Active Immunization of Murine Allogeneic Bone Marrow Transplant Donors With B-Cell Tumor-Derived Idiotype: A Strategy for Enhancing the Specific Antitumor Effect of Marrow Grafts

By Larry W. Kwak, Robin Pennington, and Dan L. Longo

Persistence of the underlying malignancy remains the major obstacle limiting the success of high-dose chemoradiotherapy with allogeneic bone marrow transplantation (BMT) for lymphomas and multiple myeloma. We used the C3H 38C13 murine B-cell lymphoma, which expresses and secretes clonally derived Ig, the idiotype of which can serve as a tumor-specific antigen, to test the principle of transfer of tumor idiotype-specific immunity with BM. BALB/c marrow donors were twice immunized with 38C13-derived Ig, or with an isotype-matched control Ig, conjugated to keyhole limpet hemocyanin. Lethally irradiated C3H recipients reconstituted with marrow from idiotype immune, but not non-specifically immune, donors demonstrated protection against subsequent lethal tumor challenge. The immunoprotective effect of immune allogeneic marrow was abrogated by T-cell depletion of the marrow graft before infusion. Low levels of serum anti-idiotypic antibody remained unaltered in recipients of T-cell-depleted immune marrow, consistent with a primary role for T-cell immunity in the cellular mechanism of this phenomenon. A modest therapeutic effect of immune allogeneic marrow was observed against 10 day, 1 cm established subcutaneous tumors, but only in combination with a booster immunization of the recipient post-BMT. These results provide the rationale for a novel strategy for enhancing the specific antitumor effect of allogeneic marrow grafts. This is a US government work. There are no restrictions on its use.

MATERIALS AND METHODS

Mice and tumor. C3H/HeN MTV- and BALB/cANNCR female mice 6 to 12 weeks of age were obtained from the Animal Production Area (National Cancer Institute-Frederick Cancer Research and Development Center [NCI-FCRDC], Frederick, MD). The carcinogen-induced 38C13 B-cell lymphoma has been previously described. These studies demonstrated the protective and therapeutic antitumor effect of immune syngeneic marrow. Based on the success of this approach, we have now explored this strategy with tumor idiotype in the model system of 38C13, a murine B-cell lymphoma that both expresses and secretes idiotypic Ig, and extended these studies to the immunization of fully major histocompatibility complex (MHC)-mismatched donors.

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for each experiment were made from the same suspension of tumor cells.

**Vaccine preparation and administration.** Idiotype IgM (38C13-Id) was rescued by somatic cell hybridization as described, and 38C13-Id and a control IgM(κ) were purified from ascites and coupled to KLH using 0.1% glutaraldehyde. Donor or recipient mice were immunized subcutaneously (sc) with 50 μg 38C13-Id-KLH or control IgM-KLH emulsified in an adjuvant formulation consisting of 2:2:1 Tween 80, 2.5% Pluronic L121, 3% saqualene, and 100 μg/mL N-acetyl-β-D-glucosaminyl-(β-1,4)-N-acetylglucosamyl-L-alanyl-D-soglutamine (GMDP). GMDP was kindly provided by P. Cooper (C-C Biotech Corp, Poway, CA) as a gift.

**Serum anti-idiotypic antibody.** Mouse serum was serially diluted over microtiter plates coated with 38C13-Id. Binding of antibodies in the serum to 38C13-Id was detected by goat-antimouse IgG-horseradish peroxidase antibodies, which had been absorbed against control IgM-KLH or from their lethally irradiated BM recipients were killed, and BM was collected from counted by trypan blue exclusion. Naive recipient C3H mice were lethally irradiated with 950 rad total body irradiation (TBI) in a CTI source irradiator (J.L. Shepherd and Associates, San Fernando, CA). Irradiated recipients were injected intravenously with 3×10^7 BM cells in 0.5 mL RPMI 1640 medium in the lateral tail vein.

For experiments in tumor-bearing recipients, C3H mice were first injected with 10^6 38C13 tumor cells sc in the right flank and monitored for tumor growth by inspection, palpation, and measurement. Ten days after tumor inoculation, all recipients received 950 rad TBI and cyclophosphamide (CY) 75 mg/kg body weight ip. One day later, conditioned recipients were injected iv with 2×10^7 BM cells. Some tumor-bearing recipients received immunizations as described above on the day of marrow infusion.

