ABSTRACT

Relapse of leukemia remains a common event after allogeneic bone marrow transplantation, despite potential donor antithost alloreactivity present in most transplants. This work examined posttransplant relapse of the DBA/2 P815 mastocytoma in a murine model of MHC-matched, minor histocompatibility antigen (mHAg)-mismatched bone marrow transplantation (BALB/c donors into DBA/2 recipients). Antithost alloreactivity was associated with reduction of posttransplant tumor burden and prolongation of survival, but posttransplant relapse commonly occurred. No evidence of acquired resistance to immune control was found in 12 relapse reisolates. Relapse tumors remained sensitive to donor antithost CTLs in vitro, suggesting continued expression of mHAggs. Reisolates also continued to express Fas. However, loss of posttransplant alloreactivity was observed at 3 weeks. This was temporally associated with the time of relapse. Antithost alloreactivity could be reactivated in stable graft-versus-host disease-free recipients by immunization with host cells. The results of this study suggest that one mechanism for relapse after bone marrow transplantation is acquired tolerance of allogeneic minor histocompatibility antigens and that posttransplant immunotherapy directed against mHAggs may induce antitumor activity.

INTRODUCTION

The rate of leukemia relapse is lower in BMT1 patients who experience acute and/or chronic GVHD and has provided evidence for an allogeneic GVL effect (1–3). However, relapse of leukemia remains a common event after allogeneic bone marrow transplantation despite potential donor antithost alloreactivity present in most transplants (4, 5). In most cases studied, tumors express mHAggs (6–12). There is a great deal of interest in developing methods that will enhance the allogeneic GVL effect associated with allogeneic BMT (13). Central to this effort is identification of the mechanism of the effect and, just as importantly, understanding the mechanism of its failure. Each could contribute to development of novel therapeutic approaches to improve the immunological control of leukemia after transplant.

Murine models can provide some insight into the immunobiology of human transplantation, given the remarkable homology between human and murine immune systems. In this work, an allogeneic, MHC-matched, mHAg-mismatched, murine BMT model (BALB/c into DBA/2) was used to study the mechanism of relapse after BMT. P815 is a poorly immunogenic DBA/2 mastocytoma (a hematological malignancy) that grows progressively in syngeneic mice, even after prior immunization (14, 15). However, it is rejected by allogeneic, H-2-matched BALB/c mice. We have observed that this tumor progresses after allogeneic BMT. In this work, we have studied potential mechanisms of tumor escape after transplant.

MATERIALS AND METHODS

Animals. Female donor BALB/c (H-2b) and recipient DBA/2 (H-2b) mice are MHC-matched, mHAg-mismatched strains. Animals were obtained from National Cancer Institute Animal Production Program (Fredrick, MD) and were used at 6–8 weeks of age. Mice were housed in conventional rooms. From the day of BMT until day 14, water was acidified (pH 2.5) and supplemented with 3 g/l neomycin sulfate (Sigma Chemical Co., St. Louis, MO).

Cell Lines. P815 is a weakly immunogenic DBA/2 mastocytoma and was obtained from American Type Culture Collection (ATCC TIB-64; Manassas, VA). When injected i.v., it forms multiple liver and spleen metastases. The cells grow in tissue culture using RPMI 1640 supplemented with 5% heat-inactivated FBS (BioWhittaker, Walkersville, MD), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine.

Immunization. BALB/c donors or BALB/c→DBA/2 recipients were injected s.c. twice at a 1-week interval in the right flank with 5 × 106 irradiated (20 Gy) DBA/2 spleen cells in 0.2 ml of HBSS. Twelve days after the second immunization, BALB/c mice were used as BMT donors. In other experiments, BALB/c donors were injected s.c. in the right flank with 5 × 106 irradiated (50 Gy) P815 cells in 0.2 ml of HBSS.

