Growth Enhancement of Established Tumors by Allogeneic Blood Transfusion in Experimental Animals and Its Amelioration by Leukodepletion: The Importance of the Timing of the Leukodepletion

By José O. Bordim, Leslie Bardossey, and Morris A. Blajchman

We had reported previously (Blood 81:1880, 1993) that allogeneic blood transfusions (ABT) administered before the infusion of tumor cells in both inbred and outbred experimental animals promote tumor growth and that this effect can be ameliorated by leukodepletion. To better reproduce the human situation, we evaluated, in this present study, the effect of ABT in animals with established tumors using enumeration of pulmonary metastatic nodules as the end point. The role of allogeneic blood component transusions in promoting tumor growth and the relative efficacy of prestorage versus poststorage leukodepletion of the ABT in preventing tumor growth enhancement were also evaluated. In an inbred murine animal model, C57Bl/6J mice were administered nonleukodepleted allogeneic (ABT), leukodepleted allogeneic (LD-ABT), or syngeneic (SBT) blood transfusions after the intravenous infusion of syngeneic methylicholanthrene-induced fibrosarcoma cells using two different protocols. A significant increase in the number of pulmonary nodules was observed in those mice that received ABT, in both protocols, compared to animals transfused with SBT or LD-ABT. Significantly higher numbers of pulmonary nodules were also seen in mice transfused with allogeneic buffy coat leukocytes compared with mice that received either nonleukodepleted allogeneic plasma or LD-ABT. In an outbred animal (rabbit) model, recipient rabbits were administered either nonleukodepleted ABT, prestorage LD-ABT, poststorage LD-ABT, or SBT on days +4 and +9 after the infusion of syngeneic epithelial tumor cells. A significant increase in the number of pulmonary nodules was seen in rabbits that received prestorage LD-ABT compared to animals transfused with SBT. Significantly lower numbers of pulmonary nodules were observed in rabbits that received poststorage LD-ABT compared with animals transfused with nonleukodepleted ABT. These studies show that ABT promote tumor growth of established animal tumors, that the ABT-induced tumor growth effect is related to the presence of donor allogeneic leukocytes, and that this effect can be ameliorated by prestorage leukodepletion. The present results also provide evidence for the lack of efficacy of poststorage leukodepletion in preventing ABT tumor growth promotion. Although data from animal models cannot necessarily be extrapolated to the clinical situation, these studies suggest that ABT may have a deleterious effect on recipients with established tumors and that the poststorage leukodepletion of allogeneic blood products may not be as effective as prestorage leukodepletion in preventing the tumor growth-promoting effect of allogeneic blood transfusions.

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MATERIALS AND METHODS

Effect of ABT on established tumor growth in mice. Two different strains of mice were used. Adult male mice of the C57Bl/6J (MHC type H-2b) and Balb/c (MHC type H-2d) strains were purchased from the Jackson Laboratory (Bar Harbor, ME). On day 0, an intravenous (IV) infusion of 2.5 × 10⁶ methylicholanthrene-induced fibrosarcoma (FSL-10) cells, syngeneic (H-2b) to the C57Bl/6J mice, were administered to each recipient C57Bl/6J animal. The viability of the tumor cells, as determined by trypan blue exclusion, before infusion, was over 95%. Blood was collected, as described previously, from both strains of mice and transfused IV directly into the tail vein of recipient animals, within an hour of collection. Two different transfusion protocols were used. In the first, recipient C57Bl/6J mice were transfused with 0.2 mL of either


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syngeneic (SBT), nonleukodepleted allogeneic (ABT), or leukodepleted allogeneic (LD-ABT) blood on days +4 and +9 after the inoculation of the FSL-10 tumor cells. In the other protocol, the transfusions took place on days +9 and +11 subsequent to the administration of the FSL-10 tumor cells. In terms of blood volume, the amount infused on each occasion (0.2 mL) to each recipient animal represents approximately 10% of the blood volume of each animal. Leukodepletion of the allogeneic blood was performed as previously described using leukocyte filters, made of cellulose acetate fiber in a polycarbonate housing, with 1- to 5-mL capacity. These filters were constructed specifically for these studies by Miles, Inc (Berkeley, CA), and provide approximately 2 log_{10} (99.9%) leukocyte removal. In the leukodepletion experiments, allogeneic whole blood was collected, as described above, and divided into two aliquots. One aliquot was transfused without leukocyte filtration, and the other aliquot was leukodepleted before transfusion. In all murine experiments, the animals were killed 21 days after tumor-cell infusion, with the number of pulmonary metastatic nodules counted after the intratracheal injection of Bouin's solution, as described previously. In all experiments, the enumeration of the pulmonary nodules was performed blindly, ie, by an individual who had no knowledge of the type of blood product received by that animal.

