Anti-Asialo GM1 Antiserum Treatment of Lethally Irradiated Recipients Before Bone Marrow Transplantation: Evidence That Recipient Natural Killer Depletion Enhances Survival, Engraftment, and Hematopoietic Recovery

By Pierre Tiberghen, Dan L. Longo, John W. Wine, W. Gregory Alvord, and Craig W. Reynolds

Natural killer (NK) cells are reported to have an important role in the resistance of lethally irradiated recipients to bone marrow transplantation (BMT). Therefore, we investigated the effects of recipient NK depletion on survival, chimerism, and hematopoietic reconstitution after lethal irradiation and the transplantation of limiting amounts of T-cell-deficient bone marrow (BM). When administered before BMT, anti-asialo GM1 (ASGM1) antiserum treatment, effective in depleting in vivo NK activity, was associated with a marked increase in survival in 3 of 3 allogeneic combinations (BALB/c into C3H/HeN, C57B1/6, or C3H/HeJ). This enhanced survival was independent of the susceptibility of each recipient strain to accept BALB/c BM. Moreover, recipient anti-ASGM1 treatment was also effective in increasing survival in recipients of syngeneic BM, suggesting that NK cells can adversely affect engraftment independent of genetically controlled polymorphic cell surface determinants. Analysis of chimerism in surviving animals 2 months post-BMT showed that recipient NK depletion significantly increased the level of donor engraftment when high doses of BM were transplanted. These studies also demonstrated that anti-ASGM1 pretreatment mainly resulted in an increase in extramedullary hematopoiesis in the second and third week after irradiation. Anti-ASGM1 treatment also dramatically accelerated the rate of appearance of donor-derived cells with a higher level of donor-cell engraftment apparent at a time when the differences in survival between NK-depleted and control BMT recipients became significant. Peripheral cell counts were also affected by NK depletion, with significantly enhanced platelet and red blood cell recovery and a moderate increase in granulocyte recovery. The overall favorable influence of anti-ASGM1 recipient treatment on hematopoietic events post-BMT suggests that, in humans, pretransplant regimens aimed toward NK depletion should be evaluated.

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The role of natural killer (NK) cells in bone marrow transplantation (BMT) has been previously studied in a variety of different experimental systems. Several lines of evidence suggest that recipient NK cells have a deleterious effect on hematopoietic reconstitution after BMT, and that at least two different mechanisms could be involved. One mechanism of BMT resistance by NK cells has been termed hybrid resistance, and derives from the observation that certain F1 hybrid mice could reject bone marrow (BM) from their parents, although they would accept other nonlymphoid tissues. Hybrid resistance is thought to be regulated through noncodominant hematopoietic-histocompatibility (Hh) genes, and because the cells that mediate this resistance appear to share a number of properties with cells having NK activity, it is likely that hybrid resistance and NK activity are mediated by the same cells. Both hybrid resistance and NK activity are under similar genetic control and are abrogated in vivo by treatment with antisera recognizing large granular lymphocytes (LGL). This unusual behavior of parental BMT was further extended to the transplantation of allogeneic and xenogeneic BM cells, and is now collectively termed genetic or hematopoietic resistance.

More recently, a second mechanism related to the normal homeostatic regulation of BM cell growth or differentiation by NK cells has been described. In humans, Percoll gradient-enriched LGL with NK activity can inhibit in vitro the development of granulocyte-macrophage colonies from either autologous or allogeneic BM. These same cells also suppress the development of autologous erythrocyte colonies. In addition, clones of human lymphocytes with NK activity have also been shown to suppress in vitro hematopoiesis in a heterogeneous but clonally stable manner. The possibility that human NK cells might act in vivo against syngeneic cells has been suggested from a patient with aplastic anemia with repeated syngeneic graft failures, where in vitro studies demonstrated that NK cells from the recipient inhibited the growth of donor BM granulocyte progenitor cells. Additional support for the possible homeostatic regulatory role of NK cells on hematopoiesis comes from a variety of diseases with LGL lymphocytosis where chronic cytopenia is a major symptom, including T-lymphoproliferative disease, cyclic neutropenia, and Felty's syndrome. In addition, it has been previously reported that a monoclonal proliferation of LGL associated with anemia and neutropenia spontaneously regressed with reversal of the anemia and neutropenia.

