The Detection of Specific Antibody Formation to Recall Antigens After Human Bone Marrow Transplantation

By Lawrence G. Lum, Nathan A. Munn, Moses S. Schanfield, and Rainer Storb

The results of this study show that donor-derived immunity can be detected and persists in long-term survivors with and without chronic graft-v-host disease (GVHD) after human marrow grafting. Seventy-one marrow recipients (60 long-term and 11 short-term survivors) were studied for the presence of specific serum IgG antibodies to tetanus toxoid (TT), and 46 marrow recipients (35 long-term and 11 short-term) were tested for antibodies to diphtheria toxoid (DT) and measles virus after marrow grafting using an enzyme-linked immunosorbent assay. Of the 60 long-term survivors, 31 were healthy and 29 had chronic GVHD. None of the recipients were immunized to the test antigens postgrafting. Most long-term healthy recipients exhibited antibody titers to the recall test antigens, whereas only a minority of those with chronic GVHD had antibody titers to recall antigens. In healthy long-term recipients (≥ one year postgrafting) whose donors were immune to the test antigens, 25 of 31 had titers to TT, 11 of 17 had titers to DT, and 12 of 20 had titers to measles. In recipients with C-GVHD, 13 of 29 had titers to TT, six of 15 had titers to DT, and six of 15 had titers to measles virus. Within 100 days postgrafting, 11 of 11 had anti-TT titers, ten of ten had anti-DT titers, and seven of eight had antimeasles virus titers.

EARLY EXPERIMENTS in mice and rats showed that the infusion of bone marrow, spleen cells, lymph node cells, or thymus cells from an immune donor could transfer specific immune reactivity.1–4 However, such adoptive transfer experiments showed only transient antibody-forming ability in the recipients. Studies in the canine marrow-grafting model showed that certain canine marrow recipients exhibited distemper titers after DLA-matched marrow transplantation without posttransplant immunizations to distemper.5 Because both the marrow donors and the recipients were immunized to distemper before marrow transplantation, the interpretation of such data was difficult.

Despite a large body of literature on immune reconstitution after human marrow transplantation (reviewed in ref. 6), information on the transfer of specific immunity from donors to recipients after human marrow transplantation is scarce. Earlier studies examined recipient’s delayed hypersensitivity responses to recall antigens and antibody responses to keyhole limpet hemocyanin, bacteriophage 4X174, and pneumococcal polysaccharide antigens.6–10 These studies showed primary and secondary responses to neoantigens by one to two years postgrafting. These studies did not address the question of transfer of specific antibody-forming ability from marrow donors to marrow recipients. One early report used keyhole limpet hemocyanin immunization of a twin donor as an immunologic marker for bone marrow graft acceptance in an identical twin transplanted for acute lymphocytic leukemia.11

This study investigates the transfer of specific antibody-forming ability from the marrow donor to the marrow recipient. The investigation shows that there is rapid development of this ability by recipient lymphocytes without postgrafting immunization to tetanus toxoid (TT), diphtheria toxoid (DT), and measles virus when the donor lymphocytes were immune to these antigens. The donor-derived immunity can be detected and persists in long-term survivors with and without chronic graft-v-host disease (GVHD). Such data on these common recall antigens will facilitate the development of immunization recommendations for the management of transplant recipients.

MATERIALS AND METHODS

Subjects. Seventy-one patients transplanted for hematologic malignancy or aplastic anemia were studied for the presence of serum antibody titers. Sixty-five received allogeneic transplants from HLA-identical siblings, five received syngeneic transplants, and one received an HLA-haploidentical transplant. In this study there were 14 chronic myelogenous leukemia, 15 acute lymphocytic leukemia, 27 acute nonlymphocytic leukemia, two Hodgkin’s lymphoma, one erythroleukemia, one lymphatic leukemia, and 11 aplastic anemia patients. Patients with hematologic malignancies were conditioned with cyclophosphamide and total body irradiation12–14 and patients with aplastic anemia were conditioned only with cyclophosphamide.15 Methotrexate, cyclosporine, or both were used as posttransplant prophylaxis for GVHD.16 Cyclosporine or antithymocyte globulin was used for the treatment of established acute GVHD, while others received steroids or a combination of the above.

