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Combined Syngeneic Bone Marrow Transplantation and Immunotherapy of a Murine B-Cell Lymphoma: Active Immunization With Tumor-Derived Idiotypic Immunoglobulin

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Recurrence of the underlying malignancy remains a major cause of treatment failure after autologous bone marrow transplantation (BMT) for patients with lymphoma. In this regard, we have developed an immunotherapeutic approach designed to induce resistance against residual tumor cells persisting after BMT. Previous studies in the model system of 38C13, a lethal B-cell lymphoma of C3H origin, have shown that active immunization with purified tumor-derived surface immunoglobulin (Id), as a tumor-associated antigen, produces resistance to tumor growth. Id immunization of lethally irradiated mice at 3 or 5 weeks after reconstitution with syngeneic bone marrow resulted in significantly prolonged survival after tumor challenge compared with nonspecifically immunized controls. Low levels of idiotype-specific antibody were also demonstrated in the sera of specifically immunized mice at this early time, when other functional studies in the literature of immunocompetence after syngeneic reconstitution might have predicted incomplete recovery. Immunization of mice *before* lethal irradiation and syngeneic marrow reconstitution also induced significant resistance to tumor challenge, suggesting the persistence of established host antitumor immunity through total body irradiation. These studies demonstrate the feasibility of Id immunization in conjunction with bone marrow transplantation.

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HIGH-DOSE chemo-radiotherapy followed by autologous bone marrow transplantation (ABMT) has shown considerable promise as a potentially curable approach to the therapy of lymphomas that are otherwise refractory to conventional therapeutic modalities.[1,2] However, despite technical improvements in "purging" the autograft of residual lymphoma cells and continuing refinement of the chemotherapy regimens, persistence of the underlying lymphoma following ABMT remains a major problem. For this reason, the addition of an immunotherapeutic approach designed to induce immune resistance against residual tumor cells after supralethal radiation and chemotherapy and marrow reconstitution would be worthwhile.

The idiotype of the surface immunoglobulin expressed by a B-cell lymphoma can serve as a tumor-specific marker, distinguishing tumor cells derived from the malignant clone from other nonmalignant B cells. Building on the results in a number of experimental models,[3-10] we have developed the approach of active immunization with purified tumor-derived surface immunoglobulin (Id) from 38C13, a B-cell lymphoma of C3H origin. Studies in our laboratory have demonstrated both an immunoprotective effect of Id immunization on survival after subsequent tumor challenge as well as a direct antitumor effect against established tumors in tumor-bearing mice.[11-13] The optimal antitumor effect required conjugation of the Id to an immunogenic carrier protein, such as keyhole limpet hemocyanin (KLH), and administration with an adjuvant. In addition, the success of Id vaccination in curing tumor-bearing animals required concomitant reduction of the tumor burden by cyclophosphamide chemotherapy. Although potentially immunosuppressive, cyclophosphamide in the doses used did not appear to significantly impede the induction of antitumor immunity.

The successful application of Id immunization in combination with ABMT would depend in large part on the recovery of relevant immune effector mechanisms posttransplant. Alternatively, if antitumor immunity were established before ABMT, this immunity would need to persist through the marrow ablative regimen to be effective in the posttransplantation period. We have now extended the 38C13 model to study the induction of tumor idiotype specific immune responses and resistance to tumor challenge following Id immunization in syngeneic mice, despite lethal irradiation and bone marrow reconstitution. The results demonstrate the effectiveness of Id vaccination in the peritransplant setting.

MATERIALS AND METHODS

Mice and tumor. C3H/HeN female mice, 8 to 9 weeks old, were obtained from Simonsen Laboratories (Gilroy, CA). The carcinogen-induced 38C13 B-cell lymphoma has been previously described.[14] Inoculation of 1,000 38C13 tumor cells intraperitoneally (ip) into syngeneic C3H/HeN mice resulted in progressive tumor growth and median survival times of approximately 21 days. Tumor cells from a common frozen stock were passaged in vitro 3 to 4 days before use. Injections for each experiment were made from the same suspension of tumor cells.