In experimental animal models, the effect of recipient NK cells on hematopoietic events after BMT have focused predominantly on the genetically restricted hematopoietic resistance, generally using spleen colony formation (CFU-S) or 125IUDR incorporation assays at relative short periods of time after BMT. A number of limitations can be found in these prior studies. First, these assays measure only the
effects of NK cells on the very early steps of hematopoietic reconstitution, and more clinically relevant information such as the role of recipient NK on overall hematopoietic reconstitution, engraftment, and survival is still lacking. Furthermore, the lack of identity between CFU-S and primitive stem cells\(^2\) cautions against interpreting the results of splenic 125IUDR or CFU-S assays as evidence for either engraftment or long-term survival. In fact, hematopoietic reconstitution can be observed in irradiated animals despite a continued profound depression of spleen CFU-S.\(^3\) In addition, the previous experimental systems have concentrated on the use of high doses of irradiation and large amounts of BM cells for reconstitution with the risk of obscuring the regulatory role of recipient NK, which may be very important in a more clinically relevant setting of limited irradiation and transfer of substantially fewer BM cells. Lastly, in many of the previous studies involving recipient NK depletion, a single strain combination has been studied with no comparison between syngeneic and different allogeneic (susceptible or resistant) strain combinations. As a result, it has been impossible to interpret the results with enhanced BM engraftment as a reflection of decreased hematopoietic resistance or a decline in hematopoietic homeostasis by NK cells acting in a genetically nonspecific fashion.

To further address the role of recipient NK cells in an experimental BMT system that might overcome some of these limitations, we have investigated several short- and long-term effects of depleting recipient NK activity in lethally irradiated mice before BMT. We first concentrated on the measurement of long-term survival and chimerism after transplantation. Limited amounts of BM were infused to quantify more sensitively the effect of recipient NK depletion. T-cell–deficient BM was chosen to eliminate graft-versus-host disease and allow for the correlation of survival with immunohematopoietic reconstitution. Importantly, the same experimental model was used with both syngeneic and allogeneic strain combinations to determine the relative contribution of genetically determined hematopoietic resistance versus genetically independent normal hematopoietic homeostasis by NK cells. We also focused on one specific allogeneic strain combination and examined the influence of recipient anti-ASGM1 treatment on early events after BMT. In particular, we examined the kinetics of hematopoietic reconstitution (spleen and BM cellularity, peripheral cell counts) and engraftment (rate of appearance of donor-derived cells in the spleen and BM) from days 4 to 40 posttransplant.

**MATERIALS AND METHODS**

**Mice.** Male, 8- to 12-week-old C57B1/6NCr (H-2b), C3H/HeNCr (H-2k), BALB/C AnNCr (H-2d), B6C3F1 (H-2k), and BALB/C AnNCr-nu (H-2d) mice were used. Mice were obtained from the NCI-FCRF animal production facility (Frederick, MD). The cell suspensions, peripheral blood mononuclear cells, and mononuclear cell suspensions (1 x 10\(^6\)) were then adjusted in 0.5 mL of PBS for subsequent experiments (10 \(\mu\)L/mouse). Anti-ASGM1 was diluted in sterile phosphate-buffered saline (PBS) and filtered through a 0.44-\(\mu\)m filter before intravenous injection (0.2 mL/mouse) 2 to 4 hours after the last irradiation. Control animals were injected with the same dilutions and volumes of filtered normal rabbit serum (NRbS) (GIBCO, Grand Island, NY).

**BMT.** Pooled BM cell suspensions were prepared under sterile conditions from the femurs and tibias of 8- to 12-week-old male BALB/C nude mice. The cell suspensions were washed in Hanks’ balanced salt solution (HBSS), and the desired amount of BM cells (from 1 x 10\(^6\) to 5 x 10\(^6\)) were then adjusted in 0.5 mL of PBS for IV administration 24 hours after the anti-ASGM1 injection. Animals were housed under laminar flow conditions and were given acid water (pH of 2.5) throughout the experiment.

**Survival.** Mice were monitored daily for survival until 60 days after BMT, when animals were euthanized by carbon dioxide asphyxiation for subsequent laboratory studies. Day 60 after BMT was chosen, because in preliminary experiments virtually stable survival levels were observed from then on. In every experiment, 10 mice received either anti-ASGM1 or NRbS and an appropriate dose of BM cells. Experiments involving each strain combination were repeated at least three times. Where possible, data from separate experiments have been pooled and are indicated as such.

**Spleen and BM cellularity.** From day 4 until day 39 after BMT, groups of randomly selected surviving NK-depleted (n = 5) and control (n = 5) recipients were periodically euthanized by carbon dioxide asphyxiation. Spleens and bones (femurs, tibias) were harvested and placed in cold HBSS. Single cell suspensions of splenocytes and BM cells were obtained from individual animals. Nucleated cells for each examined animal were enumerated with a Coulter counter (Coulter, Hialeah, FL).