Twenty-nine long-term survivors (>100 days postgrafting) developed chronic GVHD, and 20 of these were treated with either prednisone alone or in combination with cyclophosphamide, procarbazine, or azathioprine. Thirty-one healthy long-term survivors were studied. Seventy-one patients were studied pretransplant and posttransplant for IgG anti-TT titers, and 46 of these were also studied for IgG anti-DT and IgG antimeasles titers. None of the recipients were immunized to the test antigens pretransplant. None of the long-term survivors who were studied at ≥ one year postgrafting had received blood products in the six-month interval before testing. The 60 long-term survivors were studied between 320 and 3,295 days postgrafting. Twenty-five of these were studied longer than two years postgrafting. The bone marrow donors did not receive booster immunizations to the test antigens in the two-year period before marrow donation.

Serum collection. Serum samples were collected from marrow transplant recipients and their donors pretransplant and on the days posttransplant as indicated in Figs 1 through 3.

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Cytochrome. When there were differences in cytochrome sex markers, all but one of the recipients (30 out of 31) had lymphocytes of donor origin. One individual was a mixed chimera. Cytogenetics on the remaining donor-recipient pairs were not informative.

Enzyme-linked immunosorbent assay (ELISA). Serum IgG titers to TT, DT, and measles were measured using an ELISA modified from that described. Briefly, NUNC EIA plates (NUNC, Copenhagen, Denmark) were coated with 100 μl of either 1.0 Lf/mL TT or 2.0 Lf/mL DT (lots No. LP463P and DT254PD, respectively, Commonwealth of Massachusetts Department of Public Health, Boston), or a 1:100 dilution of measles antigen (lot No. X30-364, MA. Bioproducts, Los Angeles) diluted in Tris buffer, pH 9.6, 15 to 18 hours at 37°C. Plates were washed in phosphate-buffered saline (PBS) containing 0.05% polysorbate 20 (Sigma Chemical Co, St Louis) and blocked for one hour at 37°C with 10% bovine serum albumin in PBS. Plates were then washed in PBS-Tween solution and stored at -20°C. Plates were warmed and washed in PBS-Tween solution before use.

Anti-TT IgG standard was prepared from high-titer anti-TT human serum, depleted of non-IgG protein by QAE-A50 Sephadex (Pharmacia, Piscataway, NJ) and affinity purified over TT-linked Sepharose 4B (Pharmacia). The amount of specific IgG anti-TT antibody in the standard was directly calibrated in a polyclonal IgG ELISA using goat antihuman IgG specific underlayer (capture) antibody (Tago, Burlingame, Calif) and a peroxidase-conjugated goat antihuman IgG developer.

The antibody concentrations of unknown serum samples diluted on a logₐ basis for IgG anti-TT, IgG anti-DT, and IgG antimeasles were interpolated directly from the affinity-purified IgG anti-TT standard.

The optical density reading of the last positive serum dilution was compared with the linear portion of the linear regression line drawn from the human IgG anti-TT standard. Only optical density values between 0.1 and 1.0 were used for interpolation. Known dilutions of the anti-TT standard were placed into triplicate wells coated with TT on each plate. Therefore, IgG antibody units (AU) relative to the IgG anti-TT standard could be interpolated directly from the optical densities of the IgG anti-TT standard on each plate for DT and measles virus titers with 1 AU = dilution of serum x 1 ng/mL estimated by direct comparison with the IgG anti-TT standard. The goat antihuman IgG-specific peroxidase-conjugated developer would bind only with IgG bound to the specific antigen on the plate. The IgG-specific developer did not detect IgA, IgM, or IgE standards at the upper concentration of 20 ng/mL.

One hundred microliters of diluted serum was added to each microtiter well. All dilutions were done in 10% fetal calf serum in balanced salt solution. The plates were incubated for two hours at 37°C and washed with PBS-Tween solution. One hundred microliters of peroxidase-conjugated goat anti-human IgG antibody (lot No 9010401, Tago) diluted 1:1,500 in 10 mmol/L phosphate buffer was
added to each well, incubated at 37 °C for one hour, and washed with PBS–Tween. One hundred microliters of a 1.0 mg/mL solution of 2,2’-azinodi-(3-ethylbenzthiazoline sulfonic acid) in citrate buffer, pH 4, plus 0.03% H2O2 was added to all wells. The optical density was read at 414 nm after 30 minutes.

An optical density equal to 0.1 at a 1:1,000 serum dilution or above was considered a positive titer. Backgrounds were infrequently present at serum dilutions ≥1:100. Serum from agammaglobulinemic patients and from normal individuals known by history to be negative for a specific antigen had an optical density <0.1 at a serum dilution of 1:100.

Normal range for antibody titers. Forty normal individuals and 71 marrow donors ranging in age from 3 to 50 years served as controls to determine the normal interval. Normal range was defined as the interval above which 95% of the immune normal individuals studied had specific IgG titers to TT, DT, and measles virus. Titers below this interval were considered negative.

