Autoimmune and Alloimmune Phenomena in Patients With Aplastic Anemia: Cytotoxicity Against Autologous Lymphocytes and Lymphocytes From HLA Identical Siblings

By Reed P. Warren, Rainer Storb, E. Donnall Thomas, Paul J. Su, Eric M. Mickelson, and Paul L. Weiden

We have studied peripheral blood lymphocytes of 117 patients with severe aplastic anemia and 237 healthy individuals for reactivity against autologous lymphocytes and/or lymphocytes from HLA-identical siblings using a 51Cr release assay. Lymphocytes from 29% of the patients exhibited reactivity against their own lymphocytes, while only 3% of lymphocytes from normal individuals showed such reactivity. Lymphocytes from 49% of the patients showed reactivity against lymphocytes from their HLA-identical siblings compared to 4% of normal individuals. Correlation existed between allogeneic and autologous reactivities (p < 0.001), suggesting a common pathway for cytotoxicity. Both reactivities showed an association with extremely low granulocyte counts (p < 0.01) and an inability of the patient's mononuclear cells to stimulate in allogeneic mixed leukocyte culture (p < 0.05) as well as an inverse correlation with time from diagnosis (p < 0.001). At least two explanations exist for the transfusion-independent autologous and allogeneic cytotoxicities: (1) they could be epiphenomena secondary to the stem cell defect, such as the loss of a cell that suppresses or regulates naturally occurring cytotoxic cells, or (2) they may be involved in the cause of the disease.

MATERIALS AND METHODS

This study includes 117 patients with severe AA. All patients had severely hypocellular marrows and at least two of the following: platelet count <20,000/cu mm, granulocyte count <500/cu mm, and reticulocyte count (corrected) <1.0% with Hb <10 g/100 ml (before transfusion). The etiology of aplasia in 86 of these patients was unknown, in 18 the disease was drug or chemical related, in 9 it was associated with hepatitis, and 4 had Fanconi's anemia. All but three had HLA-identical siblings who subsequently served as donors for marrow transplants. Seventeen patients had not received blood products before their study (untransfused patients). The LMC assay has been described. 

Briefly, Ficoll-Hypaque-separated peripheral blood mononuclear cells (term lymphocytes) of the patient were assayed as effector cells for reactivity against the same population labeled with 51Cr (i.e., autologous targets). Control effectors were lymphocytes from healthy untransfused individuals who had never been pregnant. Lymphocytes of the patient were also tested for reactivity against lymphocytes of HLA-identical siblings as previously described. Cytotoxicity was calculated as percent 51Cr release = (experimental release - control release)/(maximum release - control release) × 100. In study for autoreactivity of patient lymphocytes, experimental release was obtained from incubating effector lymphocytes of the patient with target lymphocytes of the patient, and control was obtained from incubating effector lymphocytes of an unrelated individual or the HLA-identical sibling with target lymphocytes of the patient. In tests for LMC alloreactivity against sibling lymphocytes, experimental release was obtained from incubating lymphocytes of the patient with target lymphocytes of the HLA-identical sibling, and control release was obtained from incubating effector lymphocytes of the sibling with target lymphocytes of the sibling. Maximum release was obtained through freezing and thawing an aliquot of target cells or, alternatively, treatment of an aliquot with a detergent. As described earlier, a study of the chromium release effected by lymphocytes of 51 normal individuals against lymphocytes of HLA-identical siblings revealed a mean percentage release of 0.3 with a standard deviation of 0.9 (range 0.6-13.6%). Thus, a positive assay for this study was defined as 3.5% (i.e., mean ± 2 SD) or greater with a difference between experimental and control release significant at p < 0.05 by the t test.

Four patients could not be studied for autologous reactivity due to limited availability of lymphocytes.

The mixed leukocyte culture (MLC) assay was carried out as previously described. Patients whose lymphocytes failed to stimulate at a level of at least 50% of that of their HLA-identical sibling in parallel tests against responder lymphocytes of the same unrelated individual(s) were considered to have an impairment of ability to stimulate.