Allogeneic BMT. One day before BMT (day −1), DBA/2 BMT recipients received 850 cGy (in one fraction) or 1100 cGy (in two fractions at an 18-h interval) total body irradiation from a 60Co source. On the day of BMT (day 0), 4 × 106 donor bone marrow cells and 10–50 × 106 spleen cells were injected i.v. in a total volume of 0.2 ml of HBSS through mice tail vein (cell doses for specific experiments are noted in the text and legends).

In Vivo Tumor Challenge and Tumor Reisolation. Mice were injected i.v. via the tail vein with 2 × 105 P815 cells in 0.2 ml of HBSS on the day of BMT. In some experiments, tumors were reisolated by sterile removal of livers, and tissue was macerated between frosted glass slides, followed by short-term culture in flasks.

Enumeration of Liver Tumor Nodules. Livers were collected from mice challenged with P815 cells at 3 weeks or earlier if moribund. After fixation in formalin, macroscopic tumor nodules on the liver surface were counted.

Assessment of GVHD. Recipients were weighed weekly and observed daily for signs of GVHD, such as weight loss, alopecia, hunched posture, dermatis, or death. In some experiments, liver sections stained with H&E were examined microscopically for characteristic GVHD mononuclear infiltrates in portal triads.

Cytotoxicity Assays (CTls). Spleen cells were cultured in 10 ml of 6-well plates at 1 × 106 cells/ml in RPMI 1640 supplemented with 10% FBS (Summit Biotech, Ft. Collins, Co), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 10 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μM 2-mercaptoethanol. One hundred Gy of irradiated P815 cells (5 × 106/ml) or 30 Gy of irradiated DBA/2 spleen cells (1 × 107/ml) were used as stimulators. After 5 days, effector cells were harvested and plated with 5 × 106 Cr-labeled target cells at E:T ratios ranging from 200:1 to 12.5:1. P815 target cells were labeled with 0.1 ml (100 μCi) of isotonic sodium 51Cr (Amersham Arlington Heights, IL) at 5 × 106 cells/0.2 ml for 60 min at 37°C and 5% CO2. Con A lymphoblasts were prepared by stimulating BALB/c or DBA/2 spleen cells with 2 μg/ml Con A at 2 × 106 cells/ml in complete medium. They were labeled with 51Cr for 45 min. Labeled targets were plated at 5 × 103 cells/well with effectors in a total volume of 0.2 ml/well and incubated 4 h at 37°C and 5% CO2. 0.1 ml supernatant was removed from each
well and counted in a gamma counter (Wallac San Francisco, CA). Spontaneous release was obtained from wells containing target cells and medium only, and maximum release was derived from wells containing target cells lysed with 1% Triton X-100. Spontaneous release ranged between 13 and 30% of the maximum release. The percentage of lysis was calculated as:

\[
\text{% lysis} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100
\]

Wells were plated with triplicate replicates, and average lysis was reported.

**Flow Cytometry.** Phycoerythrin-conjugated hamster antimouse Fas (PharMingen, San Diego, CA) and phycoerythrin hamster IgG, λ isotype control (anti-keyhole limpet hemocyanin) were used to stain 1 × 10^6 P815 cells. The cells were then fixated in 2% paraformaldehyde in PBS and analyzed using a FACScan (Becton Dickinson, San Jose, CA). Ten thousand events were counted for each sample.

**Statistical Analysis.** Prism 3.0 software (Graphpad Software for Scientists, Sorrento, CA) was used for statistical evaluation of data. For survival experiments the log-rank test was used. To compare groups of liver tumor nodules, the t test was performed.

