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Characterization of effector cells of graft vs leukemia following allogeneic bone marrow transplantation in mice inoculated with murine B-cell leukemia

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Summary. It is now widely accepted that immunocompetent lymphocytes in allogeneic bone marrow grafts exert an antileukemic effect that contributes to the cure of leukemia. Graft vs leukemia (GVL) effects independent of graft vs host disease were investigated in allogeneic bone marrow chimeras tolerant of host and donor alloantigens. The role of Thy1.2, L3T4 and Lyt2 T lymphocytes as effector cells of GVL were investigated in (BALB/c × C57BL/6)F1 mice inoculated with murine B-cell leukemia and subsequently conditioned with total lymphoid irradiation and cyclophosphamide (200 mg/kg). Mice were reconstituted with C57BL/6 bone marrow cells depleted of well-defined T-cell subsets or enriched for stem cells by the soybean agglutination method. Detection of residual tumor cells, an indicator for efficacy of GVL, was carried out by adoptive transfer of peripheral blood or spleen cells obtained from treated chimeras into secondary naive BALB/c recipients at different time intervals following bone marrow transplantation. Treatment of the primary marrow inoculum with monoclonal anti-Thy1.2 or anti-Lyt2 abolished the GVL effects and all secondary BALB/c recipients developed leukemia within 60 days. On the other hand, the treatment with monoclonal anti-L3T4 did not influence the effect of GVL and all treated recipients remained without leukemia. The data suggest that T cells may mediate GVL effects in the absence of graft vs host disease and in circumstances where tolerance to conventional alloantigens is elicited. Effector cells of GVL across the major histocompatibility complex (MHC) in the murine B-cell leukemia tumor model system appear to be Thy1.2$^+$ Lyt2$^+$ L3T4$^-$. Induction of GVL effects by allogeneic cells tolerant of host MHC suggests that these effects may be independent of graft vs host disease.

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Introduction

It has been known for some time that leukemic patients receiving transplants from an identical twin have higher relapse rates compared to recipients receiving marrow from HLA-matched siblings. These data argue that the graft-dependent anti-leukemic effect occurs where the only known histocompatibility differences are in the minor histocompatibility loci. Although the incidence of clinically overt graft vs host disease (GVHD) correlates with this immunotherapeutic phenomenon of graft versus leukemia (GVL) [1, 2], the relationship of GVHD to GVL remains uncertain at the level both of effector cells and of target antigens. Similarly, relapse rates in the latter form of transplant correlate inversely with the incidence of graft vs host disease.

During the past few years we have studied the GVL effect in mice by using the B cell leukemia (BCL1) tumor model originally described by Slavin and Strober [12]. The BCL1 tumor arose spontaneously in an elderly female BALB/c mouse and serves as an experimental model for lymphocytic leukemia/lymphoma in man. The BCL1 disease is characterized by marked splenomegaly (5×10^9–10×10^9 cells/spleen) accompanied by extreme peripheral blood lymphocytosis (up to 500×10^6 cells/ml) followed by the death of all the tumor-bearing mice [14]. Our previous studies have demonstrated that about 75% of the mice

transplanted with BCL1 could be rescued by a combination of total lymphoid irradiation, cyclophosphamide and allogeneic bone marrow transplants. Tolerance of host vs graft and graft vs host was induced by total lymphoid irradiation conditioning, as previously described [8, 9, 15, 21]. Some of the "cured" chimeras (>18 months with no evidence of disease) bear dormant tumor cells which, presumably, are actively suppressed by an active immune mechanism, since a typical leukemia developed when spleen cells of treated mice were adoptively transferred to secondary BALB/c recipients. This mechanism may be responsible in part for the anti-leukemic effects observed in mice tolerant to donor alloantigens despite the lack of any clinical signs of GVHD [15, 21]. In the present study we have investigated the phenotype of cells responsible for the GVL effects in treated chimeras to develop mutual tolerance to donor and host histocompatibility antigens [8, 9]. BCL1-bearing mice were treated with a combination of total lymphoid irradiation and cyclophosphamide, as previously described [15], and grafted with allogeneic bone marrow transplants treated by different methods to remove various T-cell subsets using monoclonal antibodies and/or soybean agglutinin. Peripheral blood and spleen cells obtained from treated chimeras, reconstituted with unmodified or T-cell-depleted allogeneic bone marrow cells, were investigated at different time intervals following bone marrow transplantation for dormant residual BCL1 cells by adoptive transfer into naive BALB/c recipients.