Vaccine preparation and administration. The 38C13 tumor and its in vitro adapted cell line express immunoglobulin M (IgM) (κ) on the cell surface. 38C13-Id and a control IgM (κ) were purified from ascites[15] and coupled to KLH using 0.1% glutaraldehyde as described.[12] Mice were immunized subcutaneously (sc) with 50 μg 38C13-Id-KLH or control IgM-KLH emulsified in Syntex adjuvant formulation-1 (SAF-1) (Syntex, Palo Alto, CA) as previously described.[13] The final concentrations of the components were 0.2% Tween 80, 2.5% Pluronic L121, 5% squalane, 100 μg/mL N-acetyl-muramyl-L-threonyl-D-isoglutamine ([Thr[1]]-MDP), and 250 μg/mL 38C13-Id-KLH or control IgM-KLH. [Thr[1]]-MDP was kindly provided by A.C. Allison and N.E. Byars (Syntex).

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Serum anti-KLH assay. The KLH carrier provides a convenient internal control to establish the degree of immune competence of the animal. Enzyme-linked immunosorbent assays (ELISA) were performed using KLH coated microtiter plates. Mouse serum samples were serially diluted. Goat antimouse IgG antibody coupled to horseradish peroxidase (HRP) was used as a detector.

Serum anti-idiotypic antibody. Microtiter plates were coated with 38C13-Id and mouse serum was serially diluted. Binding of antibodies in the serum to 38C13-Id was detected by goat antimouse IgG-HRP antibodies that had been absorbed against 38C13-Id. Serum anti-idiotypic antibody levels were quantitated by comparing sera titration curves to a standard curve obtained with a known concentration of monoclonal anti-idiotype antibody. In each ELISA, sera obtained from mice immunized with control IgM-KLH were included as negative controls. Such sera never demonstrated any titratable binding activity on 38C13-Id.

Syngeneic BMT. Donor mice, 8 to 12 weeks old, were euthanized, and both left and right femurs and tibiae were removed and cleaned of all soft tissue. The ends of each bone were removed and the bone marrow was collected with RPMI-1640 medium containing 1% penicillin/streptomycin (P/S) using a 25-gauge needle and 12-mL syringe. The bone marrow was filtered through a nylon mesh and washed three times before use. Viable cells were counted as determined by trypan blue exclusion. Recipient mice, 8 to 12 weeks old, were lethally irradiated with 950 R total body irradiation (TBI) in a Philips x-ray unit (Germany; 250 kV, 15 mA). Irradiated recipients were injected intravenously with 20×10^6 bone marrow cells in 0.5 mL RPMI + P/S in the lateral tail vein. We have established 950 R as a lethal dose of radiation for the C3H strain, which led to the death of all unprotected mice within 10 to 15 days.

Bone marrow chimeras were allowed approximately 3 weeks recuperation, during which time they were maintained in a sterile microisolator cage unit and received sterile food and water. Overall postoperative mortality was less than 10%, and all surviving mice were clinically healthy.

Analysis of data. Mice were checked daily to determine the date of death. Statistical comparisons of survival were performed using the generalized Wilcoxon test of Gehan.[16] Mice surviving more than 120 days after tumor challenge were euthanized and were reported as long-term survivors.