**Chimerism determination.** Donor and recipient spleen cell phenotype was determined in BMT recipient mice by fluorescence-activated cell sorter (FACS) analysis. Single cell suspensions of splenocytes (1 x 10\(^6\) cells) were incubated with appropriate H-2 specific monoclonal antibodies (MoAbs) before washing and subsequent incubation with an FITC-labeled secondary antibody. Ten thousand cells were analyzed by a Cytofluorograf 30-H (Ortho, Diagnostic Systems Inc, Westwood, MA) cell sorter, and the percent of donor and recipient cells determined by subtracting the percent of positive cells for the H-2-specific MoAb + appropriate FITC-labeled secondary antibody from the percent of positive cells for the FITC-labeled secondary antibody alone. H-2-specific MoAbs used were 34-2-12 (anti-H-2Dd) and 5F1 (anti-H-2Kb), both a generous gift from Dr David Sachs (National Institutes of Health, Bethesda, MD) (the 5F1 was produced by Dr Linda Sherman, La Jolla, CA), and H142-23 (anti-H-2k) (Sigma, St Louis, MO). Secondary antibodies were FITC-goat antimouse immunoglobulin (lg) G2a or 2b (Sigma). The H-2-specific MoAbs for donor or recipient phenotype were not cross-reactive, and in every experiment the percent of donor + recipient cells was equal to 100% ± 10%. For long-term chimerism determination (surviving mice 2 months after BMT), mice were analyzed separately, and the number of animals analyzed depended on the survival rate for each group. The serial chimerism determinations performed from day 4 to 39 after BMT were also performed, when possible, on separate animals at each given time point after BMT. However, the chimerism data from day 4 to 11 derived from pooled spleens (n = 5) because of insufficient numbers of spleen cells, especially in the NRbS-treated recipients.

**In vivo NK depletion.** Anti-asialo GM1 (anti-ASGM1) rabbit antiserum (Wako Chemicals, Dallas, TX) was dose-titrated, and the lowest dose of the antiserum effective in depleting more than 90% of in vivo NK activity was used for subsequent experiments (10 \(\mu\)L/mouse). Anti-ASGM1 was diluted in sterile phosphate-buffered saline (PBS) and filtered through a 0.44-\(\mu\)m filter before intravenous injection (0.2 mL/mouse) 2 to 4 hours after the last irradiation. Control animals were injected with the same dilutions and volumes of filtered normal rabbit serum (NRbS) (GIBCO, Grand Island, NY).
Peripheral cell counts. Peripheral blood, 100 to 200 μL, was obtained by retro-orbital venipuncture once to twice a week from day 4 to day 35 post-BMT. At any given time, peripheral cell counts were performed on groups of randomly selected surviving NK-depleted (n = 10) and control (n = 10) recipients. White blood cell (WBC) counts were performed visually on a hemocytometer after lysis of all red blood cells (RBCs). RBC and platelet counts were performed on a Coulter counter and periodically checked visually. Differentials of the WBC were determined by examination of Wright-Giemsa-stained slides of blood smears. Retro-orbital venipuncture was usually performed only once on any given animal. On occasion, a second and final puncture was performed, but at least 2 weeks after the first one.

Statistics. Various statistical methods were used in the analysis of data in this study. Fisher exact tests were conducted to determine the hypothesized relation between anti-ASGM1- or NRbS-treated groups and survival, as well as the percent of engrafted animals at day 60. The Mantel-Haensel statistic was used to combine information from 2 × 2 tables of treatment (anti-ASGM1 or NRbS) by survival across several doses of injected BM cells. The data on the percent of donor-derived cells at day 60 were analyzed for statistically significant differences in the means between groups by two-tailed Student's t-tests. Student's t-tests were also used for comparing mean responses of anti-ASGM1 and NRbS groups on raw and log-transformed variables (eg, RBC counts). Analysis of covariance (ANCOVA) was used to compare regression surfaces with parallel response profiles across anti-ASGM1 and NRbS experimental and control groups.

RESULTS

Influence of recipient NK depletion on survival. The influence of recipient NK depletion on long-term survival (more than 60 days) after lethal irradiation and BMT was examined in four different strain combinations. In each strain combination, the same source of BM was used (BALB/c nude; H-2d). In addition to the syngeneic BALB/c strain, C57Bl/6 (H-2b), C3H/HeN (H-2k), and C3B6F1 (H-2bk) recipients were chosen. All three are H-2 allogeneic; however, they differ considerably in terms of their ability to accept BALB/c BM after lethal irradiation. CFU-S and 125IUDR incorporation studies have demonstrated that large amounts of BALB/c BM are necessary to override the C57Bl/6 "resistance." Conversely, C3H/HeN are considered more "susceptible" to the acceptance of BALB/c BM.

Because of these differences in susceptibility to BALB/c BM, it was of interest to study C57Bl/6 x C3H/HeN F1 hybrids as well and evaluate the effects of recipient NK depletion in such an F1 setting.

In all four of the strain combinations examined in the present studies, recipient NK depletion with anti-ASGM1 significantly increased long-term (more than 60 days) survival after lethal irradiation and infusion of limiting amount of BM cells. In the allogeneic-resistant BALB/c into C57Bl/6 strain combination (Fig 1), recipient NK depletion before the infusion of a limiting number of BM cells (5 × 10^5 BM cells/mouse) considerably enhanced survival, with 50% of the anti-ASGM1-treated mice surviving versus none in the NRbS-treated group (P < .0005). With a higher dose of BM cells (5 × 10^6 BM cells/mouse), NK depletion also resulted in an increase in the survival rate from 50% to 80% (P < .037). Although not statistically significant (P < .17), with 5 × 10^6 BM cells/mouse, recipient NK depletion appeared to still be able to induce a small number of animals to survive (20% v 5% in the NRbS-treated group). As expected, the 1,100-R irradiation was lethal in more than 95% of the mice if no BM cells were given.