Materials and methods

Animals. Inbred, 2–4-month-old male and female BALB/c (BALB), C57BL/6 (C57) and C_3H/He (C_3H) mice were used as bone marrow donors, and (BALB × C57)F1 or (BALB × C_3H)F1 recipients were used for the preparation of chimeras. Untreated BALB mice were used as secondary recipients in the adoptive-transfer experiments. All mice were bred at the Hebrew University-Hadassah Medical School breeding facility and kept under standard, unprotected animal-house conditions.

BCL1. BCL1 cells were maintained in vivo in BALB mice by i.v. passage of 10^6-10^7 peripheral blood lymphocytes (PBL) obtained from tumor-bearing mice. All recipients of BCL1 showed splenomegaly and marked lymphocytosis in the blood at the time they were used as donors for inoculation of BCL1 into experimental mice. PBL counts of all experimental groups were carried out weekly. Mice were considered leukemic when PBL counts exceeded 2×10^7/ml. Survival of BCL1 recipients was carefully monitored daily.

Treatment of BCL1 by cytoreduction and establishment of stable bone marrow chimeras. Adult F1 hybrid mice were inoculated with 10^7 BCL1 cells. Two weeks later the mice were treated with eight fractions of total lymphoid irradiation (200 rad/day; a total dose of 1600 rad) followed by i.v. administration of cyclophosphamide (200 mg/kg). One day later, 3×10^7 nucleated bone marrow cells obtained from normal C_3H or C57 donors were injected i.v. into the leukemic (BALB × C_3H)F1 or (BALB × C57)F1 mice, respectively. Chimerism was documented by typing PBL and spleen cells.

Depletion of T lymphocytes from bone marrow inocula. Donor bone marrow cells were adjusted to 10×10^6/ml and incubated with monoclonal anti-Thy1.2 antibody (Cedarlane Lab., Hornby, Ontario) at a ratio of 1:300 in RPMI-1640 medium (Gibco, Grand Island, NY, 1640 medium) with low-tox rabbit complement (1:10). The cell mixture was incubated for 45 min in a 37° C bath, centrifuged and resuspended in medium for injection. The lytic efficacy of the antibody was monitored by testing the residual T cells with fluorescein-isothiocyanate-conjugated monoclonal anti-Thy1.2 antibody (Bio-Yeda, Rehovot, Israel). More than 95% T cells were consistently killed in the bone marrow inocula.

Treatment of donor bone marrow cells with monoclonal anti-L3T4 and Lyt2. Bone marrow cells obtained from C57 donors were resuspended in medium at 50×10^6/ml in 50 µg/ml rat anti-(mouse-L3T4) or anti-(mouseLyt2) (both IgG2b, shown to be extremely potent for subset depletion, most likely through antibody-dependent cell-mediated cytotoxicity in vivo) [2] for 10 min on ice. Cells were resuspended at 120×10^6/ml and 0.25-ml (30×10^6 cells) inocula were injected intravenously into F1 recipients.

Depletion of T lymphocytes and enrichment of stem cells in the marrow using soybean agglutinin. Fractionation of bone marrow cells by soybean agglutinin (a gift from Dr Y Reisner, Weitzmann Institute, Rehovot, Israel) was carried out according to the method of Reisner et al. [11]. Bone marrow cells were resuspended at 2×10^8 cells in 0.5 ml phosphate-buffered saline (PBS) with 1% bovine serum albumin (containing $CaCl_2$ $6H_2O$ and $MgCl_2$ $6H_2O$ at 0.1 g/l each). Soybean agglutinin (0.5 ml, 2 mg/ml in PBS) was added for 5 min at room temperature. Agglutinating cells were gently layered with a pasteur pipette on top of a solution of 40 ml bovine serum albumin (5% wt/vol in PBS, Ca^{a+}, Mg^{a+}) in 50-ml tubes at room temperature. Within 15 min most of the unagglutinated cells had risen to the top of the bovine serum albumin solution. The bottom and top fractions were collected and resuspended in D-galactose (0.2 M) and washed once in D-galactose and twice in PBS containing 1% bovine serum albumin. The agglutinated fraction (SBA^+) is highly enriched for stem cells and depleted of T cells (more than 2 log units depletion by limiting-dilution analysis; less than 0.5% by fluorescence-activated cell sorting), whereas the unagglutinated fraction (SBA^-) is comprised mainly of T lymphocytes [11] (personal communication).

