance, irradiation of these cells with 2,000 R prior to adoptive transfer totally abolished their effectiveness in vivo. Since both treatment groups received the same number of expanded lymphoid cells with presumably equal levels of contamination with residual tumor cells, these results indicate that in vivo eradication of tumor observed with these in vitro-sensitized cells expanded in LF-TCGF was not due to the presence of adoptively transferred tumor antigen. This experiment also indicates that a cell capable of in vivo proliferation is necessary to cause eradication. The expanded cytotoxic cells used in these experiments were totally dependent on the continued presence of TCGF. We have been able to maintain these cells for this entire 2½ month period without the 'crisis' period described by others [10, 47]. Cell growth and maintenance of cytotoxicity was not dependent on the continued presence of tumor cells (antigen).

Using TCGF, we then cloned IVS cells and expanded them to large numbers. The screening technique described in detail elsewhere (T. J. Eberlein et al. 1982) enabled us to select rare, highly cytotoxic cells for further study. Only about 5% of immune lymphoid clones are more highly lytic for FBL-3 than clones from nonimmunized cultures (T. J. Eberlein et al. 1982). Suitable clones were selected and grown in LF-TCGF with maintenance of specific anti-tumor cytotoxicity for 2½ months (Table 3). Based on the probabilities of cell distribution according to the Poisson distribution, the clones we described have an 80% chance of having arisen from a single cell and we are currently in the process of recloning these cells.

Although survival was prolonged compared with that in mice treated with cytoxan alone ($P < 0.0002$), when these clones were adoptively transferred in this chemoimmunotherapy model there were few long-term 'cures' (Fig. 6) and one might speculate that these cytotoxic clones only reduced the intraperitoneal tumor burden.

The use of T cell clones offers potential advantages over treatment with bulk cultures. The cloning of these reactive cells may result in the removal of suppressor or modulator cells that might abrogate the effect of these T cells in vivo. The apparent decreased effect of these clones may be due to a variety of factors. The final expansion of these cells was conducted in LF-TCGF-EL-4 and use of this factor, as against our conventional LF-TCGF, may lead to decreased effectiveness in vivo. However, in several experiments we could find no differences in growth, survival, or cytotoxicity in vitro between cells grown in LF-TCGF and those grown in LF-TCGF-EL-4. Alternatively, it is possible that individual clones of lymphoid cells traffic in vivo in unusual patterns. Lotze et al. [22] have demonstrated an unusual distribution of injected cells in mice grown in TCGF.

A potential problem exists in selecting cells for use in adoptive immunotherapy based on in vitro reactivity in ^{51}Cr-release cytotoxicity assays. The in vivo effect may not correlate with the results of the in vitro assay we have used. The primary effector cell in vivo may not be the cytotoxic cell measured in our assay, but may instead be a helper cell. This hypothesis is supported by the work of Fernandez-Cruz et al. [16], who showed that the subset of T cells most effective in eradicating tumor in vivo was noncytotoxic in an in vitro ^{51}Cr-release assay. Loveland et al. [23] showed that skin allograft rejection in vivo was entirely dependent on the presence of Lyt-1+ cells.

In this paper we have described methods that are effective in generating and maintaining cytotoxic effector cells that can be used for the successful in vivo treatment of mice bearing a disseminated syngeneic lymphoma. Further study is under way with subcloned populations of cytotoxic and inducer clones that may be useful in elucidating important tumor-effector cell interactions in vivo.

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Received August 24/Accepted December 12, 1981