**RESULTS**

**P815 Tumor Progresses in Allogeneic BMT Recipients Despite Transfer of Alloreactive Lymphocytes.** P815 is a poorly immunogenic tumor in syngeneic DBA/2 mice that grows aggressively at doses of <1000 cells (14), even after immunization of syngeneic mice with irradiated P815 (15). This poor immunogenicity in syngeneic mice was reproduced in our laboratory. DBA/2 mice were immunized twice with 5 × 10^6 irradiated P815 cells. Seven days later, the immunized (n = 5) and control unimmunized mice (n = 5) were challenged with 5 × 10^3 cells i.v. Immunization induced no increase in survival (immune mice, 12.4 ± 0.5 days; normal mice, 12.4 ± 0.5 days). Nor did it produce any difference in hepatic tumor burden, the site in which the tumor grows after i.v. injection (immune mice, 2.6 ± 0.06 g; normal mice, 2.4 ± 0.10 g).

Although P815 is poorly immunogenic in syngeneic hosts, we hypothesized that a strong allogeneic reaction against recipient P815 tumor would inhibit tumor progression in allogeneic BMT recipients. To test this idea, H-2-matched, mHAg-mismatched BALB/c donors were immunized twice with 5 × 10^6 DBA/2 spleen cells and were used 7 days later as allogeneic BMT donors for DBA/2 mice. BALB/c mice immunized against DBA/2 spleen cells exhibit cytolytic T cell activity that kills both normal DBA/2 lymphoblasts and P815 (see Fig. 3). At the time of transplant, these mice and normal DBA/2 control mice were challenged with 2 × 10^3 P815 cells i.v. All control mice (n = 5) died by day 15, whereas no deaths were observed in the BMT group (n = 9) during this period (P = 0.003). In addition to significant differences in 3-week mortality, hepatic tumor burden was also substantially reduced in allogeneic transplant recipients. On the day of death, all control mice had >200 hepatic tumor nodules and average liver weight of 2.64 ± 0.04 g, whereas allogeneic transplant recipients sacrificed electively 3 days after the last death in the control group had an average of 29.9 ± 24.6 hepatic tumors (P = 0.001) and liver weight of 0.95 ± 0.13 g (P = 0.001). In a smaller, independent experiment, median survival of normal DBA/2 mice (n = 5) was 14 days, whereas median survival in recipients of DBA/2 spleen cell-sensitized transplants (n = 5) was 28 days (P = 0.01).

Similar results were seen in H-2-matched, mHAg-mismatched BALB/c donors allimmunized with P815 tumor cells. Allosensitized donors exhibited strong alloreactive CTL activity against both DBA/2 Con A lymphoblasts and P815 cells (data not shown), and transfer of such cells into DBA/2 mice at BMT produced hepatic GVHD lesions (data not shown). Recipients did not exhibit GVHD-related wasting or mortality. Allogeneic BMT using these allosensitized donors or naive BALB/c mice was performed, and DBA/2 recipients were simultaneously challenged with P815 cells i.v. Three weeks later, recipients were sacrificed, and livers were examined for metastatic tumor nodules. Fig. 1 demonstrates that recipients of allosensitized BMT had significantly fewer hepatic tumors. Other experiments assessed survival (Fig. 2). As expected, syngeneic DBA/2 mice not undergoing BMT died within 3 weeks of tumor challenge with or without P815 preimmunization. Again allogeneic BMT recipients of allosensitized cells exhibited significantly prolonged survival with median survival nearly double that of control mice. However, all mice in this cohort died of recurrent tumor within 2 months.

**Relapsing Tumors Retain Sensitivity to Alloreactive Cytolytic T Cells.** One possible explanation for relapse in allogeneic BMT recipients is acquired resistance of the tumors to immunological effector mechanisms. Experiments were performed to test the hypothesis that relapsing tumors were resistant to allogeneic cytolytic T cells. Tumors were reisolated from BMT recipients of allosensitized or naive allogeneic cells and after short-term culture were used as targets in chromium release assays using allogeneic T cells derived from BALB/c mice immunized against normal DBA/2 spleen cells (Fig. 3). In all cases examined, the relapsing tumors remained sensitive to these
alloreactive CTLs with sensitivity equal to the P815 tissue culture stock used to challenge the animals. The persistent sensitivity to alloreactive CTLs is compatible with continued expression of mHAgs and MHC class I and show that the tumors had not acquired intrinsic resistance to CTL effector mechanisms. In other tumor models, loss of Fas expression on tumors has been associated with tumor progression (16). We examined six tumor reisolates for Fas expression by flow cytometry and found that the relapsing reisolates continued to express Fas at a level comparable with that of the tumor cell line used to initially challenge the mice (data not shown).

