Erythropoiesis In Vitro: Effect of Normal Versus “Transfusion-sensitized” Mononuclear Cells

By Beverly J. Torok-Storb, Rainer Storb, Theodore C. Graham, Ross L. Prentice, Paul L. Weiden, and John W. Adamson

The effects of mononuclear cells from non-transfused and transfused dogs on the growth of erythroid colonies (EC) from DLA-identical littermates were studied. To this purpose, marrow samples were cultured both before and after one to three transfusions from the DLA-identical littermate. Mononuclear cells obtained before transfusions and cocultured with autologous marrow or marrow from DLA-identical littermates significantly increased the number of EC over that obtained in control cultures in 50 of 52 experiments. The magnitude of stimulation observed was dependent upon the ratio of mononuclear cells to EC precursors, suggesting that mononuclear cells interact directly with erythroid colony-forming units in some regulatory capacity. After transfusion, the effect of mononuclear cells on littermate marrow was significantly altered, resulting in most cases either in a failure to increase or an actual decrease in EC numbers. A lesser reduction in EC numbers in a smaller number of dogs was also seen with autologous mononuclear cells after transfusion. The strong reduction of littermate EC by mononuclear cells from most dogs after transfusion is presumably related to an immune reaction against minor tissue antigens, and it suggests that transfusion-induced sensitization must be considered when interpreting the effects of lymphocyte populations on the growth of erythroid colonies.

Recently it was reported that peripheral blood leukocytes from patients with aplastic anemia reduced the number of hematopoietic colonies grown in vitro from the marrow of healthy unrelated individuals. This was interpreted to suggest that the pathogenesis of some cases of acquired aplastic anemia could be attributed to lymphocyte “autoaggression.” Furthermore, autoaggression was thought to be involved in graft rejection seen in some patients with aplastic anemia after marrow transplantation from HLA-identical siblings. However, since most patients with aplastic anemia have had multiple transfusions of blood products, another possible explanation for both the reduction in vitro of colony growth by mononuclear cells and the phenomenon of graft rejection in vivo is that transfusions had “sensitized” the patients’ lymphocytes against histocompatibility antigens present on colony-forming units of the marrow donors. This explanation is supported indirectly by observations in vivo in dogs that showed that transfusions from either unrelated dogs or the intended DLA-identical littermate marrow donor were capable of sensitizing normal recipients, resulting in rejection of a subsequent marrow graft.

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The current study in dogs was aimed at detecting transfusion-induced "sensitization" in vitro as reduced erythroid colony (EC) growth from donor marrow cocultured with recipient mononuclear cells. The results indicated that as a rule mononuclear cells from nontransfused dogs significantly increased the number of EC grown from autologous or DLA-identical littermate marrow. The degree of the increase correlated with the ratio of mononuclear cells to marrow cells in culture. After transfusion, the effect of mononuclear cells on littermate marrow was altered, resulting in most cases in either a failure to increase or actual decrease in EC numbers.

MATERIALS AND METHODS

Dogs. Fourteen pairs of littermates identical for DLA and dog erythrocyte antigen I were selected as described previously. In seven cases, one member of each pair served as the blood donor and the other as the recipient. In the remaining experiments, to increase the sample size, both littermates were sensitized against each other. Recipients were given either one or three weekly transfusions of 50 ml heparinized whole blood from their matched littermate.

Bone marrow and lymphocyte preparation. Before and 10 days after the last transfusion, bone marrow cells (BMC) from both members of each pair were aspirated from the humerus head into a 10-ml syringe containing 4 ml TC-199 tissue culture medium (Microbiological Associates, Bethesda, Md.) and 20 units preservative-free heparin. Buffy coat cells were separated, washed three times, and suspended in supplemented alpha medium (Flow Laboratories, Rockville, Md.). Peripheral blood mononuclear cells were obtained from 10 ml heparinized blood by layering the blood over Ficoll-Hypaque (Lymphoprep; Nyegaard, Oslo, Norway). After they were washed three times, the cells were suspended in supplemented alpha medium. Cells were counted on a hemocytometer using trypan blue dye exclusion to measure viability.

EC assays. BMC were cultured for 2 days using the plasma clot technique described by Stephenson et al. as modified in this laboratory for dog cells, with 1 unit erythropoietin/ml culture (step III preparation of sheep plasma; Connaught Laboratories, Willowdale, Ontario). After harvesting, clots were stained with benzidine, and hemoglobin-containing EC were counted in a blind fashion with at least five replicates for each culture. Only colonies containing eight or more cells were scored. BMC from both members of each pair were cultured concurrently without mononuclear cells, with autologous mononuclear cells, and with DLA-identical littermate mononuclear cells. Dogs were tested before and after transfusions. Routine tests used 2 x 10^3 BMC with 5 x 10^3 mononuclear cells/ml culture. To determine the effect of different concentrations of mononuclear cells on EC growth, the ratio of mononuclear cells to BMC was varied as indicated.

Analysis of the data. The results were expressed as the ratio of the number of EC grown with mononuclear cells to the number obtained without mononuclear cells (Table 1). Ratios > 1 indicated that the presence of mononuclear cells increased the number of EC; ratios < 1 indicated a reduction in the number of EC. The distribution of ratios > 1 versus those ≤ 1 before and after transfusion was analyzed by Fisher's exact probability test. Least-squares regression analysis was used to interpret dose-response curves. Paired comparison regression analysis, taking into account the variation in control colony number, was also used to interpret the effect of mononuclear cells before and after transfusion.