RESULTS

Id immunization 3 and 5 weeks post-BMT. The first series of experiments was designed to determine the optimal timing of Id vaccination relative to lethal dose TBI and syngeneic marrow reconstitution. Initial experiments showed that mice prepared in this manner begin to recover the ability to make a primary antibody response to the KLH carrier protein as early as 3 weeks post-BMT. Based on this information we tested the protective effect of Id immunization against a subsequent lethal tumor challenge at two time points post-BMT. Approximately 50 C3H/HeN mice were irradiated with 950 R TBI and then reconstituted intravenously with 20×10^6 bone marrow cells from normal syngeneic donors on the same day. They were then randomly assigned to five groups of approximately 10 mice each. Mice received sc immunizations with 38C13 Id-KLH or with control IgM-KLH in SAF-1 at 3 weeks, at 5 weeks, or 38C13 Id-KLH at both 3 and 5 weeks post-BMT. Serial serum samples were obtained from each group of animals for assay of serum anti-idiotypic antibody, and 2 weeks after the last immunization they were challenged with 1,000 viable 38C13 tumor cells ip and followed for survival. The survival data from this experiment are presented in Fig 1. Transplanted mice which had been immunized with 38C13 Id-KLH in SAF-1 at 3 weeks survived significantly longer (median survival time 26 days) than control animals which had been immunized with an irrelevant IgM-KLH (median survival 18 days) ($P = .004$ by generalized Wilcoxon test of Gehan). Transplanted mice specifically immunized against tumor idiotype at 5 weeks also demonstrated significantly longer survival (median survival 28 days) after tumor challenge compared with controls (median survival 21 days) ($P = .004$ by generalized Wilcoxon test of Gehan). In addition, specific immunization at 5 weeks resulted in a significant number of long-term survivors (approximately 40%) not seen with specific immunization at 3 weeks or with either control group. This latter degree of protection was comparable with that observed in a group of 13 nontransplanted immunized mice challenged with the same preparation of tumor (77% long-term survivors, $P = .07$ by generalized Wilcoxon test of Gehan; survival curve not shown). Interestingly, two immunizations at 3 and 5 weeks did not confer any additional

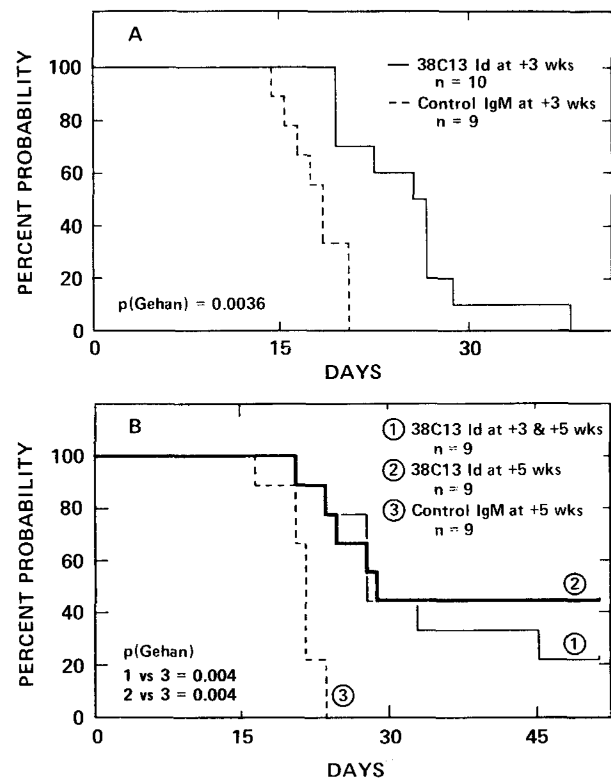

Fig 1. (A) Survival of C3H/HeN mice given a single immunization with 38C13 Id-KLH + SAF-1 or control IgM-KLH + SAF-1 3 weeks after 950 R TBI and reconstitution with syngeneic bone marrow. Mice were injected with 1,000 38C13 tumor cells ip at 5 weeks post-BMT. Survival (percent probability) was recorded in days posttumor challenge. (B) Survival of C3H/HeN mice given single immunizations with 38C13 Id-KLH + SAF-1 or control IgM-KLH + SAF-1 at 5 weeks, or a combination of specific immunizations at 3 and 5 weeks, following 950 R TBI and reconstitution with syngeneic bone marrow. All three groups of mice were injected ip with 1,000 38C13 tumor cells from the same preparation of tumor at 7 weeks post-BMT.