Assay for chimerism. Chimerism was documented by assaying spleen cells (or PBL) using an in vitro complement-dependent microcytoxicity assay with specific alloantisera and rabbit complement, and the percentage of host or donor-type cells was determined as previously described [13]. Specific alloantisera (BALB anti-C57 and C57 anti-BALB) were prepared by cross-immunizations with full-thickness skin allografts followed by six intraperitoneal injections of $30\times10^6-50\times10^6$ donor-type spleen cells given 1–2 weeks apart. Mice were bled and sera were stored at –70° C until used. Chimerism was tested by typing lymphocytes with both antisera. Lymphocytes obtained from F1 recipients showed 100% cytotoxicity with both BALB anti-C57 and C57 anti-BALB antisera, whereas lymphocytes obtained from chimeras were lysed by BALB anti-C57 and not C57 anti-BALB. The percentage chimerism was therefore calculated by the formula: 100–percentage cells lysed following treatment with C57 anti-BALB cells.

Detection of residual BCL1 cells in treated mice by adoptive transfer experiments. In order to detect the presence of the few residual BCL1 cells in the spleens of treated mice, 10^5-10^7 spleen cells obtained from each animal were adoptively transferred into secondary untreated BALB recipients. The development of leukemia was monitored by weekly PBL counts. It has previously been documented that 10 (and most likely even fewer) BCL1 cells may cause leukemia in the majority of recipients [14].

Investigation of the role of $Thy1.2^+$ cells in maintaining anti-BCL1 effects in long-term disease-free chimeras. Spleen cells obtained from long-term disease-free chimeras (>4 months) inoculated with BCL1 and treated by total lymphoid irradiation, cyclophosphamide and allogeneic bone marrow transplantation were depleted of anti-$Thy1.2^+$ cells. Intact and Thy1.2-depleted spleen cells (30×10^6 cells/mouse) were injected i.v. into normal BALB recipients to test whether the continuous presence of T cells is essential for prevention of leukemia by potentially dormant BCL1 cells.

Statistical evaluation. Statistical evaluation was carried out using Fisher's exact test.

Results

Establishment of allogeneic bone marrow chimeras in mice conditioned with total lymphoid irradiation and cyclophosphamide

Peripheral blood cells isolated from mice receiving normal C_3H bone marrow cells or the SBA+ fraction isolated from C_3H bone marrow cells showed equally high proportions of chimerism (65%–85% and 63%–91%, respectively), although the degree of chimerism following T-lymphocyte depletion varied from experiment to experiment (Table 1).

A group of (BALB × C57)F1 mice, treated with 1600 rad total lymphoid irradiation and 200 mg/kg i.v. cyclophosphamide received normal C57 bone marrow. Out of 25 mice, 24 were long-term chimeras with a high percentage of chimerism in their blood (Table 1). In a similar group of mice receiving C57 bone marrow cells depleted of T lymphocytes by monoclonal anti-Thy1.2 antibody and rabbit complement, only 15 out of 26 mice were durable chimeras with lower percentages of donor-type cells (30%–75%) (Table 1). Similarly, of the 16 (BALB × C57)F1 mice transplanted by C57 SBA+ bone marrow cells, only 8 were stable chimeras with a low percentage of chimerism (23%–60%) (Table 1). The data suggest that removal of mature T lymphocytes in the course of bone marrow transplantation may reduce the success rate of induction of chimerism.

Evidence for chimerism in BCL1-inoculated (BALB × C57)F1 recipients of fractionated C57 bone marrow allografts following conditioning with total lymphoid irradiation and cyclophosphamide

BCL1-bearing (BALB × C57)F1 mice were treated by total lymphoid irradiation (1600 rad) and cyclophosphamide (200 mg/kg) and reconstituted with normal C57 bone marrow cells, or C57 bone marrow cells depleted of anti-Thy1.2+, anti-L3T4+ or anti-Lyt2+ cells. Samples of spleen cells obtained from the different experimental groups used for adoptive transfer experiments were assayed for chimerism on the day of adoptive transfer. Chimerism of mice reconstituted with intact or T-subset-depleted C57 bone marrow cells was suggested by documenting 75%–100% donor-type (C57) cells (Table 2).

Table 1. Stable chimerism in the blood of recipients conditioned with total lymphoid irradiation (1600 rad) and cyclophosphamide (200 mg/kg) and given untreated or T-lymphocyte-depleted allogeneic bone marrow cells[a]

Bone marrow cells[b]	Recipients	Fraction chimeras	Chimerism (%) (range)
Untreated C57	(BALB × C57)F1	24/25	79–100
C57 treated by anti-Thy1.2+C	(BALB × C57)F1	15/26	30– 75
C57, SBA+ fraction	(BALB × C57)F1	8/16	23– 60
Untreated C_3H	(BALB × C_3H)F1	2/ 2	65– 85
C_3H, SBA+ fraction		8/ 8	63– 91