Statistics. For all analyses, the antibody titers were compared in a Wilcoxon rank sum test (two-tailed). For DT and measles titers, four and five patients, respectively, had donors who did not have titers and these were excluded from the statistical analysis, although they are shown in the figures. All donors had TT titers.

RESULTS

Antibody titers to TT. Figure 1 summarizes the serum IgG anti-TT titers in AU for the normal controls, the marrow donors, and the marrow recipients pretransplant and posttransplant. Each marrow graft recipient was studied only once after marrow grafting. The study was not sequential. Figure 1 shows IgG anti-TT titers on healthy recipients and those with chronic GVHD. All of their donors had normal IgG anti-TT titers. Between one and two years, 15 of 20 (75%) healthy survivors and seven of 14 (50%) recipients with chronic GVHD had anti-TT titers. After two years, ten of 11 (91%) healthy survivors and five of 15 (33%) recipients with chronic GVHD had anti-TT titers. Statistical comparis-

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A (+) signifies a titer ≥1:1,000, a (-) signifies a titer <1:1,000 by ELISA. The combination of donor (-), patient pre-tx (+), and patient post-tx (+) did not occur. Pre-tx, pretransplant; post-tx, posttransplant.
sons between the healthy long-term chimeras and the marrow-graft recipients with chronic GVHD show that the healthy chimeras had significantly higher IgG anti-TT titers than their counterparts with chronic GVHD ($P < .006$).

**Antibody titers to DT.** Figure 2 summarizes the serum IgG anti-DT in AU for the normal controls, the marrow donors, and the marrow recipients pretransplant and posttransplant. Four donors did not have detectable IgG anti-DT antibody titers. Each transplant recipient was studied once postgrafting. Ten of ten (100%) recipients 100 days postgrafting had anti-DT titers in the normal range. Between one and two years, eight of 11 (73%) healthy survivors and three of nine (33%) recipients with chronic GVHD had anti-DT titers. After two years, three of six (50%) healthy survivors and two of six (33%) recipients with chronic GVHD had anti-DT titers. There was no statistical difference between recipients with and without chronic GVHD.

**Antibody titers to measles virus.** Figure 3 summarizes the serum IgG antimeasles virus in AU for the normal controls, the marrow donors, and the marrow recipients pretransplant and posttransplant. Five donors did not have detectable IgG antimeasles virus antibody titers. Each transplant recipient was studied once postgrafting. Seven of eight (88%) recipients had antibody titers to measles virus 100 days postgrafting. Eight of 12 (67%) healthy survivors and one of nine (11%) recipients with chronic GVHD had antimeasles virus titers between one and two years after marrow grafting. After two years, four of six (67%) healthy survivors and four of six (67%) recipients with chronic GVHD had antimeasles virus titers. There was no statistical difference between the healthy long-term survivors and those with chronic GVHD.

**Chronological recovery of specific antibody titers in a single patient.** One recipient (UPN 1353) with chronic GVHD was studied 791 and 1,061 days postgrafting. She received an HLA-identical, mixed lymphocyte culture nonreactive marrow graft from her brother. The recipient had a measles virus titer of 23 AU/mL and had no titers to TT or DT 791 days postgrafting. By 1,061 days postgrafting, the recipient had 211 AU of IgG antimeasles virus, 13 AU of IgG anti-TT, and 27 AU of IgG anti-DT. Cytogenetic sex markers on both occasions showed the lymphocytes to be of donor (XY) origin. Both donor and recipient were immune to all three antigens pretransplant.

**Evidence for transfer of specific antibody immunoreactivity in donor-recipient pairs.** Data in Table 1 summarize the titer data for all recipients. Table 1 contains both informative and noninformative data; the data shown in boldfaced type are informative. Table 2 gives the specific details of the six informative cases in boldfaced type selected from Table 1. In three of the six cases shown, donors had measurable antibodies to measles or diphtheria antigens, while recipients were negative before transplantation. When tested 80 to 465 days after transplantation, all three recipients made antibodies presumably through donor-derived cells. Conversely, three donors did not make antibodies to measles or diphtheria antigens, while their respective recipients did when tested before grafting. When tested 385 to 616 days after grafting, all three recipients failed to make antibody, signifying the disappearance of host-derived immunity and the establishment of donor cells not previously exposed to these antigens.

