Ds E P I T E A D V A N C E S in supportive care and the refinement of chemoradiotherapy conditioning regimens, persistence of the underlying malignancy remains the major obstacle limiting the success of autologous bone marrow transplantation (BMT) for non-Hodgkin’s lymphomas. We used the 38C13 murine B-cell lymphoma model to explore the approach of transferring tumor antigen-specific immunity with syngeneic BM as a protective element. Mice serving as syngeneic marrow donors were twice immunized with tumor-derived surface Ig protein, the idiotype of which serves as a tumor-specific antigen, or with a control Ig of matched isotype. Naive lethally irradiated recipients reconstituted with marrow from immune donors showed serologic tumor idiotype-specific immunity, as well as protection against lethal tumor challenge. The immunoprotective effect of immune marrow was also shown in lethally irradiated recipients partially protected by specific immunization post-BMT. Combined donor and recipient immunization also replaced the requirement for the booster immunization of the donor. These results provide the rationale for active immunization with purified surface Ig from autologous tumor as an adjunct to autologous BMT in humans.

MATERIALS AND METHODS

Mice and tumor. C3H/HeN female mice 6 to 8 weeks of age were obtained from Simonsen Laboratories (Gilroy, CA). The carcinogen-induced 38C13 B-cell lymphoma has been previously described. Inoculation of 1,000 38C13 tumor cells intraperitoneally (IP) into syngeneic C3H/HeN mice results in progressive tumor growth and median survival times of approximately 19 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 IgM (κ) on the cell surface. 38C13-Id and a control IgM(κ) were purified from ascites and coupled to keyhole limpet hemocyanin (KLH) using 0.1% glutaraldehyde as described. Donor or recipient mice were immunized subcutaneously (SC) with 50 μg 38C13-Id-KLH or control IgM-KLH emulsified in Syntex adjuvant formulation-1 (SAF-1) as immunoprotection against lethal tumor challenge, with BM obtained from Id-immunized donors. Based on the success of this approach, we propose a strategy for the application of these findings to the autologous BMT setting.

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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.** As previously described, microtiter plates were coated with 38C13-Id. Mouse serum was serially diluted. Binding of antibodies in the serum to 38C13-Id was detected by goat antimouse IgG-HRP antibodies, which had been absorbed against 38C13-Id. Serum anti-idiotypic antibody levels were quantitated by comparing sera titration curves with a standard curve obtained with a known concentration of a mixture of purified monoclonal antiidiotypic antibodies. In each ELISA, sera obtained from mice immunized with control IgM-KLH were included as negative controls. Such sera never showed any titratable binding activity on 38C13-Id.

**Syngeneic BMT.** The procedure has been previously described in detail. Briefly, 8- to 10-week old donor mice, which had been immunized specifically with 38C13-Id-KLH, or nonspecifically with control IgM-KLH, in SAF-1 were killed and BM was collected from both left and right femurs and tibiae. The BM was filtered through a nylon mesh and washed three times before use. Viable cells were counted as determined by trypan blue exclusion. Eight to 10-week old naive recipient mice were lethally irradiated with 950 R total body irradiation (TBI) in a Philips x-ray unit (250 kV, 15 mA). Irradiated recipient mice were injected intravenously with 20 x 10^6 BM cells in 0.5 mL RPMI 1640 medium in the lateral tail vein. To the extent that 950 R TBI is greater than 99% marrow cell ablative for the C3H strain, it models TBI schedules used in human BMT.

BM 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, supplemented with tetracycline (400 mg/L). 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 done using the generalized Wilcoxon test of Gehan. Mice surviving greater than 90 days after tumor challenge were euthanized and were reported as long-term survivors.