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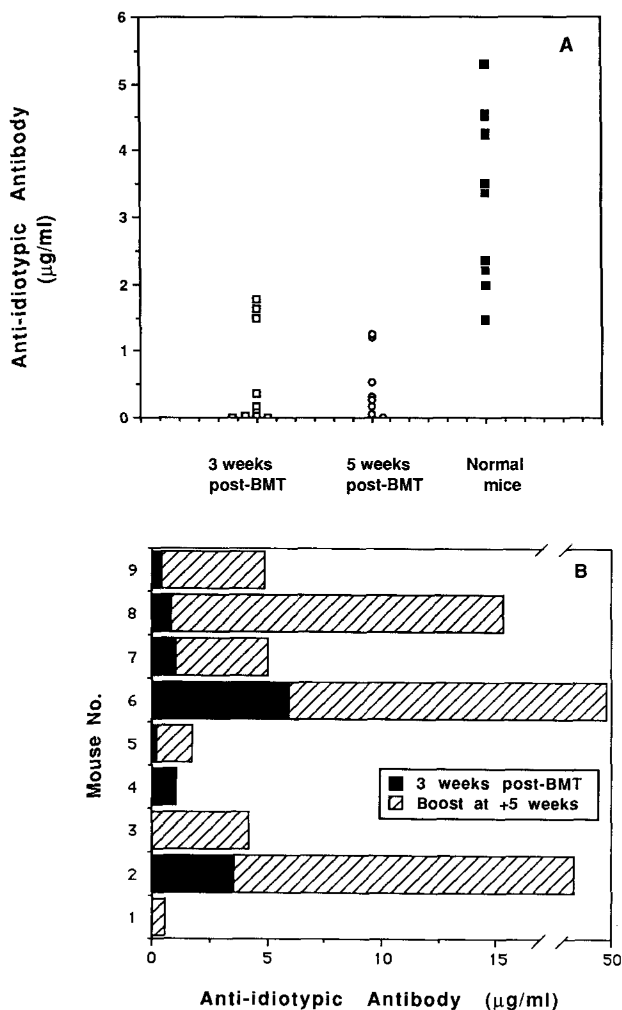

Fig 2. (A) Serum anti-38C13 Id antibody levels of the individual transplanted mice given single specific immunizations in Fig 1, and of normal mice immunized with the same preparation of 38C13 Id-KLH + SAF-1. Sera were obtained at the time of tumor challenge, 2 weeks after immunization. (B) Serial serum anti-38C13 Id antibody levels of the nine individual transplanted mice in Fig 1B that were immunized with 38C13 Id-KLH + SAF-1 3 and 5 weeks post-BMT. Serum samples for each animal were obtained 2 weeks after each immunization and assayed in a single ELISA.

survival benefit or increase in the proportion of long-term survivors over a single specific immunization at 5 weeks (Fig 1B).

Serum anti-idiotypic antibody levels were measured in these mice as another indicator of successful induction of idiotype-specific immunity. A serum sample collected from each animal at the time of tumor challenge was assayed for anti-idiotypic antibody in a single ELISA so that the range of antibody levels between animals from all five groups could be compared. As shown in Fig 2A, low but detectable levels of anti-38C13 Id antibody were found in lethally irradiated and marrow reconstituted mice that had been specifically immunized at both 3 and 5 weeks posttransplant. However, there was no significant difference in mean serum anti-idiotypic antibody levels between animals specifically immunized at 3 weeks and animals immunized at 5 weeks. No significant binding activity was detected in the sera of control mice which had been immunized with an irrelevant IgM. For comparison, serum anti-idiotypic antibody levels are also shown for the 13 normal mice 2 weeks after a single immunization with the same preparation of Id. Sera from each animal given the combination of specific immunizations were obtained 2 weeks after each immunization and assayed in parallel for anti-idiotypic antibody. Figure 2B shows the serial antibody levels of individual mice from this group following each immunization. In all but one animal, antibody levels were boosted by the second immunization. Although the magnitude of the boost was variable, in four of the animals anti-idiotypic antibody levels were boosted 10-fold or greater. Thus, the low levels of serum anti-idiotypic antibody detectable after immunization with Id protein at 3 weeks could be boosted significantly by a second immunization 2 weeks later. A nonspecific contribution of the SAF-1 adjuvant to this boost effect could not be ruled out.