**DISCUSSION**

The assumption that antibody production to diphtheria and measles beyond day 100 after allogeneic bone marrow transplantation represents immunity transferred with donor cells is based on at least four pieces of evidence: (1) recipients with antibody production after bone marrow transplantation had donors immune to the same antigens pretransplant; (2) when donors failed to make antibodies, recipients were found to be negative after bone marrow transplantation, including recipients who made antibodies before bone marrow transplantation; (3) immunoglobulin allotype production of host type ceases by day 120 after bone marrow transplantation in 97% of the recipients; and (4) in recipients with ABO incompatible transplants, host-type isohemagglutinin production ceases at a median of 40 days after bone marrow transplantation, although immunity may persist in isolated cases for up to five months postgrafting.

The majority of long-term healthy recipients developed serum IgG antibody titers to TT, DT, and measles. It is not likely that this findings represent environmental re-immunization, particularly in the short period of time postgrafting. Although one study has shown mixed chimerism in the lymphocytes of two marrow graft recipients and host-type RBC markers more than one year postgrafting, it is unlikely that host-type B cells could account for the normal levels of IgG anti-TT, anti-DT, and anti-measles antibodies in so many marrow-graft recipients. Marrow-graft recipients who did not develop titers to TT, DT, or measles virus posttransplant show that the transfused antibody did not contribute to antibody titers postgrafting.

Most marrow-graft recipients with chronic GVHD failed to develop antibody titers to recall antigens. The reasons for failure to develop specific antibody titers in this group of recipients are not clear. Both chronic GVHD and the treatment thereof with immunosuppressive regimens could suppress the development of antibody-forming cells.

Immunoglobulin allotyping confirmed donor-origin Ig allotypes in two donor-recipient pairs who were confirmed to be IgG allotype mismatched; however, we were not technically able to type affinity-purified IgG anti-TT–specific antibodies from these individuals to show allotype transfer of IgG anti-TT directly.
Mature T and B cells transferred in the marrow inoculum were probably responsible for producing specific antibodies. Recent studies using a TT-stimulated specific antibody synthesis showed that purified T cells from long-term marrow-graft recipients can help IgG anti-TT antibody production in vitro after stimulation with TT in cocultures with immune donor B cells and that recipient B cells can produce IgG anti-TT in the presence of donor helper T cells. Our findings are consistent with a recent study using human bone marrow obtained after primary and booster immunizations with TT that showed B cells could spontaneously synthesize IgG anti-TT and could be stimulated with pokeweed mitogen to produce IgG anti-TT. The presence of marrow mononuclear cells capable of producing IgG anti-TT in immune subjects and the presence of antigen-specific lymphocytes in the peripheral blood of long-term marrow recipients support the contention that mononuclear cells in the marrow inoculum are responsible for the transfer of immunity.

Persistent antigen in the reticuloendothelial system did not play a major role in the re-presentation of antigen to newly maturing cells from the transplanted marrow, since immune recipients who received marrow grafts from nonimmune donors did not develop specific antibody titers.

In the first 100 days, persistent differentiated host B cells and passive transfusion of antibody may contribute to detectable antibody. However, the levels of specific antibody detected at 100 days postgrafting suggest a major contribution by B cell engraftment, since it would be nearly impossible to attain normal antibody levels from packed red cells and/or platelet transfusions.

Insufficient numbers of donor–recipient pairs prevented an analysis of factors that might influence the formation of specific antibody, such as the relationship between identical twin transplants and healthy allogeneic recipients, the preparative regimen, the pretransplant diagnosis, and the type of posttransplant immunosuppression.

These results help to explain why more transplant recipients do not develop life-threatening infections with diseases against which their donors had been immunized. In more than 1,500 patients transplanted in our center, only one case of measles has occurred. A recipient developed measles after being exposed to his donor who developed measles postgrafting. In this case, the donor was probably not immune to measles pretransplant. These clinical observations suggest that many recipients are indeed protected by specific antibodies to recall antigens produced by donor-derived B cells. If transfer of antibody-forming capability to viruses occurred as satisfactorily as transfer of specific antibody-forming capabilities to measles, immunity to mumps, rubella, and polio may be transferred equally well. However, only follow-up on marrow recipients will determine whether their circulating levels of specific antibody are protective.

The proportion of marrow-graft recipients developing specific antibody titers or the levels of specific antibody might be increased by booster immunizations to the donor pretransplant. Healthy recipients or recipients with chronic GVHD who have not developed specific antibody titers by one year after transplant should be immunized with appropriate vaccines and evaluated for specific antibody titers. Those with chronic GVHD may not respond to immunizations and healthy individuals may respond to recall antigens as if there were “neoantigen” antigens.

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The detection of specific antibody formation to recall antigens after human bone marrow transplantation

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