[a] Chimerism was tested 30 days following transplantation and confirmed at 4–6 months following transplantation
[b] C, complement; SBA, soybean agglutinin

Table 2. Evidence for chimerism in the spleen of all BCL1-inoculated (BALB × C57)F1 mice conditioned with total lymphoid irradiation (1600 rad) and cyclophosphamide (200 mg/kg) and given various C57 bone marrow preparations on the day of adoptive transfer to secondary BALB recipients

Bone marrow cells	Chimerism (%) (range)[a]
Untreated BALB	95–100
Untreated C57	95–100
C57 treated by anti-Lyt2	90– 95
C57 treated by anti-L3T4	95–100
C57 treated by anti-Thy1.2+C	75– 96

[a] See Materials and methods for method used for detection of chimerism

The role of T lymphocytes in GVL across major histocompatibility barriers ($H\text{-}2^b \rightarrow H\text{-}2^{b/d}$ and $H\text{-}2^k \rightarrow H\text{-}2^{k/d}$)

F1 mice inoculated with 10^7 BCL1 cells were conditioned 2 weeks later with total lymphoid irradiation, cytoreduced with cyclophosphamide, and received 30×10^6 untreated or T-cell-depleted bone marrow cells into the lateral tail vein. T lymphocytes were depleted using either of two methods: (a) lymphocytoxicity with monoclonal anti-Thy1.2 antibody and rabbit complement; (b) soybean agglutinin agglutination and infusion of SBA+ cells containing T-cell-depleted stem cells [11].

Of the recipients of T-cell depleted bone marrow allografts, 15 developed leukemia within 30–44 days and died, in contrast to recipients of non-T-cell-depleted allografts ($P < 0.01$).

In order to evaluate the proportion of residual BCL1 cells in the spleens of treated bone marrow transplant recipients, blood and spleen cells (10^6, occasionally 10^5 or 10^7) from each C57→(BALB × C57)F1 chimera were transferred to 5–8 secondary naive BALB mice at 1 week, 2 weeks and 6–7 weeks after bone marrow transplantation. Representative results of one out of two adoptive-transfer experiments, in which 10^6 spleen cells obtained from chimeras reconstituted with unmodified, Thy1.2-depleted or the SBA+ fraction of C57 bone marrow cells were transferred to secondary BALB mice 1 week following bone marrow transplantation, are shown in Fig. 1. All secondary recipients of spleen cells obtained from chimeras reconstituted with T-depleted bone marrow cells, either by anti-Thy1.2 and complement or by soybean agglutination, developed leukemia and died within 60 days following cell transfer. Only 40% of secondary recipients of spleen cells obtained from recipients of normal bone marrow allografts developed leukemia within 40 days, whereas 60% showed no evidence of disease (>160 days) (Fig. 1).

The GVL effects in recipients of normal bone marrow cells were even more impressive 6–7 weeks following

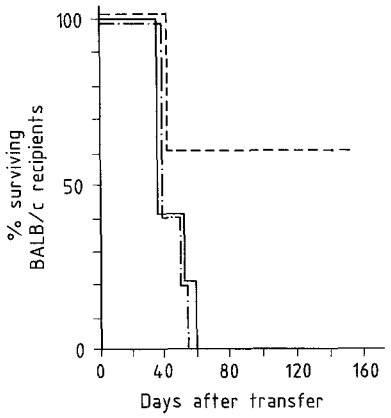

Fig. 1. Residual BCL1 in treated C57BL/6→ (BALB/c × C57BL/6)F1 (C57→F1) chimeras 1 week following transplantation by adoptive transfer of 10^6 spleen cells to secondary BALB/c recipients. Spleen cells were obtained from F1 recipients reconstituted with: – – –, normal C57 bone marrow cells; ——, SBA^+ fraction of C57 bone marrow cells; – – –, Thy1.2-depleted C57 bone marrow cells

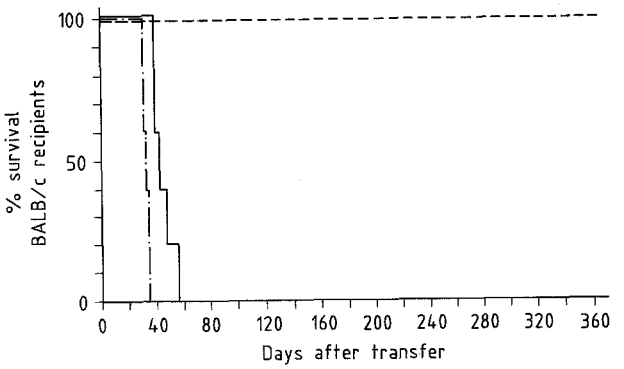

Fig. 3. Residual BCL1 in treated C57BL/6→ (BALB/c × C57BL/6)F1 (C57→F1) chimeras 6 weeks following transplantation by adoptive transfer of 10^7 spleen cells to secondary BALB/c recipients. Spleen cells were obtained from F1 recipients reconstituted with: – – –, normal C57 bone marrow cells; ——, SBA^+ fraction of C57 bone marrow cells; – – –, Thy1.2-depleted C57 bone marrow cells