The Fisher's exact test, chi-square analysis, Mann-Whitney U test, and coefficient of correlation were used to determine significance in these studies.
Differe once percent release from patient lymphocytes as compared to that from lymphocytes from normal individuals (two-sided Mann-Whitney U test).

Fig. 1. Percent chromium release from autologous lymphocytes in the lymphocyte-mediated cytotoxicity assay. Chromium-51-labeled lymphocytes from patients with aplastic anemia who had received preceding blood transfusions, from untransfused patients with aplastic anemia, and from healthy unrelated individuals were incubated with autologous effector lymphocytes.

**RESULTS**

**Autoreactivity**

Lymphocytes from 33 of 113 patients reacted against autologous lymphocytes (Fig. 1 and Table 1). Autoreactivity was found mainly with lymphocytes from patients with idiopathic aplastic anemia and with those from patients in whom the disease was thought to be due to drugs or chemicals. In contrast, lymphocytes from only 4 of 123 normal individuals had autologous reactivity of 3.5% 51Cr or greater ($p < 0.001$).

**Alloreactivity**

Lymphocytes from 56 of 114 patients reacted against lymphocytes from HLA-identical siblings (Fig. 2). The mean percent 51Cr release from lymphocytes of all 114 patients was 5.5% with a range of −6.9%–28.2%. The mean percent 51Cr release of the positive assays (i.e., > 3.5%) was 10.7. Seventeen of the 114 patients were untransfused. The mean percent 51Cr release from their lymphocytes was 8.8% with a range of −3.8%–28.2%. Ten of the 17 had reactive lymphocytes with mean 51Cr release of 13.8%. In contrast to findings with the aplastic patients, lymphocytes from only 5 of the 114 normal siblings had significant cytotoxicity against lymphocytes of the patients.

Because of the limitations in lymphocyte numbers and the fact that the patients underwent marrow transplantation soon after admission, only a few additional studies were carried out. The results of one

<table>
<thead>
<tr>
<th>Table 1. Autologous Cytotoxicity of Lymphocytes of Patients With Aplastic Anemia</th>
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</thead>
<tbody>
<tr>
<td><strong>Individuals Studied</strong></td>
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<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Patients with aplastic anemia</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Normal HLA-identical siblings</td>
</tr>
<tr>
<td>Normal unrelated controls</td>
</tr>
</tbody>
</table>

$^*$ Difference between percent release from patient lymphocytes as compared to that from lymphocytes from normal individuals (two-sided Mann-Whitney U test).
study in an untransfused patient is illustrated in Fig. 3. An increase in $^{51}$Cr release from cells of the HLA-identical sibling was found with an increasing effector/target cell ratio.

Another study involved an untransfused patient whose lymphocytes repeatedly failed to react against autologous lymphocytes but did react against lymphocytes of the HLA-identical sibling. Further study with lymphocytes of this patient revealed strong reactivity against the lymphocytes of both parents and two healthy unrelated individuals (Table 2). Cells of one of the unrelated individuals shared two HLA antigens with the patient and cells of the other unrelated individual shared one.

**Correlation Between Autoreactivity and Alloreactivity**

Correlation existed between autoreactivity and alloreactivity ($p < 0.001$). Lymphocytes from 27 patients were reactive against both autologous and sibling lymphocytes and those from 52 were negative against both. Lymphocytes from 4 patients were reactive against autologous lymphocytes only, and those from 27 were reactive against sibling lymphocytes only.

**LMC Reactivity and Time From Diagnosis**

The median time from diagnosis to study for patients with lymphocyte reactivity was 1 mo as compared to 3 mo for patients with lymphocytes lacking reactivity ($p < 0.01$). The median time from diagnosis for patients with lymphocyte reactivity against autologous lymphocytes was 0.75 mo as compared to 2.5 mo for patients without autologous lymphocyte reactivity ($p < 0.001$). For 10 untransfused patients with lymphocyte reactivity against autologous lymphocytes or lymphocytes of allogeneic siblings, the median time from diagnosis was 0.6 mo as compared to 1 mo in 7 untransfused patients without lymphocyte reactivity (no significant difference, $p < 0.3$).