RESULTS

Normal mononuclear cells. Before transfusion, autologous mononuclear cells increased EC numbers in 24 of 26 experiments while littermate mononuclear cells increased EC in all cases. The degree of stimulation varied from experiment to experiment, ranging from 1.1 to 9.0. This variation was attributed to differences between experiments in the ratio of mononuclear cells to EC precursors. Figure 1 shows that with both autologous and littermate mononuclear cells,
stimulation decreased as the number of control EC increased. Such an association was further supported by the finding of a positive correlation between the degree of stimulation and the mononuclear cell:BMC ratio (Fig. 2). Mononuclear cells cultured without BMC did not form EC.

"Transfusion-sensitized" mononuclear cells. Paired comparison regression analysis of data on the same dogs before and after transfusion showed a significant reduction in stimulating ability of posttransfusion mononuclear cells ($p < 0.001$ with either autologous or littermate mononuclear cells) (Table 1). This analysis, using the relationship in Fig. 1, showed that the reduction in stimulation could not be attributed to differences between pre- and posttransfusion control colony numbers. A more descriptive analysis (Table 2) showed that greater reduction in stimulating ability and actual inhibition of EC growth were observed with littermate mononuclear cells. Specifically following transfusion, autologous mononuclear cells failed to stimulate in 2 and decreased EC growth in 5 of 16 experiments (31.25%). The number of control EC was decreased in 3 of 16 experiments (18.75%).
The probabilities were determined by Fisher's exact probability test, with which we compared the number of ratios > 1 with the number of ratios 1.

Table 1. Effect of Lymphocytes on Growth of EC From Marrow of Dog B398

<table>
<thead>
<tr>
<th>Time of Test</th>
<th>Source of Lymphocytes</th>
<th>No. of EC/2 x 10^6 BMC (Mean ± SEM)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before transfusion</td>
<td>None</td>
<td>150 ± 10.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autologous</td>
<td>204 ± 10.8</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>Littermate</td>
<td>188 ± 11.4</td>
<td>1.25</td>
</tr>
<tr>
<td>After transfusion</td>
<td>None</td>
<td>76 ± 4.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Autologous</td>
<td>116 ± 3.0</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>Littermate</td>
<td>30 ± 2.0</td>
<td>0.39</td>
</tr>
</tbody>
</table>

in 4 of 21 experiments. In comparison, littermate mononuclear cells after transfusion failed to stimulate in 4 and decreased EC in 10 of the remaining 17 cases.

DISCUSSION

These experiments show that when normal mononuclear cells from untransfused dogs are cocultured with either autologous or DLA-identical littermate marrow they significantly increase the number of EC over that obtained in control cultures.

Previous work by several investigators showed that normal lymphocytes with T cell characteristics can stimulate the growth of human erythroid colonies in vitro and promote erythropoiesis in vivo in mice. The exact nature of the lymphocyte–marrow cell interaction responsible for stimulation either in vivo or in vitro has not been defined. The stimulation of erythroid colony growth in vitro by the addition of lymphocyte-conditioned media suggests that lymphocytes produce a factor that can mediate this response.

In our study, the magnitude of stimulation observed from a given number of mononuclear cells was dependent upon the number of EC precursors present, suggesting that mononuclear cells interact directly with erythroid colony-forming units in some capacity. Unfortunately, since it is not possible to control the number of EC precursors in a given suspension of BMC, optimum ratios could not be maintained from experiment to experiment. Despite this limitation, the assay was sensitive enough to determine that the effect of transfusion-sensitized mononuclear cells on donor marrow was different from that of mononuclear cells obtained before transfusion.

Following exposure of a normal dog to one or three transfusions of blood from a DLA-identical littermate, the ability of mononuclear cells to stimulate

Table 2. Incidence of Nonstimulation or Inhibition (Ratios < 1) of EC by Autologous and Littermate Lymphocytes Before and After Transfusion

<table>
<thead>
<tr>
<th>Lymphocyte Source</th>
<th>Time of Test</th>
<th>Mean ± SEM</th>
<th>Ratios ≤ 1/Total No. of Dogs</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous</td>
<td>Before transfusion</td>
<td>1.86 ± 0.19</td>
<td>2/26</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>After transfusion</td>
<td>1.55 ± 0.20</td>
<td>6/21</td>
<td></td>
</tr>
<tr>
<td>Littermate</td>
<td>Before transfusion</td>
<td>2.05 ± 0.79</td>
<td>0/25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>After transfusion</td>
<td>1.09 ± 0.10</td>
<td>14/21</td>
<td></td>
</tr>
</tbody>
</table>

The probabilities were determined by Fisher's exact probability test, with which we compared the number of ratios > 1 with the number of ratios ≤ 1.
growth of autologous EC was somewhat reduced. This reduction in the degree of autologous stimulation remains unexplained. The same “sensitized” mononuclear cells, when cocultured with marrow from the littermate transfusion donor, inhibited EC growth in 10 of 21 experiments. This reduction of littermate EC by posttransfusion mononuclear cells is presumably related to an immune reaction against minor tissue antigens. Whether or not inhibition in vitro of EC growth has any mechanism in common with marrow graft rejection in vivo is only speculative. Both occur after exposure and presumably sensitization to minor histocompatibility antigens.

The potential use of lymphocyte-BMC cocultures to predict graft rejection after marrow transplantation, even in this relatively controlled system, is limited by the difficulty in preparing optimum lymphocyte:target cell ratios. However, since marrow graft rejection is a considerable clinical problem, the question merits further study. In patients with aplastic anemia, when comparisons between the same lymphocyte source before and after transfusion and sensitization are not possible, or when HLA-identical sibling marrow is not available, interpretations from coculture studies should be made with caution. The potential effects of transfusion sensitization on EC growth in vitro would appear to limit the value of such culture techniques in elucidating the pathogenesis of marrow failure in multiply transfused patients.

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