Previous studies in our laboratory have suggested that anti-idiotypic antibody plays the predominant role in resistance to tumor growth.[17] However, there was no correlation between anti-idiotypic antibody levels and the survival times of individual mice in this experiment (Fig 3).

Id immunization pre-BMT. We then explored the effect of Id immunization before lethal dose TBI and syngeneic marrow reconstitution, testing the possibility that some component of established antitumor immunity would persist through the lethal dose TBI. In a pilot experiment, five mice administered a single Id immunization 2 weeks before lethal irradiation and syngeneic marrow reconstitution were shown to have detectable levels of serum anti-idiotypic antibody at the time of BMT as well as 3 to 4 weeks post-BMT (data not shown). This finding was then confirmed in a larger experiment. Approximately 30 mice were randomly assigned to receive a single immunization with 38C13 Id-KLH or control IgM-KLH in SAF-1. Two weeks later both groups were tail bled and then received 950 R TBI followed by syngeneic marrow reconstitution. After 3 weeks' recuperation from the BMT procedure both groups again were tail bled and then

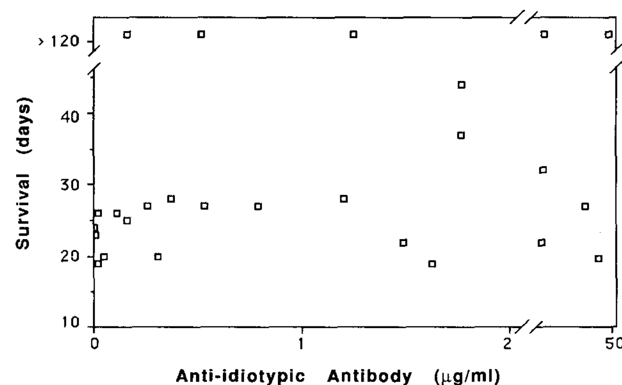

Fig 3. Correlation of anti-idiotypic antibody levels at the time of tumor challenge with the survival times of individual mice. All three groups of specifically immunized transplanted mice in Fig 1 are included in this analysis.

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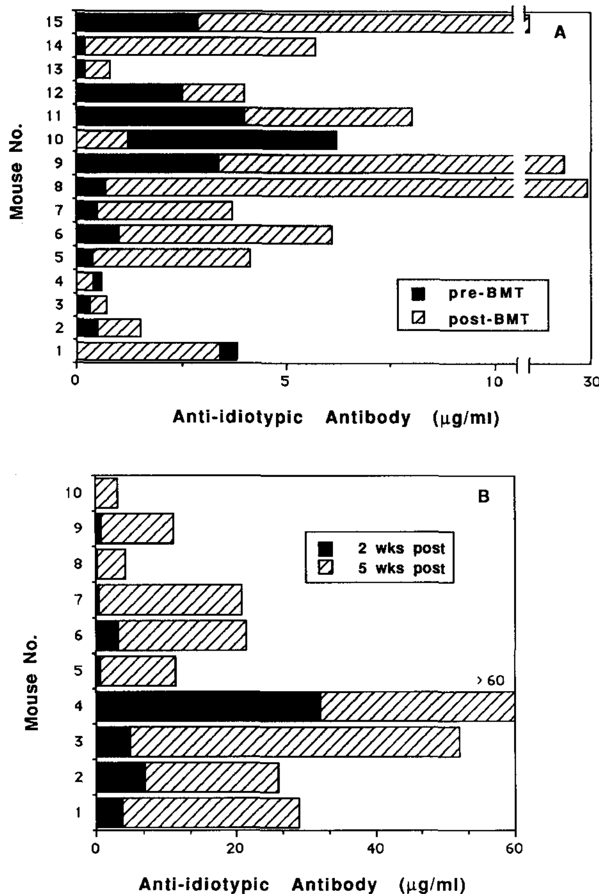