Reactivity of the patient’s lymphocytes at specific times following diagnosis was also analyzed. Mean percent $^{51}$Cr releases and standard deviations, calculated from the reactivity of each patient’s lymphocytes against either autologous or allogeneic sibling lymphocytes (whichever was greater), are illustrated in Fig. 4. A significant inverse correlation between LMC reactivity and time from diagnosis was found ($p < 0.001$ by coefficient of correlation).

The data illustrated in Fig. 4 are a reflection of the average magnitude of $^{51}$Cr release at various times after diagnosis. In Fig. 5, the frequency of reactivity against autologous lymphocytes at the various times is illustrated. A significant inverse correlation with time from diagnosis was found with this analysis

![Figure 3. LMC reactivity at different effector/target ratios of lymphocytes from an untransfused patient against $^{51}$Cr-labeled lymphocytes from an HLA-identical sibling.](image)

![Figure 4. Mean percent releases and standard deviations of the lymphocyte reactivity from each patient against either autologous lymphocytes or those from HLA-identical siblings (whichever was greater). The shaded area above the broken line indicates means in excess of 3.5%.](image)

**Table 2. LMC Reactivity of Lymphocytes From an Untransfused Patient Against Lymphocytes of Family Members and Unrelated Control Individuals**

<table>
<thead>
<tr>
<th>Reactivity* Against Lymphocytes of</th>
<th>Self</th>
<th>Sibling</th>
<th>Father</th>
<th>Mother</th>
<th>Unrelated 1</th>
<th>Unrelated 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-Identical</td>
<td>2.1</td>
<td>23.0</td>
<td>42.8</td>
<td>16.7</td>
<td>36.2</td>
<td>36.8</td>
</tr>
</tbody>
</table>

*Percent chromium release.
Similarly, lymphocytes from 24 of 33 (73%) patients studied within 1 mo of diagnosis had reactivity against HLA-identical sibling lymphocytes compared to those from 32 of 81 (39%) patients studied 1 mo or later ($p < 0.001$).

**LMC Reactivity and Ability to Stimulate in MLC (Table 3)**

We have previously reported that cells from 28 of 53 (40%) patients with severe AA showed impaired ability to stimulate lymphocytes from unrelated individuals in MLC. Of the patients in this report, 4 were among the 53 patients previously tested in MLC for the ability of their cells to stimulate. In confirmation of our earlier findings, cells from 72 of 110 (65%) of the additional patients in this report failed to stimulate allogeneic cells at a level of at least 50% of that of their HLA-identical siblings.

An inverse correlation was found between ability to stimulate and LMC reactivity (Table 3). Lymphocytes from only 7 of 33 (21%) patients with autologous reactivity could stimulate, while those from 31 of 80 (39%) of patients without autologous reactivity could stimulate ($p < 0.05$). Lymphocytes from only 9 of 56 (19%) patients with allogeneic reactivity could stimu-

### Table 3. Patient Characteristics and LMC of Autologous Lymphocytes and Those From HLA-Identical Siblings

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Cytotoxicity of Lymphocytes of 17 Untransfused Patients Against Sibling Lymphocytes</th>
<th>Cytotoxicity of Lymphocytes of 113 Patients Against Autologous Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (10)</td>
<td>Negative (7)</td>
</tr>
<tr>
<td>Ability to stimulate in MLC*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. patients having lymphocytes with ability to stimulate</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Sex of patient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Age (median yr)</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Blood cell values (median/cu mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total WBC</td>
<td>2,100</td>
<td>2,700</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>150</td>
<td>700</td>
</tr>
<tr>
<td>Platelets</td>
<td>13,000</td>
<td>10,600</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1,900</td>
<td>1,800</td>
</tr>
<tr>
<td>HCT (mean %)</td>
<td>20</td>
<td>22</td>
</tr>
<tr>
<td>Percent of patients with infection at admission</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Percent of patients with history of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androgen treatment</td>
<td>30</td>
<td>57</td>
</tr>
<tr>
<td>Prednisone treatment</td>
<td>80</td>
<td>57</td>
</tr>
<tr>
<td>Infection</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

*Ability of lymphocytes from patients with AA to stimulate lymphocytes of healthy unrelated individuals when tested in mixed leukocyte culture: patients whose lymphocytes were able to stimulate at a level of at least 50% of that of their HLA-identical siblings.