In the allogeneic susceptible strain combination (BALB/c nude BM into C3H/HeN), similar results were observed (Fig 2). However, because C3H/HeN mice are more capable of accepting BALB/c BM cells, lower doses of BM cells were infused. As with C57Bl/6 recipients, depletion of NK activity with anti-ASGM1 antiserum before the infusion of low doses of BM cells (2 × 10^5 and 5 × 10^5 BM cells/mouse) was associated with a dramatic increase in survival compared with NRbS-treated controls (70% v 20% [P < .035] and 90% v 20% [P < .003], respectively). Although not statistically significant (P < .1) at higher doses of BM (1 × 10^6 BM cells/mouse), recipient anti-ASGM1 treatment still appeared to increase survival (100% v 70% in NRbS-treated

![Graph](https://www.bloodjournal.org/)

Fig 1. Influence of recipient anti-ASGM1 treatment on the percent of surviving C57Bl/6 mice after lethal irradiation (500 + 600 R) and transplantation of BALB/c nude BM cells (pooled data). (■—■), Anti-ASGM1; (○—○), NRbS.
animals). All control mice irradiated without BMT died within a time range consistent with hematopoietic toxicity (ie, between days 8 and 15).

As in previous allogeneic combinations, NK depletion in C3B6F1 mice (Fig 3) before an appropriate dose of BALB/c BM cells (1 × 10⁶) was associated with enhanced survival when compared with NRbS-treated recipients (85% v 40%) (P < .004). At a lower dose of BM cells (2 × 10⁵), anti-ASGM1 did not significantly increase survival (20% v 0%, P < .24). As expected, when no BM was infused, less than 10% of the animals survived.

Recipient NK depletion had an almost identical beneficial effect in the syngeneic BALB/c model (Fig 4). The infusion of 1 × 10⁶ BM cells into anti-ASGM1–treated recipients translated into a significant advantage in survival over NRbS-treated animals (95% v 60%) (P < .01). When a lower dose of BM was administered (3 × 10⁵), the increased survival induced by recipient NK depletion was even more dramatic: 80% versus 30% in the NRbS-treated animals (P < .002). When no BM followed the irradiation, lethality was greater than 90% in both the anti-ASGM1 and NRbS-treated groups.

When we considered the effects of recipient NK depletion in terms of the relationship between number of surviving mice 2 months after BMT and the amount of BM cells infused (Fig 5), several observations could be made. First, survival after lethal irradiation and BMT was clearly a dose-dependent phenomena in all four strain combinations examined. As expected, in a syngeneic setting, a relatively small number of BALB/c BM cells was necessary for survival. In the allogeneic settings, C3H/HeN recipients were clearly more susceptible to BALB/c BM than were C57B1/6 mice, and B6C3F1 recipients expressed an intermediate susceptibility when compared with C3H/HeN and C57B1/6 recipients. In addition, although recipient NK depletion significantly reduced the amount of BM cells
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necessary for survival in both the allogeneic and the syngeneic strain combination, the doses of infused BM at which this enhanced survival was maximum varied depending on the recipient strain used (2 to 5 x 10^5 BM cells in the syngeneic setting, 0.5 to 2 x 10^5 BM cells in the C3H/HeN allogeneic susceptible setting, and 0.5 to 5 x 10^6 BM cells in the C57B1/6 allogeneic resistant as well as with the B6C3F1 recipients). Recipient NK depletion clearly reduced the number of BALB/c BM cells necessary for the survival of BALB/c (P < .006), C3H/HeN (P < .0001), and C57B1/6 (P < .02) recipients. A similar trend is obvious for B6C3F1 mice without, however, reaching statistical significance. The favorable impact of anti-ASGM1 treatment appeared to result in a 1 to 2 log reduction in the number of BM cells necessary to achieve the same level of survival seen in NRbS-treated recipients.

**Influence of recipient NK depletion on long-term chimerism.** The level of donor- and recipient-derived cells in the spleens of surviving recipients 2 months after allogeneic BMT was determined by H-2 phenotyping (FACS analysis) (Table 1). The persistence of recipient cells in various proportions was observed at all doses of BM in both strain combinations examined (BALB/c into C3H/HeN and C57B1/6, respectively). The degree of chimerism was clearly dependent on the number of BM cells infused, as well as the susceptibility or resistance of the recipient strain to BALB/c BM. With 1 x 10^6 BM cells, a significant level of engraftment was observed in C3H/HeN recipients. However, in C57B1/6 mice, a similar number of BM cells resulted in very little to no splenic donor cells 2 months after BM transplantation. Also, levels of engraftment varied considerably within groups of mice having received identical irradiation and number of BM cells. Yet these animals survived lethal irradiation because of the allogeneic BM cells exerting effects in the early period after BMT. Within an experimental group, one could detect individual animals with nearly...
100% donor cells, some with a large majority of recipient-type cells and some mixed chimeric animals with equal numbers of donor and recipient origin.