Fig 4. Serial serum anti-38C13 Id antibody levels of individual C3H/HeN mice that were immunized with the same preparation of 38C13 Id-KLH in SAF-1 and subsequently (A) transplanted 2 weeks later as described in Materials and Methods or (B) not manipulated further. Serum samples for all animals were obtained at 2 weeks postimmunization and again 3 weeks later at the time of tumor challenge (pre- and post-BMT, respectively, in [A]), and assayed in a single ELISA.

challenged ip with 1,000 viable 38C13 cells. Twenty nontransplanted mice that had been randomly assigned to receive single immunizations with 38C13 Id-KLH or control IgM-KLH in SAF-1 at the same time as the subsequently transplanted mice were also tail bled and challenged with the same preparation of tumor. Stored serial serum samples from each specifically immunized animal were assayed for anti-idiotypic antibody in a single ELISA. The results, shown in Fig 4A, clearly demonstrate that in every animal significant levels of anti-idiotypic antibody were found after BMT. In fact, a direct comparison of pre- and post-BMT antibody levels showed that in nearly all of the animals post-BMT antibody levels were actually higher than those obtained immediately pre-BMT. This finding was in accordance with the kinetics of the anti-idiotypic antibody response in normal mice at 2 and 5 weeks after Id vaccination, as shown in Fig 4B for the 10 normal specifically immunized mice in this experiment. Although there was some variation in the absolute levels of anti-idiotypic antibody between different animals, for each individual animal the levels 5 weeks postimmunization were higher than those 2 weeks postimmunization (mean magnitude of difference 16.9-fold). In comparison, the mean magnitude of the difference between pre- and post-BMT antibody levels in transplanted mice was 6.7-fold ($P = .096$ *v* nontransplanted mice by Student's *t*-test), and in three of these individual animals antibody levels were lower post-BMT. Thus, the ongoing anti-idiotypic humoral response was somewhat attenuated, but certainly not ablated, by TBI.

The survival of all four groups of mice after tumor challenge is shown in Fig 5. Lethally irradiated and syngeneic marrow reconstituted mice that had been specifically immunized demonstrated significantly prolonged survival (median survival 27 days) compared with their controls that had been immunized with irrelevant IgM (median survival 22 days). In addition, specific immunization pre-BMT resulted in a significant proportion of long-term survivors (approximately 30%). Similarly, nontransplanted specifically immunized mice demonstrated significantly prolonged survival compared with controls (median survival 16 days for the control group). As this immunoprotective effect resulted in 60% long-term survivors, the median survival time was not reached in the immunized group. Although the proportion of long-term survivors was greater in the nontransplanted group, the Kaplan-Meier survival curves of the two specifically immunized groups were not significantly different ($P = .48$ by generalized Wilcoxon test of Gehan). Thus, the protective effect of a single Id immunization pre-BMT against subsequent tumor challenge was not significantly compromised by TBI.

Again, there was no significant correlation between the serum anti-idiotypic antibody levels obtained at the time of tumor challenge and the survival times of individual transplanted or nontransplanted mice (correlation coefficient = .05; data not shown).

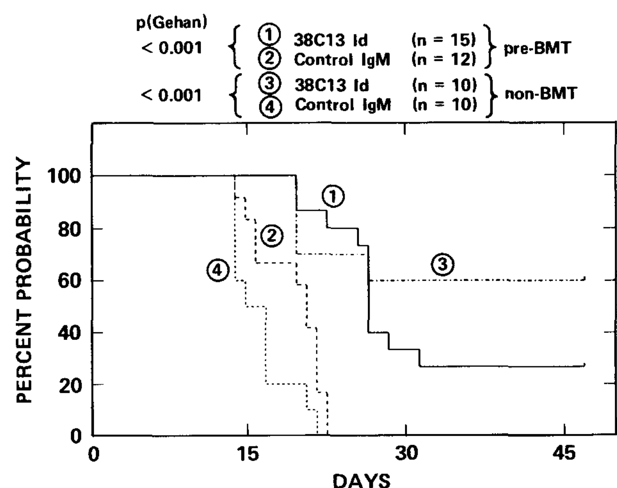

Fig 5. Survival of C3H/HeN mice given a single immunization with 38C13 Id-KLH + SAF-1 or control IgM-KLH + SAF-1 and either transplanted or not further manipulated 2 weeks later. All mice were challenged ip with 1,000 38C13 cells from the same preparation of tumor after 3 weeks recuperation from syngeneic BMT.