**BM recipients.** BM recipients were maintained in sterile microisolator cage units in a laminar flow hood for approximately 3 weeks, during which time they received sterile food and acidified water. Overall postoperative mortality was approximately 10% (maximum 23% in a single experiment), and all surviving mice were clinically healthy without evidence of graft-versus-host disease (GVHD; weight loss, alopecia, diarrhea, hunching, or skin erythema). In addition, random mice were killed and subjected for histopathological examination performed by the Pathology/Histotechnology Laboratory, NCI-FCRDC. No histologic evidence of GVHD was observed in any recipients. The absence of lethal GVHD in the BALB/c → C3H strain combination has been observed by others. 24

Ex vivo T-cell depletion (TCD) of BM. TCD of BALB/c BM cells was performed according to methods previously described by others. 25-28 BM cells were adjusted to a concentration of 10^7 cells/mL in RPMI-1640 and incubated with 50 μg/mL anti-Thy-1.2 for 30 minutes at 4°C (30-H-12, rat IgG2b; Hazelton Biotechnologies Co, Vienna, VA). Cells were then pelleted, resuspended at 10^7 cells/mL in a 1:10 dilution of Low Tox-M rabbit complement (C; Accurate Chemical and Scientific Corp, Westbury, NY), and incubated at 37°C for 45 minutes. Cells were then washed twice before use. Aliquots of BM cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD4 (YTS 191.1, rat IgG2b) and -CD8 (YTS 169.4, rat IgG2b) monoclonal antibodies (MoAbs; both from Caltag, South San Francisco, CA) after treatment with C' alone or anti-Thy-1.2 plus C' and analyzed by flow cytometry to monitor the efficiency of TCD. These studies generally showed a reduction of T cells from approximately 5% to 10% of lymphocytes to less than 1%.

**Analyses of lymphoid chimerism.** Chimerism of splenocytes obtained post-BMT was analyzed by single-color flow cytometry using FITC-labeled anti-H-2^d (SF1-1.1, mouse IgG2a) or anti-H-2^k (36-7-5, mouse IgG2a) specific MoAb (both from Pharmingen, San Diego, CA). Briefly, splenocytes were treated with red blood cell ACK lysing buffer (Sigma, St Louis, MO), washed, and preincubated with 2,4G2 antimouse Fcy receptor MoAb (rat IgG2b, American Type Culture Collection [ATCC] HB 197; ATCC, Rockville, MD) to block nonspecific binding of the primary antibodies. Cells were incubated with primary antibodies for 15 to 30 minutes at 4°C, washed, and fixed in 1% paraformaldehyde. All results were obtained using an EPICS Profile flow cytometer (Coulter Corp, Hialeah, FL). Forward and side scatter settings were gated to exclude any remaining red blood cells and debris. Ten thousand cells were analyzed for each determination.

**Results.** Mice were checked daily to determine the date of death. Tumor-bearing mice with sc tumors were inspected by palpation, and tumor diameters were measured on opposing axes every other day. Statistical comparisons of survival were performed using BMDP IL software (BMDP Statistical Software, Inc, Los Angeles, CA) to generate nonparametric Mantel-Cox logrank P values. Mice surviving greater than 90 days after tumor challenge were killed and were reported as long-term survivors.

**Immune syngeneic BM dose response.** Before studying the immunization of MHC-mismatched donor-recipient pairs, we first performed several experiments in syngeneic mice to determine the optimal dose of immune marrow for transfer of tumor idiotype-specific immunity. C3H mice served as marrow donors were immunized with either 38C13-Id-KLH or control IgM-KLH in adjuvant, and then again with the respective immunogen 2 weeks later. One to 2 weeks after the booster immunization, graded numbers of BM cells pooled from these idiotype immune or nonspecifically immune donors were used to reconstitute lethally irradiated (950 rad TBI) normal C3H recipients. After 3 weeks' recuperation, these recipients were challenged ip with a lethal dose of 10^7 38C13 tumor cells and monitored for survival. Recipients reconstituted with 2×10^7 immune BM cells demonstrated a median survival time (MST) of 29 days, compared with recipients reconstituted with an equivalent number of nonspecifically immune marrow cells (MST 25 days), as well as 24% long-term survival (P = .0001). However, lower doses of immune marrow (5×10^7 and 10×10^7 cells) failed to confer any significant survival benefit on recipients, compared with nonspecifically immune marrow (data not shown). Thus, for subsequent experiments, a marrow inoculum of 2×10^7 cells was routinely used.