transplantation; spleen cells obtained from T-cell-depleted C57→F1 chimeras (both $Thy1.2^-$ and SBA^+) contained tumorogenic BCL1 cells, since leukemia developed in all secondary recipients within 60 days. In contrast, C57→F1 chimeras reconstituted with normal bone marrow remained disease-free for an observation period >300 days ($P<0.01$) (Fig. 2).

In order to test whether BCL1 was indeed eliminated in recipients of normal bone marrow, we have extended the sensitivity of the adoptive transfer system by increasing the number of cells transferred to 10^7/recipient. As shown in Fig. 3, no leukemia developed in any of the secondary recipients of spleen cells obtained from recipients of normal bone marrow cells, whereas all recipients of spleen cells obtained from chimeras reconstituted with T-cell-depleted bone marrow allografts developed leukemia and died (<60 days). It appears that depletion of T lymphocytes by either soybean agglutinin or monoclonal anti-Thy1.2 antibodies and complement leads to an identical loss of the GVL effect (P <0.01).

Significant GVL effects in mice reconstituted with normal bone marrow cells as compared with marrow depleted of immunocompetent T lymphocytes by soybean agglutination was also confirmed in C_3H→(C_3H × BALB)F1 chimeras by transferring either spleen cells (Fig. 4) or peripheral blood cells (Fig. 5) obtained from the respective experimental chimeras into naive BALB recipients 6–7 weeks following bone marrow transplantation.

The adoptive transfer of Thy-1.2-depleted T cells obtained from the spleen of four long-term chimeras (pooled) reconstituted with non-depleted bone marrow obtained from C57 mice showed no evidence of leukemia in 6 secondary recipients.

The effects of selective depletion of L3T4 or Lyt2 from the bone marrow prior to transplantation on the GVL effect

Tumor-bearing (BALB × C57)F1 mice (10^7 BCL1 cells/mouse) were treated with total lymphoid irradiation and cyclophosphamide, as previously described, followed

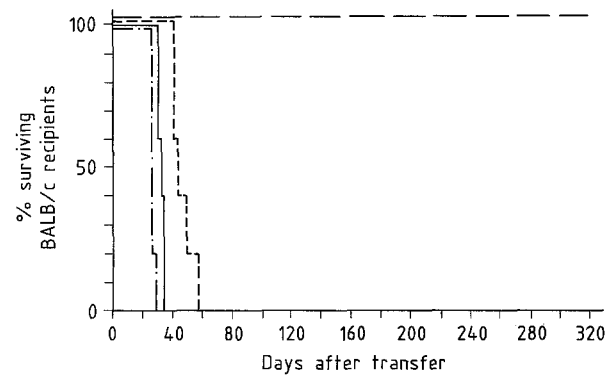

Fig. 2. Residual BCL1 in treated C57BL/6→ (BALB/c × C57Bl/6)F1 (C57→F1) chimeras 6 weeks following transplantation by adoptive transfer of 10^6 spleen cells to secondary BALB/c recipients. Spleen cells were obtained from F1 recipients reconstituted with: — —, normal C57 bone marrow cells; – – –, SBA^+ fraction of C57 bone marrow cells; ——, Thy1.2-depleted C57 bone marrow cells; – – –, normal BALB bone marrow cells

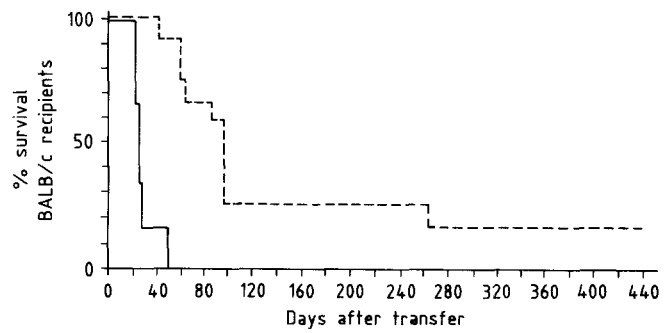

Fig. 4. Residual BCL1 in treated C_3H/He→ (BALB/c × C_3H/He) F1 chimeras 7 weeks following transplantation by adoptive transfer of 10^5 spleen cells to secondary BALB/c recipients. Spleen cells were obtained from (BALB/c × C_3H/He)F1 recipients reconstituted with: – – –, normal C_3H bone marrow cells; ——, SBA^+ fraction of C_3H bone marrow cells