The effects of recipient NK depletion on chimerism appeared to be dependent on the dose of BM infused. Not surprisingly, large numbers of BM cells were generally associated with a higher rate of donor cell engraftment. With 1 x 10^6 BM cells, a significant increase in the overall percent of donor derived cells among C3H/HeN recipients was apparent (78% ± 5.5%, P < .039). Interestingly, the increase in engraftment was related to the near-absence of mice without engraftment among anti-ASGM1–treated animals (94% engrafted). Indeed, when one considered the percent of engrafted animals (defined as expressing more than 60% of donor-derived cells in the spleen), the trend in favor of the NK-depleted recipients persisted, but the difference between anti-ASGM1- and NRbS-treated mice was observed from day 7 to 18 (ANCOVA, P < .0001). By day 25, spleen cellularity was identical in both groups, and thereafter appeared to be on a plateau. This considerable increase in spleen cellularity from day 12 to day 18 in NK-depleted recipients is striking and was readily observable macroscopically (data not shown).

Recipient NK depletion was associated not only with an increase in total cellularity, but also with an accelerated rate of donor cell engraftment in the spleen (Fig 7A). An increase in the percent of donor cells was apparent as early as day 4 after BMT and persisted through day 18, with 84% donor determination of leukocyte, granulocyte, erythrocyte, and platelet counts.

At day 3 after BMT, spleen cellularity (Fig 6) was considerably decreased when compared to pretreatment levels (103 x 10^6 ± 6.1) with similar values in anti-ASGM1– and NRbS–treated mice. However, by day 12 post-BMT, NK-depleted recipients demonstrated a significantly larger number of cells than control mice. This twofold to 10-fold difference between anti-ASGM1– and NRbS–treated mice was observed from day 7 to 18 (ANCOVA, P < .0001). By day 25, spleen cellularity was identical in both groups, and thereafter appeared to be on a plateau. This considerable increase in spleen cellularity from day 12 to day 18 in NK-depleted recipients is striking and was readily observable macroscopically (data not shown).

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Table 1. Influence of Recipient NK Depletion on the Level of Engraftment in Surviving Animals Two-Months Postallogeneic BMT

<table>
<thead>
<tr>
<th>Recipient Strain</th>
<th>Dose of BM Cells (x 10^6)</th>
<th>Anti-ASGM1</th>
<th>NB of Mice</th>
<th>% of Donor Cells</th>
<th>% of Engrafted Mice</th>
<th>% of Donor Cells Among Engrafted Mice</th>
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<tr>
<td>C3H</td>
<td>10</td>
<td>+</td>
<td>16</td>
<td>78 ± 2 *</td>
<td>94 ± .05</td>
<td>80 ± 1 *</td>
</tr>
<tr>
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<td>10</td>
<td>-</td>
<td>15</td>
<td>58 ± 9</td>
<td>67 ± .05</td>
<td>80 ± 2 *</td>
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<tr>
<td>C3H</td>
<td>2</td>
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<td>5</td>
<td>59 ± 14</td>
<td>80 ± .05</td>
<td>73 ± 3 *</td>
</tr>
<tr>
<td>C3H</td>
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<td>-</td>
<td>5</td>
<td>63 ± 18</td>
<td>80 ± .05</td>
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<td>76 ± 3 *</td>
</tr>
<tr>
<td>C3H</td>
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<td>4</td>
<td>34 ± 17</td>
<td>25 ± .05</td>
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</tr>
<tr>
<td>C57B1/6</td>
<td>50</td>
<td>+</td>
<td>4</td>
<td>74 ± 1</td>
<td>100 ± .05</td>
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<tr>
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<tr>
<td>C57B1/6</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>0 ± .05</td>
<td>0 ± 2 *</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>0.5</td>
<td>+</td>
<td>2</td>
<td>1</td>
<td>0 ± .05</td>
<td>0 ± 2 *</td>
</tr>
<tr>
<td>C57B1/6</td>
<td>0.5</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>0 ± .05</td>
<td>0 ± 2 *</td>
</tr>
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</table>

Two-month postlethal irradiation, anti-ASGM1, or NRbS administration and infusion of Balb/c nude BM cells; the percent of splenic donor and recipient-derived cells in surviving C3H or C57B6 recipients were determined by FACS analysis. In all cases, the percent of donor-derived cells + the percent of recipient-derived cells equaled 100% ± 10%.

*Animals with over 60% donor-derived cells.

†Standard error to the mean.