Effect of allogeneic blood component transfusions on established tumor growth in mice. Allogeneic whole blood was collected from Balb/c mice as described above. Shortly after collection, one aliquot was transfused as whole blood. The other aliquot was centrifuged at room temperature at 10,000g for 3 minutes. The supernatant plasma and the buffy-coat were then removed. Recipient C57Bl/6J mice were administered either allogeneic whole blood (0.2 mL), plasma, or buffy-coat on days +4 and +9 after the inoculation of the FSL-10 tumor cells. The volume of plasma or buffy-coat transfused on each occasion was estimated to represent the amount of each component found in 0.2 mL of whole blood. All mice were killed 21 days after the tumor cell infusion and the number of pulmonary metastatic nodules counted as described above.

Effect of ABT on established tumor growth in rabbits. Two different outbred strains of rabbits were used for these studies. California Black (CB) rabbits were used as the allogeneic blood donors and New Zealand White (NZW) rabbits were used as the recipients, as described previously. Because the NZW rabbits used for these studies were often littermates or siblings, we have chosen for the purpose of these experiments to regard donor blood from such animals as being "syngeneic" to the recipients. All animals were purchased from suppliers by the Animal Care Facility at McMaster University Medical Centre. On day 0, 10^6 tumor cells derived from a spontaneously occurring rabbit epithelial tumor (VX-2 tumor cells) were administered IV via a marginal ear vein to each recipient NZW rabbit, as described previously. Blood was collected from animals of both strains of rabbits using the main auricular artery into the anticoagulant, citrate phosphate double-dextrose solution (CP2D), in a triple additive (AS-3) set with collection bag. The fresh whole blood was thoroughly mixed and then divided into three aliquots. One aliquot was transfused without leukodepletion. The second aliquot was leukodepleted before storage (prestorage leukodepletion) using a leukocyte-depletion system (Leukotrap Red Cell Storage System; Miles Inc) that provides 3 log_{10} (99.9%) leukocyte removal, and then stored at 4°C for 1 week. The third aliquot was stored for 1 week at 4°C and then leukodepleted (poststorage leukodepletion) using the same leukocyte depletion system. Recipient NZW rabbits were administered 10 mL of either the nonleukodepleted allogeneic red blood cell (RBC) suspension, the prestorage leukodepleted allogeneic RBC suspension, the poststorage leukodepleted allogeneic RBC suspension, or the syngeneic RBC suspension on days +4 and +9 after the infusion of the VX-2 tumor cells. The amount (10 mL) of the RBC suspension infused on each occasion to each recipient animal represents approximately 10% of the total blood volume of each animal. All rabbits were killed 28 days after the infusion of the tumor cells, and the number of pulmonary metastatic nodules counted as described for the murine experiments. Again, the enumeration of the pulmonary metastatic nodules was performed by an observer who had no knowledge of the type of blood transfusion received by that animal.

Hematologic techniques and statistical methods. In both animal models, leukocyte counts were performed microscopically before leukodepletion as described previously. To evaluate leukocyte removal efficiency, white blood cell (WBC) counts were performed after leukodepletion, using the Nageotte hemocytometer (Paul Marienfeld, Bad Mergentheim, Germany). Statistical analyses were performed by comparing the median numbers of pulmonary metastatic nodules in the different groups of animals using the Mann-Whitney U-test. The statistical significance level was chosen to be 0.05.

RESULTS

Effect of blood transfusions on growth of established tumors in mice. Table 1 shows the results of the effect of blood transfusions on tumor growth as well as the efficacy of leukodepletion in preventing the ABT-related growth enhancement of established tumors in mice. Mice transfused with nonleukodepleted ABT on days +4 and +9 after the infusion of the FSL-10 tumor cells had a significantly higher (P = .0003) number of pulmonary metastatic nodules than animals that had received syngeneic blood. A statistically significant difference (P = .002) was also seen in experiments in which mice were transfused 9 and 11 days after tumor cell inoculation.

The leukodepletion of the fresh allogeneic donor mouse whole blood was 99.6% effective, resulting in a median leukocyte count reduction from 2.890 to 0.01 x 10^9/L. A statistically significant (P = .0001) reduction in the numbers of pulmonary metastatic nodules was observed in animals that received leukodepleted ABT on days +4 and +9 compared to animals transfused with nonleukodepleted ABT. Leukodepletion of ABT before transfusion also prevented the ABT-related enhancement of tumors in mice transfused on days +9 and +11 (P = .0003). The pooled data from three separate experiments performed to examine the effect of each type of blood transfusion on growth of established tumors in mice (n = 75) are also shown in Table 1. Animals transfused with nonleukodepleted ABT had higher numbers of pulmonary metastatic nodules than those that had received either leukodepleted ABT or SBT. There was not a difference between the median numbers of pulmonary nodules observed in mice that received leukodepleted ABT compared to mice transfused with syngeneic blood.