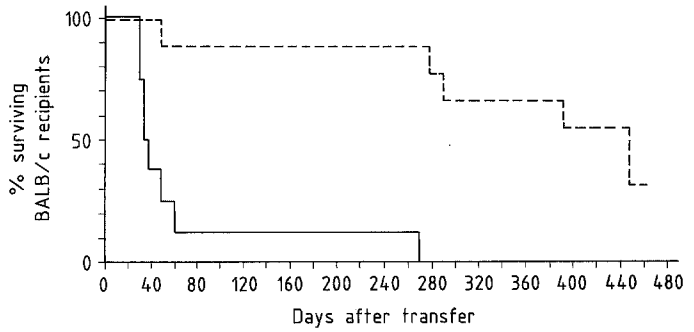

Fig. 5. Residual BCL1 in treated $C_3H/He \rightarrow$ (BALB/c × C_3H/He)F1 chimeras 6 weeks following transplantation by adoptice transfer of 10^5 peripheral blood cells into secondary BALB/c recipients. Peripheral blood cells were obtained from (BALB/c × C_3H/He)F1 recipients reconstituted with: – – –, normal C_3H/He bone marrow cells; ———, SBA^+ fraction of C_3H/He bone marrow cells

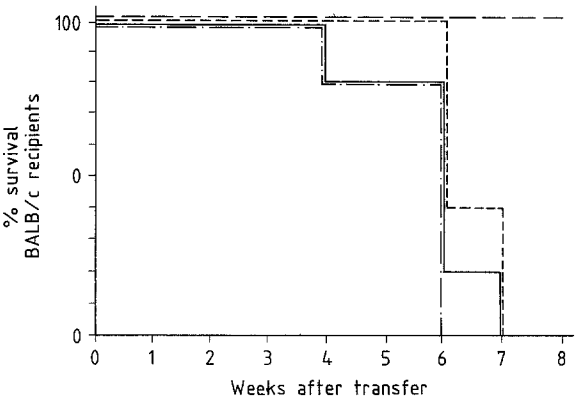

Fig. 6. Residual BCL1 in treated C57BL/6→ (BALB/c × C57BL/6)F1 (C57→ F1) chimeras 3 weeks following transplantation by adoptive transfer of 10^6 spleen cells into secondary BALB/c recipients. Spleen cells were obtained from F1 recipients reconstituted with: – – –, normal BALB/c bone marrow cells; ———, Thy1.2-depleted C57 bone marrow cells; – – –, Lyt-2-depleted C57 bone marrow cells; — —, L3T4-depleted C57 bone marrow cells

by infusion of C57 bone marrow cells treated by different monoclonal antibodies against helper (L3T4) T cells, cytotoxic/suppressor (Lyt2) cells and all Thy1.2 T lymphocytes.

Monitoring of residual BCL1 cells in the spleens of treated chimeras was assayed by adoptive transfer into secondary BALB recipients, as described in the previous paragraphs. As shown in Table 3, all secondary recipients of spleen cells obtained from chimeras reconstituted with Lyt2-depleted bone marrow cells at 2, 3 and 4 weeks following transplantation developed leukemia within 25–31 days and died, as did recipients of spleen cells obtained from mice reconstituted by syngeneic bone marrow cells. In contrast, none of the adoptive recipients of spleen cells obtained from chimeras reconstituted with L3T4-depleted bone marrow developed leukemia for an observation period of >300 days (P <0.01, Table 3). The survival curves of a typical adoptive transfer experiment carried out 3 weeks following transplantation are shown in Fig. 6. Some chimeras (4 or 5 in each group) that served as the source of spleen cells for the adoptive transfer experiments underwent hemisplenectomy and therefore were not sacrificed. Development of leukemia in these chimeras was carefully monitored by weekly peripheral blood counts. As shown in Table 4, all recipients of Lyt2, as well as most recipients of Thy1.2-depleted bone marrow cells, developed leukemia as readily as recipients of syngeneic bone marrow cells, whereas all recipients of L3T4-depleted bone marrow showed no evidence of disease >300 days following transplantation (P <0.01).

The results of adoptive transfer experiments were reproduced using another group of mice (data not shown) confirming anti-tumor effects of allogeneic spleen cells: no leukemia developed in a total of 8 F1 recipients grafted with anti-L3T4-treated C57 bone marrow cells, and none of 12 secondary recipients of 10^5 of their spleen cells developed leukemia (>12 months). In contrast, 7 out of 7 recipients of Lyt-2-treated C57 bone marrow cells died of leukemia.