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DISCUSSION

Relapse of the underlying lymphoma, due to persistence of small amounts of residual disease after supralethal dose radiation and chemotherapy and/or incomplete purging of the autologous marrow graft, remains the major factor limiting the success of ABMT. Unlike allogeneic BMT, where a biologically significant graft-versus-leukemia effect may develop in the course of graft-versus-host disease (GVHD),[18] such an immune mechanism producing resistance to a residual tumor burden cannot be expected to occur in ABMT. Adjunctive therapeutic modalities designed to induce resistance to residual tumor cells post-ABMT must therefore be sought. One possibility that has been tested in a rat transplant model is the use of a passively administered monoclonal antibody (MoAb), which binds leukemia cells.[19] In the current study, we have tested the approach of active immunization with purified tumor-derived idiotypic IgM in the peritransplant setting using the 38C13 B-cell lymphoma model. Although there are other well-characterized animal models of active immunization against B-cell malignancies with purified surface or secreted immunoglobulin, the experiments presented in this report represent the first extension of this approach to the BMT setting.

We have shown that as early as 3 weeks post-BMT primary antibody responses directed against the idiotype of the tumor could be induced. Immune recovery at this time was sufficient such that Id immunization resulted in an immunoprotective effect against a lethal dose tumor challenge (Fig 1A), although near full resistance against the tumor was not elicited until 5 weeks post-BMT (Fig 1B). The anti-idiotypic humoral response generated at 3 weeks post-BMT could be boosted by a second immunization 2 weeks later (Fig 2B), but this boost did not result in any additional protection against the tumor (Fig 1B). This apparent lack of correlation between anti-idiotypic antibody level and protection against tumor challenge may reflect the presence of a threshold level, above which excess antibody confers no additional survival benefit. Indeed, it has been shown that very low concentrations of less than 1 μg/mL of syngeneic monoclonal anti-idiotype antibody are sufficient to perform antibody-dependent cell-mediated cytotoxicity in vitro in this model, and passively administered MoAb in a total dose as low as 1 μg per animal is sufficient to produce protection in vivo.[20] Alternatively, this lack of correlation may reflect a limitation of the assay system used, that it does not detect antibody of the IgM isotype, which may comprise a significant component of this primary humoral response. It is also possible that antibody affinity, rather than absolute levels, may be a more important factor.

In autologous or syngeneic BMT the nature and severity of transplant-related immune depression and time course of subsequent recovery can be expected to be determined in large part by the marrow ablative chemo-radiotherapy regimen. Studies of functional recovery of the immune system after syngeneic BMT using the same strain of mice and lethal dose TBI in the range of 750 R have suggested that certain helper T-cell functions in peripheral lymph nodes, as well as antibody production to a protein antigen studied, remain significantly depressed for up to 3 months post-BMT.[21,22] In a separate study, C3H mice treated with 800 R TBI and infused with syngeneic bone marrow were incapable of producing normal amounts of interleukin-2 or responding to concanavalin A for up to 2 months post-BMT.[23] These mice also did not demonstrate an active cytotoxic T-lymphocyte response until at least 4 weeks posttransplant.[24] Taken together, these functional studies would have predicted that immunization against Id would not have been effective in the early posttransplant period (before 6 weeks posttransplant). However, contrary to these studies, the unequivocal protective effect of Id immunization on survival as early as 3 and 5 weeks posttransplant indicates that the relevant immune effector mechanisms for protection against a tumor challenge were already intact at this time. Although the exact mechanism of antitumor immunity in the 38C13 Id vaccination model remains a subject of active investigation, anti-idiotypic antibodies play the major role in the destruction of 38C13 tumor cells, in part shown by the passive transfer of tumor immunity to naive mice with immune serum but not with immune cells.[17] We suggest that the kinetics of recovery of immunologic function after BMT are complex. Indeed, functional studies may be influenced by the lymphoid compartment under study, as splenocytes from lethally irradiated C3H mice infused with syngeneic marrow in the study cited above[22] demonstrated no reduction in immune function compared with nonirradiated controls at any time point post-BMT.