Alloreactivity in BMT Recipients Is Substantially Down-Regulated 3 Weeks after Transplant. Analysis of the relapsing tumors failed to identify any intrinsic change in the tumors that would allow them to escape from donor immune attack. Therefore, an alternate possibility for a mechanism of relapse was loss of donor alloreactivity some time after BMT. The experiments examining tumor numbers 3 weeks after transplant as well as the survival experiments indicated that significant donor antihost, antitumor activity may have been present early after BMT. Experiments were designed to test the hypothesis that donor alloreactivity would be significant in the early post-BMT period but substantially down-regulated later. DBA/2 mice underwent BMT using allosensitized BALB/c donors and were challenged with P815 cells at transplant. One and 3 weeks after BMT, donor cytolytic activity against DBA lymphoblasts, P815 cells, and negative control targets BALB/c Con A blasts was assessed (Fig. 4). One week after BMT, there was strong allospecific cytolytic activity in recipients. However, 3 weeks after transplant, no cytolytic activity against either DBA/2 lymphoblasts or P815 was detected. Similar results were observed in an independent experiment in which transplant donors were sensitized with DBA/2 spleen cells. Four weeks after transplant, 0% anti-P815 cytolytic activity (E:T ratio 200) was seen in transplant recipients, whereas 56 ± 2.5% P815 cytolytic activity (E:T ratio 200) was observed in the positive control BALB/c donors sensitized to DBA/2 spleen cells. In a third independent experiment, transplant donors were sensitized with P815 cells, but transplant recipients were not challenged with P815 cells at the time of transplant. Again, little anti-P815 cytolytic activity was observed in the transplant recipients (13 ± 0.4%; E:T ratio 200) compared with 32 ± 3% in BALB/c donor mice sensitized with P815 cells (P = 0.005). No cytolytic activity was seen against BALB/c control targets.

BMT Recipients Harbor Potential Alloreactive Cells That Can Be Induced by Immunization with Recipient Splenocytes. One explanation for the gradual loss of alloreactivity after BMT is clonal exhaustion. This has been observed in experimental systems in which persistent, high-dose antigen exposure is present (17). Alloimmune BMT may be analogous in that recipient alloantigens are ubiquitous continuously after BMT. To test the hypothesis that these BMT recipients had experienced clonal exhaustion, stable GVHD-free mice 7 weeks after allogeneic BMT were immunized twice with recipient strain-irradiated splenocytes, and cytolytic activity against DBA/2 lymphoblasts, P815 cells, and donor strain BALB/c lymphoblasts was measured (Fig. 5). In the absence of specific immunization, the BMT recipients did not exhibit measurable alloreactivity. However, after immunization with recipient-strain splenocytes, significant allospec-
cific cytolytic activity was observed against the DBA/2 lymphoblasts and P815 cells, both of hematopoietic origin. This result is incompatible with global clonal exhaustion toward mHAgs.

DISCUSSION

In this murine allogeneic bone marrow model, BMT recipients of alloreactive T cells experienced relapse of host P815 tumor after an apparent 3–4-week prolongation of survival. No evidence of antigen loss, intrinsic resistance to cytolytic cells, or loss of Fas expression was found in the reisolated tumors to account for relapse. Rather, evidence of loss of alloreactivity was found in the BMT recipients, and the time to this loss correlated with the prolongation of survival. Immunization of GVHD-free BMT recipients suggested that some degree of alloreactivity could be regenerated despite the early down-regulation of alloreactivity.