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Fig 6. Influence of recipient anti-ASGM1 treatment on spleen cellularity after lethal irradiation and allogeneic BMT (Balb/c nude into C3H/HeN) (●), normal value. (●—●), Anti-ASGM1; (○—○), NRbS.
NK depletion enhances BM transplantation

The rate of recovery of peripheral blood cell counts after lethal irradiation and allogeneic BMT was also influenced by the presence or absence of recipient NK cells at the time of irradiation (Fig 9). In our model, the effects of NK depletion were most dramatic when assessing erythroid and platelet recovery, but more modest for leukocyte recovery. The total number of peripheral WBCs (Fig 9A) was very low from day 4 to 18, after which WBC counts progressively increased to near normal values by day 42. As observed in the BM, NK depletion did not modify cell counts until day 18 after BMT. However, from day 18 on the kinetics of total WBC recovery appeared to be faster in anti-ASGM1-treated mice. This accelerated recovery rate was modest and did not reach statistical significance (ANCOVA, \( P < .052 \)). Examination of peripheral smears for absolute granulocyte counts (AGC) (Fig 9B) showed a similar pattern with enhanced recovery in the NK-depleted mice that did reach significance between day 20 to 27 (ANCOVA, \( P < .04 \)), indicating the increase in WBC numbers is predominantly due to an increase in granulocytes.

The pattern of peripheral RBC recovery was statistically more significant (Fig 9C). In both the anti-ASGM1- and NRbS-treated mice, RBC counts declined until day 20. In NRbS-treated recipients, a nadir of \( 2 \times 10^6 \pm 0.8/\text{mm}^3 \) was reached at day 29, at which point the RBC counts progressively increased before stabilizing around \( 4.5 \times 10^6/\text{mm}^3 \), values approaching that of normal animals. In contrast, RBC recovery in NK-depleted recipients was significantly enhanced at several time points (day 14, \( P < .015 \); day 16, \( P < .035 \); day 22, \( P < .042 \)). However, as noted previously in the spleen and BM, the cell counts in NK-depleted and control animals were similar beyond day 25 after post-BMT. Recipient NK depletion was associated with a clear increase in platelet numbers observed in the NK-depleted recipients (Fig 9D). Platelet numbers in the NRbS-treated recipients reached a nadir of \( 25,000 \pm 3,000 \) platelets/\text{mm}^3 at day 10 before slowly increasing to approximately normal values by day 35. Similarly, in anti-ASGM1-treated recipients, platelet numbers reached a nadir at day 10, but recovered at a significantly faster rate from day 14 to 24.
DISCUSSION

Our results clearly demonstrate a favorable impact of recipient NK depletion on long-term survival after lethal irradiation and transplantation with limiting amounts of BM. Because survival after BMT is the ultimate consequence of an efficient multi-step immunohematopoietic reconstitution, the enhanced survival observed here is of clinical importance and is possibly more relevant than earlier measurements of splenic events such as CFU-S or 125IUDR incorporation.

Interestingly, in every strain combination examined, NK depletion was associated not only with an increase in long-term survival but also with a marked reduction in the number of BM cells required for survival. This effect was seen irrespective of the “BM resistance” expressed by the recipient, and was even observed in syngeneic recipients. Although previous studies measuring early events after BMT have shown that recipient NK depletion with anti-ASGM1 or NK1.1 in allogeneic strains could result in the abrogation of hematopoietic resistance, the effects of NK depletion on allogeneic or syngeneic control animals were either not examined or were not significant at the doses of BM infused. Therefore, the conclusion drawn from these prior studies was that genetically restricted hematopoietic resistance, mediated via NK cells, was the major mechanism responsible for the inhibition of CFU-S and 125IUDR incorporation after BMT in resistant animals.

The present studies are not entirely compatible with this conclusion. Anti-ASGM1 did effectively improve the survival of resistant C57B1/6 recipients to a degree similar to susceptible C3H/HeN recipients, thus confirming and extending the earlier studies measuring genetically restricted hematopoietic resistance. However, in the present studies, NK depletion in the recipient had a similar impact on survival in the susceptible (C3H/HeN) and syngeneic (BALB/c) recipients as well. The result is a persistent difference between C3H/HeN and C57B1/6 recipients in terms of the amount of BALB/c BM cells necessary for survival, even after recipient NK depletion. The basis for this persistent difference in the ability of C3H/HeN and C57B1/6 strains to accept allogeneic BM is not clear, but appears not to be entirely NK dependent. Perhaps there are genetically restricted interactions between marrow stromal cells and hematopoietic cells that could account for the NK-dependent differences.