In accordance with our findings, Skorski and Kawalec [25] were able to successfully induce resistance to L1210 leukemia when immunization with chemically treated whole leukemic cells following lethal irradiation and syngeneic marrow grafting was started 2 weeks post-BMT and continued at weekly intervals for 3 additional weeks. Thus, tumor-specific immunity could be successfully induced by active immunization soon after recovery from lethal irradiation and syngeneic BMT in this model as well, although the nature of the revelant tumor-specific antigen on L1210 cells has not been as precisely characterized. Recovery of immunologic function after ABMT in humans, although less well studied than its allogeneic counterpart, appears to proceed at a more rapid rate, and the prolonged depression of immune function developing as a consequence of GVHD and its treatment are not observed.[26-28] Nevertheless, whether immune depression post-ABMT poses an obstacle to Id immunization in humans remains to be determined. The future use of cytokines and growth factors accelerating immunologic recovery post-BMT may impact favorably in this regard.

We have also demonstrated that a single Id immunization before syngeneic BMT resulted in lasting protection against challenge with tumor post-BMT (Fig 5). Immune mice demonstrated an ongoing humoral anti-idiotypic response that was in fact greater post- than pre-BMT in almost all individual cases (Fig 4A). Nevertheless, the magnitude of this response post-BMT did appear to be attenuated when compared with nontransplanted control animals. It is important to note that the conditioning regimen (950 R TBI), analogous to TBI schedules used in human BMT, was truly bone marrow stem cell ablative. Specifically, it has been

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shown that even a slightly lower dose of 750 R TBI in C3H mice results in greater than 99% stem cell reduction, as assayed by standard in vitro granulocyte-macrophage colony assay (CFU-GM).[21] Moreover, C3H mice prepared with 800 R TBI and reconstituted with marrow from allogeneic donors were subsequently shown to be full chimeras.[23] However, the 950 R TBI that was insufficient to eradicate an ongoing immune response, unlike conditioning regimens used in humans, may be insufficient to eradicate a tumor. Nevertheless, there are a number of examples of persistence of host immunity in humans. Supporting this possibility are data showing the persistence of host isohemagglutinin in ABO-incompatible marrow graft recipients for up to 4 months postgrafting,[28] and in one study antibodies against multiple viral or bacterial antigens did not show any reduction after ABMT when compared with pretransplant levels.[27] Furthermore, recipients successfully immunized with tetanus toxoid 1 week pre-BMT showed persistence of the antibody response following TBI.[29] Our conclusion from the data is that a major component of antitumor immunity, if successfully established in the recipient before lethal dose TBI, does persist post-BMT. There remains the possibility that this immunity post-BMT may be derived from repopulating syngeneic donor cells stimulated by antigen still present in the recipient, but the kinetics of the anti-idiotypic humoral response was most consistent with that of an ongoing established immune response (Fig 4A). Definitive proof that this antibody response is not entirely donor-derived would require the availability of allotype congenic strains on the C3H background.

We are currently extending these studies to the setting of established tumor, in which pre- and post-BMT Id vaccination are being tested in combination in tumor-bearing mice. Previous studies of chemoimmunotherapy of established transplanted tumor in this animal model have established that concurrent reduction of the tumor burden by cyclophosphamide is required for the optimum effect of active immunotherapy.[13] One may speculate that supralethal TBI, representing an even more potent cytoreductive therapy, may further enhance the antitumor effect of Id vaccination. The experiments presented here demonstrate the feasibility of active Id immunization following this marrow ablative regimen, and they form the basis for a model for peri-BMT Id vaccination in humans.

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