Discussion

It has been suggested by several independent clinical studies that GVHD associated with allogeneic marrow grafting

Table 3. Development of leukemia in secondary BALB recipients of spleen cells obtained from treated, leukemia-inoculated C57 → (BALB × C57)F1 chimeras reconstituted with Lyt2- or L3T4-depleted marrow allografts

Time interval from BMT[a] to adoptive transfer (weeks)	Fraction of secondary mice with leukemia after bone marrow grafts comprising		
	BALB whole marrow	C57 bone marrow	
		Lyt2-depleted	L3T4-depleted
2	5/5 (31)[b]	5/5 (31)	0/5 (>300) P <0.01[c]
3	5/5 (19)	5/5 (25)	0/5 (>300) P <0.01
4	5/5 (20)	5/5 (30)	0/5 (>300) P <0.01

[a] BMT, bone marrow transplant
[b] Median day of onset of leukemia
[c] Statistical significance of the difference between bone marrow cells treated by anti-Lyt2 and those treated with anti-L3T4

Table 4. Leukemia relapse in treated (BALB × C57)F1 mice previously inoculated with 10^7 BCL1 cells, following conditioning with total lymphoid irradiation and cyclophosphamide, and given different fractions of C57 bone marrow (BM) cells

Exp.	Bone marrow cells used for BMT	Total no. of mice[a]	No. of mice with leukemia[b]	Statistical evaluation
1	BALB, untreated BM	5	5	
2	C57, untreated BM	4	1	
3	C57, Lyt2-treated BM	4	4	3 vs 4 $P<0.01$
4	C57, L3T4-treated BM	5	0	4 vs 2 NS[c]
5	C57, Thy1.2-treated BM	4	3	

[a] Mice underwent hemisplenectomy for adoptive transfer experiments. Splenectomized mice surviving >30 days were included in the present analysis of the role of various T-lymphocyte subsets in the bone marrow inoculum on graft vs leukemia effects

[b] Leukemia was determined in the peripheral blood (lymphocytes >20000/mm^3)

[c] NS, not significant

may be associated with a significant reduction in the incidence of relapse of leukemia, as compared with syngeneic, autologous or allogeneic bone marrow transplant procedures not associated with GVHD [3, 20]. These studies have raised the possibility that GVL effects may be mediated by alloimmune effector mechanisms of donor origin, recognizing host residual tumor cells of host origin as target cells, similar to the mechanism of GVHD. Nevertheless, the effector and target cells of GVL have never been clearly defined in human or animal experimentation. Moreover, several experimental studies have clearly demonstrated that potent GVL effects may operate in the absence of clinically overt GVHD [1, 7, 10, 15, 17, 18]. We have previously shown that BCL1-inoculated (BALB × C57)F1 mice cytoreduced with 200 mg/kg cyclophosphamide immediately following total lymphoid irradiation, given for induction of transplantation tolerance to C57 BM allografts, could be "cured" of leukemia, although recipients of BALB marrow grafts all showed relapse of leukemia and died. Semiallogeneic C57 → (BALB × C57)F1 chimeras showed no clinical evidence of GVHD and spleen cells obtained from stable chimeras, all being of C57 origin, were totally unresponsive against BALB alloantigens [8, 9], indicating that GVL could be evoked by allogeneic cells that were to become tolerant of the histocompatibility antigens of the tumor-bearing host [15]. Our data suggest that in the experimental system used, GVL was independent of GVHD. In the present study, we have further extended our original observations, attempting to investigate the phenotype of effector cells involved in GVL across the major histocompatibility complex (MHC).