Effect of allogeneic blood component transfusions on growth of established tumors in mice. The results of a different set of experiments performed to examine the impact of allogeneic blood component transfusions on growth of established tumors in mice (n = 133) are described in Table 2. The median leukocyte number present in the blood products used in these studies was 0.42 x 10^9/L in the whole blood, 0.61 x 10^9/L in the buffy-coat, and 0.005 x 10^9/L in the fresh plasma. Mice transfused with buffy-coat leukocytes had a significantly increased number of pulmonary meta-
either nonleukodepleted plasma or leukodepleted plasma. Pretransfusion leukodepletion produced a median leukocyte count reduction from 1.638 to 0.006 x 10^9/L, whereas poststorage leukodepletion was 99.6% effective, providing a median leukocyte count reduction from 1.638 to 0.007 x 10^9/L. Significantly lower numbers of pulmonary metastatic nodules were observed in rabbits that had received pretransfusion leukodepleted ABT compared with those detected in animals that had been transfused with either nonleukodepleted ABT (P < .0001), or poststorage leukodepleted ABT (P < .0001). In contrast, no difference was seen in the numbers of pulmonary nodules observed in rabbits that received poststorage leukodepleted ABT compared to those seen in animals transfused with nonleukodepleted ABT.

**Table 1. Effect of SBT, ABT, or LD-ABT on Numbers of Pulmonary Metastatic Nodules in C57Bl/6J Mice With Established Tumors**

<table>
<thead>
<tr>
<th>Time of Blood Transfusion After the Infusion of the Tumor Cells (d)</th>
<th>SBT</th>
<th>ABT</th>
<th>LD-ABT</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Mice per Group</td>
<td>Median (range) No. of Pulmonary Metastatic Nodules</td>
<td>No. of Mice per Group</td>
<td>Median (range) No. of Pulmonary Metastatic Nodules</td>
</tr>
<tr>
<td>+4 and +9</td>
<td>14</td>
<td>6.5 (0-100)</td>
<td>15</td>
</tr>
<tr>
<td>+9 and +11</td>
<td>14</td>
<td>9.0 (1-100)</td>
<td>12</td>
</tr>
<tr>
<td>Both protocols</td>
<td>28</td>
<td>8.5 (0-100)</td>
<td>27</td>
</tr>
</tbody>
</table>

On day 0 each animal was administered with 2.5 x 10^5 FSL-10 tumor cells intravenously. Transfusions were administered either on days +4 and +9 or on days +9 and +11 after the infusion of the tumor cells.

Effect of blood transfusions on the growth of established tumors in rabbits. Table 3 shows the pooled data of two experiments in which NZW rabbits with established tumors were transfused with either SBT, nonleukodepleted ABT, prestorage ABT, or poststorage ABT. A significant (P < .0001) increase in the number of pulmonary nodules was observed in rabbits that received nonleukodepleted ABT compared to animals transfused with SBT. Prestorage leukodepletion produced 99.7% WBC removal resulting in a median leukocyte count reduction from 2.025 to 0.006 x 10^9/L, whereas the poststorage leukodepletion was 99.6% effective, providing a median leukocyte count reduction from 1.638 to 0.007 x 10^9/L. Significantly lower numbers of pulmonary metastatic nodules were observed in rabbits that had received prestorage leukodepleted ABT compared with those detected in animals that had been transfused with either nonleukodepleted ABT (P < .0001), or poststorage leukodepleted ABT (P < .0001). In contrast, no difference was seen in the numbers of pulmonary nodules observed in rabbits that received poststorage leukodepleted ABT compared to those seen in animals transfused with nonleukodepleted ABT.

**DISCUSSION**

Previous studies in both inbred (mice) and outbred (rabbits) animals from our laboratory provide evidence that unmodified ABT have a tumor growth-promoting effect when compared to animals transfused with SBT. Pretransfusion leukodepletion produced 99.7% WBC removal resulting in a median leukocyte count reduction from 2.025 to 0.006 x 10^9/L, whereas the poststorage leukodepletion was 99.6% effective, providing a median leukocyte count reduction from 1.638 to 0.007 x 10^9/L. Significantly lower numbers of pulmonary metastatic nodules were observed in rabbits that had received prestorage leukodepleted ABT compared with those detected in animals that had been transfused with either nonleukodepleted ABT (P < .0001), or poststorage leukodepleted ABT (P < .0001). In contrast, no difference was seen in the numbers of pulmonary nodules observed in rabbits that received poststorage leukodepleted ABT compared to those seen in animals transfused with nonleukodepleted ABT.