**RESULTS**

Protective effect of immune marrow requires boosting of the donor. We first performed a pilot experiment, designed to test the ability of BM pooled from immune donors to transfer specific immunity against the 38C13 idiotypic protein. Single immunization of normal syngeneic C3H/HeN mice with 50 μg 38C13-Id-KLH in SAF-1 reproducibly induces serum anti-idiotypic antibody levels of 10 to 20 μg/mL and results in approximately 70% protection when these mice are given a lethal tumor challenge. Because our studies in this model have suggested a primary role for the humoral anti-idiotypic response in the underlying mechanism of protective immunity, we first measured the production of anti-idiotypic antibody in lethally irradiated recipients of marrow from these immune donors. Five lethally irradiated recipients were reconstituted with marrow prepared from syngeneic donor mice that had been given a single SC immunization with 38C13-Id-KLH in SAF-1 2 weeks earlier. Three weeks postsyngeneic BMT, the production of anti-idiotypic antibody by the recipients was assessed by determination of serum anti-idiotypic antibody levels by ELISA. None of these recipient mice demonstrated detectable titers of serum anti-idiotypic antibody. A parallel experiment was performed to test the ability of immune marrow to transfer protective immunity against 38C13. Twenty-four lethally irradiated C3H/HeN mice were randomly assigned to receive marrow prepared from donors that had been immunized either with 38C13-Id-KLH or with control IgM-KLH in SAF-1 2 weeks earlier. After 3 weeks’ recuperation, both groups of recipient mice (12 mice per group) were challenged with 1,000 viable 38C13 tumor cells and monitored for survival. Although two recipients of immune marrow survived this lethal tumor challenge, the median survival times of both groups were identical (21 days), and there was no significant difference in their log rank survival curves (P = .47, data not shown). Analysis of serum samples from recipients of immune marrow obtained at the time of tumor challenge in this experiment also showed no detectable levels of either anti-idiotypic or anti-KLH antibodies. The exogenous KLH carrier protein can serve as an informative internal control antigen, and the lack of transfer of immunity to KLH from donor to recipient suggested that perhaps single immunization of donor mice was not sufficient for the successful transfer of antigen-specific immunity under the experimental conditions used. We tested this hypothesis by boosting immune donor mice against 38C13-Id in the following experiment. Syngeneic mice serving as marrow donors were immunized with either 38C13-Id-KLH or control IgM-KLH in SAF-1 and then again with the respective immunogen 2 weeks later. One week after this booster immunization, marrow pooled from these immune or nonspecifically immune donors was used to reconstitute lethally irradiated C3H/HeN recipients. After 3 weeks of recuperation, these recipients of immune or nonspecifically immune marrow were challenged IP with 1,000 38C13 tumor cells and monitored for survival. At the time of tumor challenge, serum samples were obtained from recipients of immune marrow and assayed for anti-idiotypic antibody. Serum anti-idiotypic antibody titers of five representative recipients are shown in Fig 1A. Low but detectable levels of anti-idiotypic antibody (mean antibody level, 0.3 μg/mL ± 0.1 μg/mL) were shown in all samples. Control sera from recipients of nonspecifically immune marrow showed no binding to 38C13-Id (complete lack of absorbance of undiluted serum; data not shown). This low level of humoral immunity in recipients of immune marrow was associated with full protective immunity against the 38C13 tumor. As shown in Fig 1B, recipients of immune marrow showed significantly prolonged survival after tumor challenge (70% long-term survivors) compared with recipients of nonspecifically immune marrow (median survival, 20 days; P < .005).

**Protective effect of immune marrow in 38C13 immune recipient mice.** We next performed experiments designed to test the protective effect of immune BM in the setting of post-BMT immunization of the lethally irradiated recipient. C3H/HeN mice serving as marrow donors were immunized SC with 38C13-Id-KLH or control IgM-KLH in SAF-1 and given a booster immunization with the respective immunogen 2 weeks later. One week later, marrow pooled from these immune or nonspecifically immune
mice is shown in Fig 2B. The tumor challenge was uniformly lethal in control nonimmune recipients of nonimmune marrow (group 3, median survival 21 days). Specific immunization of recipients of nonimmune marrow on the day of transplantation was associated with prolongation of survival (median survival, 25 days; \( P = .05 \)) and resulted in 25% long-term survivors (group 2). The enhanced tumor resistance of group 2, compared with group 3, confirms and extends our prior results showing the protective effect of Id immunization of the BMT recipient in the early posttransplant period. Of primary interest, specifically immunized recipients of immune marrow (group 1) demonstrated superior survival compared with the other two groups (\( P = .039 \) vs group 2; 70% long-term survivors). Thus, the independent immunoprotective effect of immune marrow could be demonstrated, even in lethally irradiated recipients that had been afforded an increased level of protection by Id immunization alone posttransplantation.

We next tested the ability of a single Id immunization of the donor to transfer protection in the setting of an immune recipient. C3H/HeN mice serving as marrow donors received a single SC immunization of 38C13-Id-KLH or control IgM-KLH in SAF-1. Two weeks later, lethally irradiated syngeneic recipient mice were randomly allo-

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The excerpt above contains technical scientific content, including figures and tables, describing experiments and results related to immunology and tumor challenge. The text discusses the effects of Id immunization on survival rates and the potential for transferring protection from donor to recipient in the context of bone marrow transplantation. The figures illustrate data on survival rates and antibody titers, showing statistical comparisons and survival plots for different groups of mice.
transfer of an anti-tumor immunity

Fig 3. Combined donor and recipient Id immunization using singly immunized syngeneic donors. Lethally irradiated recipients were reconstituted with either immune or nonspecifically immune marrow. Both groups of recipients then received a single specific immunization on the day of BMT. Shown is the survival of both groups following IP challenge with a single preparation of 38C13 tumor cells.