In the first set of experiments, the role of T lymphocytes present in the marrow inoculum was investigated by comparing residual BCL1 leukemia cells in mice inoculated with BCL1 cells prior to cytoreduction with cyclophosphamide, total lymphoic irradiation and transplantation of T-lymphocyte-depleted marrow allografts. T-cell depletion was accomplished by either monoclonal anti-Thy1.2 antibodies or by infusing a soybean-agglutinin-positive fraction that is enriched for stem cells depleted of lymphocytes [11]. All experiments indicated that immunocompetent donor T lymphocytes play a major role in GVL. In subsequent experiments, bone marrow inocula were further treated by monoclonal antibodies against cytotoxic/suppressor cells (Lyt2) and helper cells (L3T4). The anti-tumor effects following each of the bone marrow transplant procedures were assayed by adoptive transfer of spleen cells obtained from treated mice at different time intervals following transplantation into naive BALB recipients. We have previously demonstrated that the in vivo adoptive transfer system is the most sensitive one for detecting residual tumor cells [14, 15, 21]. Lack of development of leukemia in treated mice may not necessarily indicate lack of BCL1 cells, because there may be in vivo mechanisms that block leukemogenesis of dormant BCL1 cells [15, 21]. In contrast, adoptive transfer of even a few residual BCL1 cells into secondary naive recipients is the most reliable indicator for the presence of BCL1 cells [21]. Our studies indicate that most of the GVL effect is mediated by Lyt2-positive cells and not by L3T4-positive cells. Since immunocompetent T lymphocytes capable of exerting alloimmune reactions are present in the initial inoculum of normal bone marrow, it is conceivable that initiation of the GVL effects may occur within the first few days following bone marrow transplantation by alloantigen-responsive T cells as a result of events occurring during the time that tolerance is developing. Interestingly, removal of Thy1.2-positive cells from spleen cells obtained from stable chimeras reconstituted with non-depleted C57 bone marrow cells without leukemia did not result in leukemia following adoptive transfer into secondary BALB recipients, suggesting that the GVL in long-term chimeras is not maintained by tolerant Thy1.2-positive cells. The nature of the effector cells of GVL has also been studied in other tumor model systems in mice. Bortin et al., working with an AKR leukemia model in mice, have shown that marked anti-leukemia effects could be generated without augmentation GVHD following immunization of MHC-compatible donor mice against alloantigens in vivo [1]. In addition, it was previously shown by the same group that leukemic AKR mice, decontaminated actively in a protective environment, exhibited no GVHD to MHC-incompatible allografts containing bone marrow and lymph node cell mixtures but demonstrated a lower incidence of leukemia relapse as compared with AKR mice reconstituted with syngeneic or MHC-compatible marrow donors [18]. They could show that T lymphocytes do play an essential role in the generation of GVL effects in vivo [17]. Our GVL-mediating cell subset was shown to be Thy1.2$^+$, Lyt1$^+$ Lyt2$^+$, in agreement with the previously published results [17]. Truitt et al. have suggested that effector cells of GVL may be heterogeneous and belong to either one of the following groups: (a) MHC-restricted

against determinants outside the H-2 complex; (b) MHC-nonrestricted, class-I-specific Thy1+, Lyt1−, Lyt2+ cytotoxic T lymphocytes; and (c) neoplastic cell-"specific", or rather -"selective", cytotoxic effector cells [19].

Meredith and O'Kunewick documented GVL effects in SJL/J mice receiving B10.S bone marrow allografts without inducing overt GVHD [7]. Effector cells of GVL in their tumor model systems were also shown to be Lyt1-positive T lymphocytes, most of them being L3T4-positive helper cells and hence most likely Lyt2-negative T cells [10].

It appears that all investigators, as well as our own studies, are in agreement that T lymphocyte depletion of donor marrow has a negative effect on GVL competence. More specifically, the effector cells of GVL may vary in different experimental systems and in relationship with the nature of the histoincompatibility between donors and hosts. The crucial role of Lyt2 cells across major histocompatibility barriers is suggested by our own data, whereas Truitt et al. have indicated the role of cytotoxic T lymphocytes specific for a class-I-like MHC molecule, Qa-1^b, across minor histocompatibility barriers B10.BR ($H\text{-}2^K$) → AKR [5]. In this regard it is hard to interpret O'Kunewick's data as indicating a definite role for helper T lymphocytes, since most if not all T lymphocytes are Lyt1+, most of them being L3T4 cells. It should be kept in mind that different cell subsets may play different roles in different tumor model systems in mice and therefore that data on mechanisms of GVL should not be overinterpreted in drawing conclusions for man.

Since marked, even curative, GVL effects may be mediated without overt GVHD, the possibility of unrestricted cytotoxic or inhibitory T cells cannot be excluded. Indeed, natural killer and lymphokine-activated killer lymphocytes ("LAK" cells) potent against leukemia cells have been documented in mice [16] and man [4, 6]. One hypothesis that is currently being investigated in our laboratory is that interleukin-2, produced in the course of clinical or subclinical GVHD, activates natural killer cells, resulting in lymphokine-activated killer cells mediating GVL in vivo. Understanding the effector cells of GVL in comparison with effector cells of GVHD seems an important issue in designing future adoptive imunotherapy in conjunction with allogeneic bone marrow transplantation. The feasibility of activating GVL effector cells that do not recognize alloantigens in normal tissues might provide a clue for more innovative immunotherapy for cancer treatment.

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