**Table 2. Effect of Allogeneic Blood Component Transfusions on Numbers of Pulmonary Metastatic Nodules in C57Bl/6J Mice With Established Methylcholanthrene-Induced Fibrosarcoma Tumors**

<table>
<thead>
<tr>
<th>Allogeneic Blood Product Transfusion</th>
<th>No. of Mice per Group</th>
<th>Median (range) No. of Pulmonary Metastatic Nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonleukodepleted whole blood (ABT)</td>
<td>55</td>
<td>31.0 (2-150)</td>
</tr>
<tr>
<td>Buffy-coat (BC)</td>
<td>29</td>
<td>41.0 (6-150)</td>
</tr>
<tr>
<td>Nonleukodepleted fresh plasma (FP)</td>
<td>29</td>
<td>12.0 (1-150)</td>
</tr>
<tr>
<td>Leukodepleted blood (LD-ABT)</td>
<td>20</td>
<td>5.0 (0-15)</td>
</tr>
</tbody>
</table>

Transfusions were administered on days +4 and +9 after tumor cell infusion.

*Statistical significance (Mann-Whitney U-Test): ABT v LD-ABT, P = < .0001; ABT v FP, P = .01; ABT v BC, P = .5; LD-ABT v FP, P = .002; LD-ABT v BC, P = .0001; FP v BC, P = .007.

**Table 3. Effect of Syngeneic (SBT), Nonleukodepleted Allogeneic (ABT), Prestorage Leukodepleted Allogeneic (PRE-LD-ABT), or Poststorage Leukodepleted Allogeneic (POST-LD-ABT) Blood Transfusions on Numbers of Pulmonary Metastatic Nodules in Recipients NZW Rabbits With Established Tumors**

<table>
<thead>
<tr>
<th>Blood Transfusion</th>
<th>No. of Rabbits per Group</th>
<th>No. of Pulmonary Metastatic Nodules Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syngeneic (SBT)</td>
<td>12</td>
<td>17.5 (5-28)</td>
</tr>
<tr>
<td>ABT</td>
<td>20</td>
<td>50.0 (5-86)</td>
</tr>
<tr>
<td>PRE-LD-ABT</td>
<td>20</td>
<td>20.0 (2-50)</td>
</tr>
<tr>
<td>POST-LD-ABT</td>
<td>18</td>
<td>39.0 (22-86)</td>
</tr>
</tbody>
</table>

On day 0 each animal was inoculated with 10^6 VX-2 tumor cells. Transfusions were administered on days +4 and +9 after the infusion of the tumor cells.

* Statistical significance (Mann-Whitney U-Test): SBT v ABT, P < .0001; ABT v PRE-LD-ABT, P < .0001; ABT v POST-LD-ABT, P = .06; PRE-LD-ABT v POST-LD-ABT, P < .0001.
administered before the infusion of the tumor cells. These observations were made in animals who received ABT before the tumor cells. To better simulate the human situation, we explored, in these present studies, the role of ABT in animals with established tumors. The present data show clearly that ABT significantly enhance tumor growth also in animals with established tumors. Interestingly, the detrimental ABT tumor growth-promoting effect appears to be unrelated to the timing of ABT and occurred both in mice transfused on days +4 and +9, as well as days +9 and +11 after tumor cell inoculation. Moreover, another transfusion protocol (on days +1 and +4) in the mouse model gave similar results (data not shown). This is in contrast to data from a recent study, using inbred animals (mice) only, which indicated that multiple ABT administered after tumor cell engraftment can promote tumor growth. In that study some groups of animals that received two transfusions between 1 and 10 days after the infusion of B16 melanoma cells did not develop larger tumors than mice administered syngeneic blood or saline. However, it is important to note that in the latter report some groups consisted of only a small number of animals (<10).

It has been shown that the presence of methylcholanganthrene-induced fibrosarcoma tumors causes phenotypic and functional alterations in T lymphocytes in tumor-bearing mice. Such alterations are related to an increase in the percentage of highly suppressive CD8+ cells and splenic macrophages lacking major histocompatibility complex (MHC) class II antigens (Ia+ cells) associated with cytokine dysregulation. Thus, mice bearing a tumor for more than 26 days have been shown to develop CD8+ T cells with impaired cytotoxic function and decreased ability to mediate an antitumor response in vivo. In the mouse experimental model used in the present study, mice were killed 21 days after the tumor cell inoculation and thus probably before the appearance of the immunosuppressive effect caused by tumor growth. Therefore, this would suggest that the growth enhancement of established tumors observed in our experimental animals represents the effect of the ABT rather than the immunosuppression caused by the presence of the tumor, which has been observed in tumor-bearing animals for long periods.