Comparison of normal syngeneic and allogeneic BM and protective effect of immune allogeneic marrow. We next performed experiments designed to evaluate the effect of normal allogeneic, compared with syngeneic, BM on tumor
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Recipient anti-idiotypic antibody and the effect of marrow TCD. In an effort to explore the cellular mechanism of the observed protective effect of immune allogeneic marrow, we first determined whether the anti-idiotypic humoral response elicited in donors was transferred to lethally irradiated recipients. Normal BALB/c donor mice showed vigorous anti-idiotypic antibody responses after two immunizations at the time of marrow harvest (mean serum antibody level > 500 μg/mL, data not shown). Serum samples were obtained from the recipients of idiotype-immune or nonspecifically immune marrow in the experiment in Fig 2 at the time of tumor challenge and assayed for anti-idiotypic antibody. Serum anti-idiotypic antibody titers of five individual randomly selected recipients from each group and from five recipients of immune BM from a second identical experiment are shown in Fig 3A. In contrast to donor levels, extremely low but detectable levels of anti-idiotypic antibody were observed in each C3H recipient of immune marrow (mean antibody levels 0.3 ± 0.2 and 1.7 ± 3.0 μg/mL, experiments 1 and 2, respectively). The consistent lack of binding to 38C13-Id by control sera from recipients of nonspecifically immune marrow (complete lack of absorbance of undiluted serum) showed the sensitivity of this ELISA.

We then tested the effect of in vitro marrow TCD on the protective effect of immune marrow. Pooled BM from BALB/c donors twice immunized with either 38C13-Id-KLH or control IgM-KLH in adjuvant was subjected to treatment with either anti-Thy 1.2 plus rabbit C' or with C' alone. Lethally irradiated C3H recipients were then randomly allocated to receive one of the four resulting pools of marrow. After 3 weeks, these recipients were challenged ip with 5 × 10⁵ tumor cells and monitored for survival. The protective effect of immune allogeneic marrow was again evident, as demonstrated by comparison of the survival of recipients of immune with recipients of nonimmune marrow treated with C' alone (P = .001, 83% long-term survival in recipients of immune marrow at this tumor challenge dose). Treatment

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of immune marrow with anti-Thy 1.2 plus C' alone-treated marrow was demonstrated in each of the individual recipients of immune marrow treated with C' alone (mean antibody level 0.9 ± 1.1 µg/mL), compared with control recipients of nonimmune marrow (Fig 3B). Of primary interest, low levels of antibody of comparable magnitude were also shown in recipients of TCD immune marrow (mean antibody level 0.9 ± 1.6 µg/mL). Thus, the anti-idiotypic antibody alone is not responsible for the antitumor effects seen after transfer of idiotype-immune allogeneic marrow.

**Therapeutic effect of allogeneic immune marrow against established tumor.** To more closely model the clinical status of a human patient bearing an established tumor burden, we performed a series of experiments designed to test the potency of this approach against well-established tumors in mice. The 38C13 lymphoma grows rapidly, and 10 days after the sc implantation of 10⁶ tumor cells in naive syngeneic mice, 100% of mice develop macroscopic, 1-cm tumor
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masses. Preliminary experiments in 10-day tumor-bearing mice showed that 950 rad TBI was largely ineffective at reducing tumor size or prolonging survival (data not shown). The addition of CY ip at doses ranging from 25 to 75 mg/kg to TBI followed by rescue with normal BALB/c marrow was effective at reducing tumor masses and prolonging survival without effecting cures (data not shown). Based on these results, a conditioning regimen of 950 rad TBI plus CY 75 mg/kg was used for experiments in tumor-bearing recipients.

Ten-day tumor-bearing C3H recipients were treated with TBI plus CY and received pooled marrow from BALB/c donors that had been twice immunized with either 38C13-Id-KLH or control IgM-KLH in adjuvant. Initial experiments failed to demonstrate a survival advantage for recipients of idiotype-immune, compared with nonspecifically immune marrow. In an effort to more rapidly expand the adoptively transferred T cells in their adoptive hosts, recipients from each group were given a booster immunization with either 38C13-Id-KLH or control IgM-Id-KLH in adjuvant on the day of marrow infusion. Data pooled from two such experiments are shown in Fig 5. Recipient mice injected with 10⁶ tumor cells s.c. in the right flank developed progressively growing visible tumor masses, and by day 10 all of the mice had 1-cm tumors (100% tumor incidence). The administration of TBI plus CY produced temporary remissions in all recipients (1.2% tumor incidence, day 16), but within 10 days tumors started to recur. Ultimately, all but one of the control mice that had received nonimmune BALB/c marrow and a nonspecific immunization post-BMT relapsed (95% tumor incidence). The kinetics of tumor relapse were not significantly different in recipients of nonimmune marrow given a specific boost or in recipients of immune marrow given a nonspecific boost, compared with this control group, with nearly all of the recipients eventually relapsing with tumor. Relapsing tumors resulted in progressive tumor growth and death of the hosts. However, a modest delay in tumor relapse rate was observed for recipients of immune marrow who had also received a specific booster immunization post-BMT. Although the majority of these recipients also eventually relapsed, a significant proportion (32%) remained free of tumor during the period of observation and were apparently cured (Table 1, P = .02 compared with the control group). Thus, the therapeutic effect of immune marrow transfer against established tumors required the addition of a specific booster immunization in the recipient post-BMT.