Fig 4. Strategy for autologous BMT combined with active specific immunotherapy against lymphomas. Id immunization before marrow harvest is achieved as a means to generate immune marrow used to reconstitute the host after high-dose chemotherapy. Post-BMT Id immunization complements this approach.

DISCUSSION

The individual experiments presented here clearly demonstrate that specific anti-tumor immunity can be transferred to lethally irradiated mice with syngeneic BM. Sufficient immunization of marrow donors with purified Id protein from the 38C13 lymphoma was associated with the successful transfer of both serologic anti-idiotypic immunity, and of primary interest, protective immunity against challenge with the tumor. It is important to note that in each experiment donor BM cells were washed extensively before reconstitution of recipients (see Materials and Methods); therefore, the detection of anti-idiotypic antibody in the serum of naive recipients 3 weeks post-BMT represented true antibody production in the recipient and not simply passive transfer of serum antibody from the immune donor (Fig 1A). Of interest, the relatively low serum levels of anti-idiotypic antibody in both naive and immune recipients of immune marrow (see Results) were associated with full protective immunity (Fig 1B). Although the underlying mechanism of protective immunity in this model is believed to be antibody-dependent, this lack of correlation between anti-idiotypic antibody levels and the survival times of individual mice challenged with tumor has been noted and discussed previously.

The direct transfer of antitumor immunity with donor marrow conflicts with recent studies in human BMT suggesting that the expression of donor-derived antigen-specific immunity requires both an immune donor and recipient. Nevertheless, the protective effect of immune marrow was clearly demonstrated even in recipients that were partially protected from tumor challenge by Id immunization post-BMT (Figs 2 and 3). Furthermore, when donor and recipient Id immunization were combined, successful transfer of donor immunity was accomplished without a booster immunization of the donor (Fig 3). In this setting, differentiated antibody-producing cells transferred in the marrow from singly immunized donors may have added to a subsequent immune response initiated in the recipient by a posttransplant immunization.

These experiments were performed with whole, unfractionated marrow from immune donors. Efforts to characterize the mature and/or progenitor lymphoid subpopulations in immune marrow capable of transferring specific anti-tumor immunity may be worthwhile. In vitro depletion studies of immune marrow with pan-B and T-cell subset specific antibodies may further elucidate the mechanism of protective immunity in the 38C13 model. Furthermore, the use of purified subpopulations of immune lymphocytes may facilitate the determination of the requirements for optimal Id immunization of the marrow donor and precise dose of transferred BM cells required. Complementary studies exploring the requirements for successful secondary in vitro Id immunization of bone marrow cells in short- or long-term culture are also planned.

Taken together, our results provide a rationale for Id immunization in conjunction with human BMT for lym-
phoma. In this regard, our initial studies of active immunization of 12 chemotherapy-treated patients with lymphoma with autologous tumor-derived Id have demonstrated the successful induction of tumor idiotype-specific humoral and cellular immunologic responses, as well as associated objective tumor regression. The observations presented here are in principle directly applicable to Id immunization of the syngeneic marrow donor of an identical-twin BMT. The report of successful transfer of anti-KLH immunity from a KLH-immunized marrow donor to her identical twin recipient with leukemia supports this idea. The application of immune marrow as a transfer element to autologous BMT could be accomplished by Id immunization of the patient before BM harvest. If successful immunization of the host is accomplished, immune marrow used to reconstitute the patient after host conditioning would be spared the immunosuppressive effects of high-dose chemoradiotherapy. Inherent assumptions are that specific immunity can be preserved throughout ex vivo marrow purging and cryopreservation. The potential impact of the former can be evaluated in the animal model by the in vitro studies using pan-B and T-cell subset specific monoclonal antibody plus complement treatment of immune marrow proposed above. Figure 4 outlines one such strategy for this approach, in combination with post-BMT Id immunization.

We are extending our studies in the 38C13 model to establish the therapeutic effect of Id immunization combined with syngeneic BMT in mice bearing an established tumor. The demonstration that specific antitumor immunity can be transferred with immune BM also provides the opportunity to explore Id immunization of an allogeneic marrow donor in this model. Such an approach may hold promise for enhancing the GVL effect of allogeneic BMT.

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