The immunosuppressive effects caused by ABT have been postulated to be immunologically mediated. The presence of passenger leukocytes bearing class II MHC antigens in the transfused allogeneic blood may be important in modifying host immune defense. Although the mechanism involved in this phenomenon has not been clearly elucidated, results from experiments in C57Bl/10 mice indicate that the immunosuppressive effect of ABT may be caused by the antigen-presenting B cells downregulating naive T cells, inducing tolerance to tissue allografts. In the present experiments, using allogeneic blood component transfusions to evaluate the relevance of transfused leukocytes in the ABT-promoting tumor-growth enhancement, we showed, in both inbred and outbred animal models, that animals transfused with allogeneic blood components containing WBCs consistently develop higher numbers of pulmonary metastatic nodules compared to animals transfused with allogeneic leukodepleted blood components.

To confirm and extend our previous observations that the prestorage leukodepletion of ABT reduces the growth enhancement of nonestablished animal tumors, we also showed in the present study that WBC reduction shortly after collection (prestorage leukodepletion) of ABT significantly ameliorates the ABT-induced growth enhancement of experimentally established tumors in both inbred and outbred animals. Interestingly, we showed, in the rabbit model, that WBC reduction after storage just before transfusion (poststorage leukodepletion) of ABT did not prevent the ABT tumor-growth promotion, even though the number of WBCs removed was similar in both instances. In this context, it has been shown that platelet transfusions may be accompanied by acute febrile nonhemolytic transfusion reactions associated with the transfusion of cytokines actively synthesized and released by leukocytes in the donor blood during storage. Prestorage leukodepletion decreases cytokine production in stored platelet concentrates and thus possibly prevents febrile transfusion reactions. It is possible that in addition to the removal of immunologic active cells, prestorage leukodepletion might also prevent the accumulation of soluble biologic mediators that may also be involved in the ABT immunomodulatory effect.

The effect of ABT on the recurrence of human malignancies has not yet been established. The available clinical data do not provide definitive conclusions as to whether ABT influence the rates of tumor recurrence and survival of patients with malignancy undergoing curative surgery. Thus, whether individuals with such clinical conditions should receive leukodepleted blood products has not yet been established. The data examining the relationship between ABT and colorectal cancer recurrence have been derived mostly from retrospective studies published over the last decade. These data have been subjected to meta-analysis by two groups of investigators. One such analysis indicates that the cumulative odds ratios of colorectal carcinoma recurrence, cancer-associated death, and death from any cause in ABT-transfused patients are 1.80, 1.76, and 1.63, respectively. These investigators concluded that the results support the hypothesis that perioperative blood transfusion is associated with an increased risk of colorectal carcinoma recurrence and death from this malignancy. The second group of investigators concluded that the transfusion effect might increase the relative risk of colorectal cancer recurrence by 37% (95% confidence interval: 20% to 56%). Therefore, both analyses suggest that ABT adversely affect the prognosis of patients with colorectal cancer. In contrast, a recently reported prospective randomized study in colorectal carcinoma patients concluded that the risk of colorectal cancer recurrence was increased in patients transfused with either homologous or autologous blood transfusions compared with patients who did not require such transfusions. However, a similar prospective randomized study (reported only in abstract form) concluded that homologous (allogeneic) blood transfusions in colorectal cancer patients were an independent prognostic factor on cancer recurrent rate. Thus, whether or not ABT affect tumor growth in humans is still an open issue.

In conclusion, this report shows clearly that ABT signifi-
cantly enhance the growth of established animal tumors and that this effect can be ameliorated by the prestorage leukodepletion of the allogeneic blood products. The data also provided evidence for the lack of efficacy of poststorage leukodepletion in preventing this ABT tumor-growth promotion effect. Although results obtained from experimental animals cannot necessarily be extrapolated to the clinical situation, these studies suggest that the bedside (poststorage) leukodepletion of allogeneic blood products may not be effective in preventing the tumor growth-promoting effect of ABT. Properly designed prospective clinical studies are necessary to provide data for decision making about the appropriate use of leukodepletion in patients with a malignant tumor.

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Growth enhancement of established tumors by allogeneic blood transfusion in experimental animals and its amelioration by leukodepletion: the importance of the timing of the leukodepletion

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