All long-term survivors were found to contain hematopoietic cells dominantly of donor origin and were free of any histologic evidence of GVHD (data not shown). Under the experimental conditions used, no significant difference in survival was observed between controls receiving nonimmune BALB/c marrow and other recipients reconstituted with nonimmune syngeneic C3H marrow in parallel (MST 43 and 42 days, respectively; 0 of 12 survivors in the latter; data not shown). Thus, allogeneic MHC recognition of host tumor cells by donor lymphocytes was not sufficient to produce significant antitumor effects under these conditions.

DISCUSSION

Exploiting the potential antileukemic effect of marrow grafts remains an attractive concept. A GVL effect has been inferred for most leukemias by the retrospective analyses of higher rates of relapse after human syngeneic, compared with allogeneic, BMT.¹⁶ and a similar GVL effect may exist for lymphomas¹ and multiple myeloma. Efforts to manipulate the GVL effect have met with variable degrees of success, largely limited by the difficulty of dissociating GVL reactions from GVHD.²⁷-²⁹ Perhaps the most definitive evidence of a GVL effect in humans has been provided by the observations of cytogenetic remissions in patients with chronic myelogenous leukemia (CML) receiving allogeneic buffy coat mononuclear cells after relapse from allogeneic marrow transplantation.³⁰,³¹

The experiments we have presented provide an alternative strategy, designed to enhance the graft antitumor response in a specific manner against a defined tumor antigen. C3H recipient mice conditioned with 950 rad TBI, which had been previously shown by others to be marrow ablative in

Table 1. Survival of Tumor-Bearing Recipients in Figure 5

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. Survivors/ Total No. Mice</th>
<th>Logrank P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nonimmune BM + control Id boost</td>
<td>1/22</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>Nonimmune BM + 38C13-id boost</td>
<td>0/12</td>
<td>.66</td>
</tr>
<tr>
<td>3</td>
<td>Immune BM + control Id boost</td>
<td>1/22</td>
<td>.96</td>
</tr>
<tr>
<td>4</td>
<td>Immune BM + 38C13-id boost</td>
<td>8/25</td>
<td>.02</td>
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* Compared with group 1.
this strain, and infused with unfractionated BALB/c BM were fully reconstituted with donor type cells 3 weeks after BMT (see Results) and were more resistant to challenge with 38C13 lymphoma, compared with recipients of syngeneic marrow. The principal finding was that this basic effect of allogeneic marrow on syngeneic tumor resistance could be augmented significantly by active immunization of allogeneic marrow donors with Id-KLH. In addition, we have investigated the potential cellular mechanism of this phenomenon.

The mechanism of immunoprotection against the 38C13 lymphoma in normal syngeneic C3H mice immunized with Id-KLH remains to be fully elucidated but appears to involve both humoral and cellular arms of immune response. For example, protection could be transferred with immune serum containing anti-idiotypic antibodies in a Winn assay. However, protection is abrogated by in vivo depletion of CD4+ or CD8+ T cells postimmunization, and in such mice anti-idiotypic antibody levels remain unaltered (L.W. Kwak, manuscript in preparation). In the BCL-1 lymphoma model, protection after immunization with idiotypic Ig was primarily antibody mediated. In contrast, Id-induced resistance to MOPC-315 appeared to be predominantly a result of cell-mediated immunity, as postimmunization thymectomy abolished tumor resistance and had no effect on serum anti-idiotypic antibody levels. Id-induced resistance to MOPC-11 could not be attributed to the predominance of either antibody- or cell-mediated immunity. In the current study, TCD of immune allogeneic marrow before infusion clearly abolished the transfer of the enhanced protective effect of immune allogeneic marrow to syngeneic recipients (Fig 4).