The possibility exists that anti-ASGM1 antiserum depleted cell populations other than NK cells in our experimental system. In particular, the hypothesis that anti-ASGM1 treatment before irradiation and BMT could have depleted cytotoxic T lymphocytes or macrophages was of concern. In fact, we have considered two parameters when assessing the in vivo specificity of the antiserum for NK cells. First, the effects of anti-ASGM1 antiserum are very dose-dependent, and we have previously demonstrated that these low doses of the antiserum (10 μL) are effective in depleting NK activity without affecting T-cell or macrophage function. Second, the antiserum administration schedule...
is an important parameter to consider. Previous studies have shown that alloantigen-activated T cells appear to be anti-ASGM1\(^+\) and can be depleted in vivo with repeated large amounts of the antiserum (100 \(\mu\)L \(\times\) 3), whereas 100 \(\mu\)L administered 2 days before to allogeneic challenge (timewise consistent with our experimental setting) fails to prevent the subsequent proliferation of alloreactive T cells.\(^{27}\) It is of interest to note that in vitro generated alloreactive T cells are reported to be resistant to anti-ASGM1 plus complement treatment in vitro.\(^{34}\) Therefore, we feel confident that anti-ASGM1 antiserum at the dose used throughout this study (10 \(\mu\)L/mouse) and administered 24 hours before BMT selectively depleted the recipient NK cells. However, further studies currently underway using more NK specific MoAbs (NK1.1, LGL-1) are required to definitively answer this question.

The favorable impact of anti-ASGM1 in the BALB/c syngeneic strain combination is of major interest when one tries to dissect how NK cells influence hematopoietic events after BMT. As mentioned previously, NK cells appear to have an overall inhibitory physiologic role in the regulation of hematopoiesis.\(^3\) It has been suggested that once in contact with HLA-DR\(^+\) hematopoietic cells, NK are activated and produce a colony-inhibiting factor (NK-CIA).\(^{33}\) The present data, as well as previous experimental animal studies using syngeneic BM, would be consistent with this negative regulatory role for NK cells after BMT. For instance, mice infected with lymphocytic choriomeningitis virus reject syngeneic BM, and this resistance is completely abolished by anti-ASGM1 antiserum.\(^{34}\) More recently, recipient NK depletion before syngeneic BMT has been shown to significantly increase the number of CFU-S.\(^{35}\) In contrast, several other studies have failed to demonstrate any effect of NK cells in syngeneic BMT.\(^{44,46-49}\) One of the reasons for this apparent discrepancy might be that these experimental models used a large number of BM cells that quantitatively overcome the inhibition by NK cells.

The capacity of hematopoietic stem cells to survive lethal doses of irradiation\(^46\) and induce varying degrees of autologous reconstitution after BMT is well-established in animal models\(^{44}\) as well as in humans.\(^{45}\) Therefore, it was of interest in the present experiments to determine the influence of recipient NK depletion on chimerism after lethal irradiation and allogeneic BMT. NK depletion was associated with a significant increase in engraftment when administered before relatively high doses of BM. Interestingly, this advantage in engraftment was related to an increase in the fraction of animals that engrafted rather than to an increase in the fraction of donor cells in the animals. The absence of significant differences in engraftment when lower doses of BM were infused could be due, at least in part, to the increase in survival related to recipient NK depletion.

To further address the mechanisms by which NK cells affected engraftment and hematopoietic reconstitution post-BMT, we focused on one specific allogeneic combination (BALB/c into C3H/HeN) and examined, in some detail, early events post-BMT. At the dose of BM cells chosen (1 \(\times\) 10\(^8\)), NK depletion induced a significant advantage in survival over NRbS-treated controls occurring mainly between day 10 and 25. Most NRbS-treated controls died during this period. In addition, NK depletion induced profound modifications in the kinetics of engraftment and hematopoietic recovery after lethal irradiation and allogeneic BMT. However, NRbS-treated controls surviving beyond day 25 were not very different from surviving mice treated with anti-ASGM1, presumably because all animals had reached a critical level of hematopoietic function to survive. Thus, comparisons between NK-depleted and control animals after day 25 were considered unlikely to show any important differences in the mechanism of NK enhancement of BM reconstitution.

In these experiments, recovery of spleen cell numbers increased considerably faster (as soon as day 7) in NK-depleted recipients. Interestingly, a difference in cell recovery was not observed in the BM of NK-depleted and control animals until a later time point (day 12). This difference in kinetics between the enhancement of spleen versus BM recovery could have at least three explanations. First, the murine spleen could play a more important role than the BM in early hematopoietic recovery after irradiation.\(^{43}\) Alternatively, such differences in cell recovery could reflect differences in the susceptibility of extramedullary versus medullary hematopoiesis to NK-mediated regulation. Consistent with this hypothesis, Hansson et al\(^{44}\) recently demonstrated that mice depleted of NK activity since birth showed an increase in the number of myeloid precursor cells only in the spleen and not in the BM. Lastly, the difference in kinetics of cell recovery between spleen and BM may simply reflect the different types of hematopoietic activity observed in the two organs after BMT. Some elements resulting in early hematopoiesis (pluripotent CFU) may be observed rapidly in the spleen. In contrast, development of more committed erythroid, granulocyte, monocyte, and lymphoid elements in the BM may take longer to be observed. Our results, which demonstrated an effect of NK depletion on the development of the RBC, platelet, and granulocyte hematopoietic populations, but only after day 20 (the time when NK-related effects were observable in the BM), would be consistent with this hypothesis.