One limitation of some of the experiments in which donors were allogeneically immunized with P815 tumor cells is that in addition to reactivity to allografts, some degree of reactivity toward tumor-specific antigens may have been induced. Although this is a possibility in these experiments, it is also clear such reactivity was also down-regulated in concert with the alloreactivity. Any tumor-specific reactivity was not sufficient to induce any changes in survival or tumor burden in syngeneic settings. On the other hand, in other experiments in which donors were allogeneically immunized with DBA/2 spleen cells in which only alloreactivity can be induced, substantial increases in survival and reductions in tumor burden were observed. The changes induced were very similar to the results of experiments in which P815 was used to induce alloreactivity.

Although clinical and preclinical studies have clearly demonstrated an allogeneic GVL effect that is statistically associated with GVHD, the mechanisms of the effect are not known with certainty. Nor is it clear that GVL is mechanistically or antigenically distinct from GVHD. However, a variety of studies including the one reported here and elsewhere demonstrate that the donor alloreactivity that causes GVHD can itself produce a significant antitumor effect (6, 7). This could be explained by the expression of host mHAgs on tumor cells.

If host mHAgs are significant targets on tumor cells, it is paradoxical that tumors could survive in a microenvironment in which there is potent alloreactivity. In other tumor systems in which syngeneic hosts have antitumor reactivity, antigen loss is often observed in tumor variants that progress or metastasize (18–24). In human transplantation, reduced sensitivity of relapse leukemia cells to donor CTLs has been observed in some cases of allogeneic BMT (25). It is interesting that in a detailed study of P815 tumor progression in syngeneic mice, antigen loss occurred regularly and accounted for growth of tumor after a period of immunological control (20). In contrast, in our allogeneic system all relapsing variants examined remained sensitive to lysis by alloreactive donor T cells, and this suggests that the variants have neither lost target antigens nor acquired intrinsic resistance to lysis. The failure to observe antigen loss in this setting of strong immune selection suggests the hypothesis that mHAgs are derived from cellular proteins that are essential for tumor cell viability, whereas other antigens (perhaps those serving as the targets for a syngeneic response) may be derived from proteins the tumor cell can live without. If this is indeed the case, mHAg may represent good immune targets on tumor cells if an acceptable therapeutic index between GVL and GVHD can be established.

Although no evidence of antigen loss was observed in relapsing tumors, loss of donor alloreactivity was observed at a time shortly before relapse. One credible interpretation of this observation is that one mechanism of relapse after allogeneic transplant is acquired donor tolerance of host mHAgs. Unlike solid organ transplantation, in BMT immunosuppression can be withdrawn after 6–12 months if a tolerant state has been achieved (26). Through incompletely unknown mechanisms donor alloreactivity is down-regulated in this period. One could hypothesize that the “normal” acquisition of tolerance to host mHAgs after transplant removes the immune inhibition of leukemia cell proliferation that may occur in the early post-BMT period and promotes relapse. Recurrent leukemia still sensitive in vitro to pre-BMT donor T cells has been observed in GVHD-free human BMT recipients (27). Compatible with this hypothesis is the clinical observation that the majority of relapses occur within 1–2 years after BMT and the experimental observation that tumor relapse is infrequent in mice with active GVHD (7).

The observations suggest the hypothesis that in transplants in which there is a high risk of relapse but no significant GVHD clinically apparent, induction of limited alloreactivity may restore an important antileukemia effect. One experimental approach to test this hypothesis would be active immunization of BMT recipients with normal or malignant host cells. In this model, immunization of GVHD-free chimeras 7 weeks after BMT did reactivate alloreactive cells, which could lyse hematopoietic tumor cells in vitro, suggesting that the post-BMT tolerant state is not attributable to global clonal deletion of all potentially alloreactive T cells. Rather, such hosts may retain latent alloreactivity directed to some mHAgs, possibly those with restricted tissue distribution. If so, activation of such alloreactive cells may provide an additional avenue of therapy.

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Down-Regulation of Antihost Alloreactivity after Bone Marrow Transplant Permits Relapse of Hematological Malignancy

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