Furthermore, no effect of marrow TCD was observed on serum anti-idiotypic antibody levels in these recipients (Fig 3B). These observations, taken together, suggest a primary role for antigen-specific T cells in the effector phase of this phenomenon and that the transfer of humoral anti-idiotypic immunity from donor to recipient at the low levels determined is not sufficient to mediate enhanced protection. This finding was rather unexpected, given the fact that the percentage of mature T cells in murine BM is extremely low (approximately 5%). This fact may also explain the requirement for a relatively large marrow inoculum: specifically, the protective effect of immune marrow, compared with nonimmune, marrow was not observed at doses less than 20 X 10^6 cells (see Results). Nevertheless, the abrogation of protection by TCD was not complete, as a slight but statistically significant difference between immune and nonimmune marrow recipients was still evident in the two anti-Thy 1.2 plus C'-treated groups. It is tempting to speculate that this residual degree of protection may be caused by the low levels of anti-idiotypic antibody demonstrated in the former.

The precise mechanism by which allogeneic BALB/c T cells specific for idiotype mediate protection against the 38C13 tumor is under further investigation. T-cell recognition of idiotypic peptide presented by the tumor may be occurring either in the context of a self-MHC molecule cross-reactive for an H-2^k molecule expressed by the tumor target or in direct association with an H-2^k molecule (allo MHC). It is noteworthy that T-cell responses specific for allogeneic MHC + antigenic peptide have been described previously. Alternatively, indirectly activated effector mechanisms such as non–antigen-specific effectors (eg, macrophages, natural killer cells) recruited by cytokines secreted by antigen specific CD4+ T cells, are also being considered. For example, tumor-derived idiotype may be taken up and processed by antigen-presenting cells of donor haplotype and subsequently presented to specific T cells. A precedent for such an indirect antitumor effect of T cells in vivo was provided recently by the demonstration that CD4+, MHC class II–restricted T-cell clones, which were specific for myeloma idiotype, protected against the MOPC-315 myeloma, which lacks MHC class II molecules.

The therapeutic effect of immune allogeneic marrow could be demonstrated against rapidly growing, macroscopic tumors, in which setting a slight delay in tumor relapse rate and a modest cure rate were observed (Fig 5 and Table 1). The full expression of this therapeutic effect required the addition of a specific booster immunization against idiotype in the recipient post-BMT, as neither immune marrow alone, nor a specific recipient immunization alone were sufficient to produce any survival benefit compared with nonimmune allogeneic marrow. Although the potency of immune marrow could be demonstrated, by comparison, it is noteworthy that against these same established tumors no survival advantage was observed for recipients of nonimmune allogeneic, compared with syngeneic, BM (see Results). Future studies aimed at quantitating the magnitude of this effect, ie, log tumor cell elimination by immune marrow, may be worthwhile.

It is unclear why BALB/c marrow did not mediate significant GVHD, despite MHC differences between donor and recipient. Evidently, the small number of T cells that were present in the marrow inoculum to transfer protection were not sufficient to induce clinically or histologically evident GVHD (see Materials and Methods). Furthermore, as the goal of the current study was to establish the principle of transfer of protection with idiotype immune allogeneic marrow under controlled experimental conditions, no additional attempts were made to actively induce GVHD in marrow recipients. Inasmuch as the transfer of protection with immune allogeneic marrow is predominantly T-cell mediated, it may be worthwhile to explore the following: (1) the addition of T cells from other, relatively enriched peripheral sources (blood or splenocytes) from immune marrow donors to increase potency, and (2) assessing the potential offsetting effect of accompanying GVHD on immune transfer. We have recently transferred a human tumor idiotype-specific T-cell response with unmanipulated marrow from an HLA-matched sibling donor, who had been immunized with myeloma idiotype protein, to a myeloma patient who experienced grade II GVHD. Thus, GVHD and its prophylaxis may not be ineluctable barriers to the transfer of donor-derived T-cell immunity.

Finally, it is acknowledged that the experimental setting of fully MHC-mismatched differences between donor and recipient is not reproducible in humans and that some findings may not be generally applicable. Furthermore, the requirement for isolation of idiotypes from individual patients'
lymphomas or myelomas for formulation into safe, refined donor vaccines may limit the routine application of this strategy in humans. Nevertheless, the clinical translation of these results would probably best be performed using non-TCD allogeneic marrow (unless subsequent infusion of donor T cells was planned). The dose of BM infused may not be a limiting factor, as human BM grafts are most often contaminated with significant amounts of peripheral blood containing T cells. Finally, the experiments in tumor-bearing recipients suggest that recipient booster immunizations post-BMT may be necessary. A precedent for this observation exists in human allogeneic BMT, in which the transfer of T-cell immunity to VZV or PPD and humoral immunity to KLH required both donor and recipient to be immune. In principle, this therapeutic approach could be applied in the future to other malignancies for which BMT has a role in treatment and for which defined tumor antigens have been identified (eg, CML).

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