The most striking difference in the spleen induced by NK depletion is the accelerated kinetics of donor cell engraftment in the spleen. In fact, donor cells appeared very early in the spleens of both anti-ASGM1- and NRbS–treated animals; however, the increase in the percent of donor-derived cells is significantly faster in NK-depleted recipients and reaches a maximum of 84% by day 18. If one considers the total number of donor-derived spleen cells, the difference induced by NK depletion is considerable, with a 10-fold increase at day 18 in the number of donor-derived cells. Interestingly, the NRbS-treated recipients who did survive never reached such a high level of donor cell engraftment at any given time. These results also favor an important role of recipient NK cells in the early events after BMT. Accordingly, experiments involving the injection of radiolabeled BM cells into irradiated allogeneic recipients have shown that repression of allogeneic engraftment occurs within the first 24 hours after BMT, and that anti-ASGM1 administration reduced such resistance.\(^{45}\)
Interestingly, all NK-depleted recipients analyzed at day 18 have a substantial percentage of donor-derived cells. For some of these animals, this predominant donor engraftment is transient and, as soon as day 25, close to half of the NK-depleted recipients exhibit a predominant (greater than 50%) autologous reconstitution. At day 25, no significant differences can be observed between the two experimental groups. Apparently, two types of allogeneic cell rejection have to be considered: first, an early rejection that appears NK-mediated and is related to the survival of BMT recipients; second, a late rejection mediated by other immune mechanisms (T cell, antibody?) at a time when autologous reconstitution can take place.

Recipient NK depletion also significantly influenced the kinetics of peripheral cell recovery. Interestingly, the most significant effects were observed on the RBC, platelet, and granulocyte recovery rate. Again, the early inhibitory role of NK cells on all three cell types favors the suggestion of a preferential effect of such cells on early pluripotent or multipotent stem cells, as previously suggested by CFU-S or 125IUDR incorporation assays. In fact, several in vitro experiments have demonstrated that NK cells can have an inhibitory role on granulocyte and erythroid progenitors. In addition, neutropenia and anemia are predominant features in various human diseases where NK cells are thought to play a pathologic role. The effects of NK cells on megakaryocytopoiesis are well-understood and may be at variance with the results from patients with T-lymphoproliferative disease, where increased NK activity is not commonly associated with thrombocytopenia. Recently, NK cells were also shown to stimulate megakaryocytopoiesis in an in vitro system. The reasons for this apparent discrepancy are not yet known.

How does recipient NK depletion reduce the number of BM cells necessary for survival after lethal irradiation? The observed modifications of the RBC, platelet, and granulocyte counts related to anti-ASGM1 pretreatment are probably sufficient to explain the enhanced survival. Indeed, by significantly reducing the time period after BMT during which recipient cells are severely cytopenic, NK depletion should reduce the susceptibility of the BMT recipients to lethal complications such as disseminated infection, severe hemorrhage, or anemia. It is important to note that the differences in the kinetics of engraftment and cell recovery between NK-depleted and control recipients take place precisely at the time when the differences in survival between both groups become most pronounced. However, the present experimental system can only suggest, but not prove, a relationship between survival and the observed hematologic data. Alternatively, the enhanced survival could be due to other factors possibly influenced by NK depletion and not evaluated in the present experiments. Indeed, NK cells produce a wide variety of cytokines, and are cytolytic for early thymocytes, and are capable of suppressing antibody production both in humans and mice. Alternatively, anti-ASGM1 could enhance the survival of host-derived hematopoietic cells. However, the difference in survival between anti-ASGM1- and NRbS–treated irradiated controls (no BM), as well as the very early appearance of important numbers of donor-derived cells in anti-ASGM1–treated mice, argue against a “radioprotective” effect as being a significant component of the enhanced effect on survival.

Another question not yet answered is how NK cells recognize and inhibit BM engraftment. There is evidence that bone marrow cells can be recognized and lysed by NK cells. In addition, among the cytokines produced by NK cells, tumor necrosis factor and transforming growth factor-β are associated with the growth inhibition of pluripotent stem cells. Overall, the present results confirm the important role that NK cells have in determining the outcome of hematopoietic events after BMT. The favorable influence of recipient NK depletion on survival in allogeneic-resistant and susceptible as well as syngeneic strain combinations suggests that NK cells can function to inhibit hematopoiesis in genetically unrestricted fashion. The reduction in the number of BM cells necessary for survival could be of particular clinical importance, especially in transplantation situations where the number of stem cells infused could be a critical limiting factor. The design of pretransplant regimens aimed at NK depletion could be beneficial in humans.


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Anti-asialo GM1 antiserum treatment of lethally irradiated recipients before bone marrow transplantation: evidence that recipient natural killer depletion enhances survival, engraftment, and hematopoietic recovery

P Tiberghien, DL Longo, JW Wine, WG Alvord and CW Reynolds