†Chi-square analysis.

‡Mann-Whitney U test.
late as compared to those from 30 of 58 (52%) of patients without alloimmune reactivity \( (p < 0.001) \). However, lymphocytes from 2 of 10 (20%) untransfused patients with reactivity could stimulate, which is not significantly different from the 4 of 7 (57%) untransfused patients without lymphocyte reactivity whose lymphocytes could stimulate.

**Correlation of LMC Reactivity With Clinical Features**

A number of patient characteristics was studied for possible association with LMC reactivity (Table 3). Only autologous and alloimmune tests of untransfused patients were considered in these analyses to eliminate any influence of sensitization due to preceding blood transfusions. Granulocyte numbers were significantly lower in patients with autoreactive lymphocytes than in those without \( (p < 0.01) \) and were also significantly lower in untransfused patients who had alloimmune lymphocytes \( (p < 0.1) \). However, granulocyte numbers were extremely low in all of these patients. The percentage of patients who had received androgens prior to study was significantly lower in those who had autoreactivity than in those who did not \( (p < 0.05) \). None of the other patient characteristics in Table 3 was associated with LMC reactivity. HLA-A and B antigens and ABO types of the patients were also studied for correlations with LMC, and no association was found (data not shown).

**DISCUSSION**

Lymphocytes from approximately one-third of the 117 patients in the present study were reactive in LMC against autologous lymphocytes compared to lymphocytes from 3% of 123 healthy individuals. Similarly, lymphocytes from half of the 117 patients showed cytotoxicity to lymphocytes from their healthy HLA-identical siblings, a phenomenon observed with lymphocytes from only 4% of 114 healthy individuals. This indicates an alteration in immunity in one-third to one-half of lymphocytes of patients with AA that is only rarely seen with lymphocytes of healthy individuals. Autologous and alloimmune reactivities were concordant on a statistical basis, though approximately one-third of the patients had lymphocytes reactive in one assay and not the other. This suggests a common pathway for cytotoxicity in many instances. Further support for a common cause for autologous and alloimmune lymphocyte reactivities is provided by the fact that both showed an association with extremely low granulocyte counts and an inability of the cells to stimulate in MLC as well as an inverse correlation with time from diagnosis. Transfusion-induced sensitization to non-HLA antigens may have been responsible for some of the alloimmune reactivity, as suggested by the observation that lymphocyte reactivity to cells from the HLA-identical marrow donor and marrow graft rejection were correlated in patients studied more than 1 mo after diagnosis. The current data fail to show an apparent correlation between a history of preceding transfusions and alloreactivity in the LMC assay. Lymphocytes from 56% of untransfused and 47% of transfused patients were reactive in this assay. It must be pointed out that lymphocytes from most untransfused patients in the present study were tested for LMC reactivity within 1 mo of diagnosis, while those of most transfused patients were tested beyond 1 mo of diagnosis. More transfused and untransfused patients need to be studied at comparable time intervals after diagnosis to determine the contribution of transfusions to the alloreactivity in the LMC assay. The effect of transfusions cannot explain the autologous reactivity by lymphocytes from transfused patients nor both autologous and alloimmune reactivities by lymphocytes from untransfused patients. Clearly, other explanations must be sought.

One explanation is that autologous and alloimmune reactivity could be epiphenomena secondary to the stem cell defect of AA. The lack of stem cells would lead not only to reduced or absent production of granulocytes, platelets, and red blood cells but also of mononuclear blood cells. The rates of their disappearance from the peripheral blood would be a function of their natural lifespans. Early in the course of AA, there might be disappearance of mononuclear subpopulation(s) suppressing (or regulating) naturally occurring cytotoxic cells. Monocytes can function as suppressor cells and are also known to be efficient stimulators in MLC.\(^{10-13}\) The lack of suppression would lead to the observed cytotoxicity against autologous mononuclear cells and those of the HLA-identical siblings. The nature of the target cell is unknown. The finding of cytotoxicity against lymphocytes from the HLA-identical sibling in the absence of autologous cytotoxicity can perhaps be explained by disappearance of the target cell population from the blood of some patients with AA. As time after diagnosis increases, the incidence and magnitude of autologous and/or alloimmune cytotoxicities decreases, which, according to this hypothesis, would most likely to due to a gradual disappearance of the effector cell population from the circulation as well. The naturally occurring cytotoxicity is not due to natural killer (NK) reactivity,\(^{14}\) since 26 patients of the present study were tested for NK activity, and no correlation with autocytotoxicity was found.\(^{15}\)

Another explanation for our findings is that the autoimmune and alloimmune phenomena are in some
way involved in the etiology of AA in either of two ways: (1) The disappearance of suppressor cells could be the primary event in AA. The lack of suppressor cells would lead to emergence of naturally occurring cells cytotoxic not only to differentiated mononuclear cells but also to hemopoietic stem cells. (2) The cytotoxicity seen could represent immunity to antigens of hemopoietic cells modified by extrinsic agents such as viruses or drugs. The cytotoxicity would lead to destruction of the modified hemopoietic target cells, thus causing AA. It is somewhat difficult to explain with this theory the allotoxicity observed, since the allogeneic target cells would presumably be unmodified. However, in vitro studies in mice showed that sensitization by trinitrophenol-modified "self" can extend to unmodified allogeneic targets by virtue of as yet undefined cross-reactivity.  

In recent years, several in vitro studies have provided evidence that some cases of AA may be mediated by immunologic mechanisms. Following the original report by Kagan et al., several laboratories have reported that lymphocytes from patients with severe AA are capable of inhibiting both committed granulocytic (CFU-C) and erythrocytic (CFU-E) stem cell growth. However, a study by Singer et al. showed that lymphocytes from multiply transfused patients tested against cells from unrelated individuals were probably reactive against histocompatibility differences related to sensitization by transfusion rather than to the etiology of the disease. Inhibition of CFU-E formation by mononuclear cells "sensitized" by transfusion was also demonstrated in studies involving health DLA-identical canine littermates. However, Singer et al. subsequently studied 16 untransfused patients and found that three possessed lymphocytes that significantly inhibited CFU-C growth of marrow from HLA-matched siblings. Ascensao et al. in a study of the patient described by Kagan et al., found that CFU-C growth of the patient's marrow was described by the addition of antithymocyte globulin, suggesting that T cells might be involved. Torok-Storb et al. have recently found that erythroid colony growth (BFU-E) from peripheral blood cells of 6 of 34 patients with aplastic anemia failed to grow until T cells from the patients had been removed. Moreover, growth of the colonies was inhibited when T cells from the patients were added back to the culture. These data suggest that an immune mechanism may play a role in the pathogenesis of AA in a small proportion of the patients.

Evidence from in vivo observations also suggest that some cases of AA may be mediated by an immunologic mechanism. Patients with severe AA given a simple infusion of marrow from a monozygous twin usually recover, suggesting that the disease is due to a stem cell defect caused by a toxic mechanism no longer present in the patient. However, several patients have now been observed who did not recover after a simple infusion of marrow from a monozygous twin but did recover following immunosuppressive therapy either with or without an infusion of allogeneic marrow. Finally, it has been known for a long time that a small fraction, perhaps 10%, of patients will undergo a "spontaneous" remission after a few weeks or months. Evidently, in these patients, the pathogenetic mechanism must have been transient. Perhaps an immunogenic stimulus, which might have been the patients' own stem cells with a viral or chemical hapten, disappeared so that the immunologic reaction faded away. If enough normal stem cells escaped the immunologic assault, recovery could then ensue.

The data presented here indicate a cytotoxic reaction, which was most evident soon after diagnosis and progressively less evident thereafter. A transient cytotoxic insult of this type would help to explain some of the contradictory information cited above. There is an obvious need for study of more patients at the time of onset of the disease. Unfortunately, symptoms do not usually develop until extensive marrow damage has occurred. Physicians should be particularly alert for those patients who do seek medical help early in the course of the disease so that appropriate studies can be carried out before transfusions have to be given.

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