Immunologic Response of Patients With Acute Leukemia to Platelet Transfusions

By Francisco Tejada, Wilma B. Bias, George W. Santos, and Philip D. Zieve

Nine leukemic patients in aplasia received platelet concentrates from random donors over a period of 5 to 32 wk (an average of 31 transfusions per patient). All nine developed lymphocytotoxic activity (presumably HL-A antibodies) in their serum against cells from a panel of 40 selected donors within 20-50 days after transfusions were begun. Lymphocytotoxic activity markedly diminished in the sera of seven surviving patients over a period of 8-24 wk despite continued occasional platelet transfusions. When lymphocytotoxic activity was present in the serum of these patients, survival of transfused platelets was significantly reduced. Sera in which lymphocytotoxic activity was detected released endogenous serotonin (26 ± 5% SEM) from washed human platelets (but not from dog or rabbit platelets) when incubated at 37°C for 30 min. In contrast, sera from these patients, obtained when lymphocytotoxic activity was not detected, released minimal amounts of serotonin (6 ± 2%) and sera from nine healthy subjects released none (0 ± 1%). The ability of serum to release serotonin was abolished, and lymphocytotoxic activity was markedly diminished, after preincubation with allogeneic platelets.

The incidence of hemorrhage in aplastic patients with acute leukemia has been markedly reduced by platelet transfusions.1 Although the antigenicity of platelets has been clearly established,2,3 these cells are generally not typed prior to transfusion. It has, however, been shown that platelets carry on their surface histocompatibility (HL-A) antigens3 and that, after several weeks of transfusions, patients who are refractory to nonmatched platelet donors do respond normally to HL-A “compatible” platelets.4 This observation suggests that the development of HL-A antibodies shortens the survival of HL-A incompatible platelets. Alloimmunization in immunosuppressed patients...
with acute leukemia has been reported to be variable\textsuperscript{1,5} and, in fact, sensitization
to Rh incompatible red cells has been said to be reduced.\textsuperscript{6} In the present
studies we measured the immune response of leukemic patients, made aplastic
by chemotherapy, to repeated transfusion of platelets from random donors,
demonstrating invariable development of multiple lymphocytotoxic (presum-
ably HL-A) antibodies and showing that the antibodies in these sera could be
detected by their release of endogenous serotonin from washed human platelets.

MATERIALS AND METHODS

Nine patients, 18 to 60 yr old, with acute leukemia were studied. Three patients had lympho-
blastic leukemia, three had myeloblastic leukemia, and three had myelomonocytic leukemia. There
were five male patients and four females; none of the latter had been pregnant. Platelet trans-
fusions were administered prophylactically when the patients’ platelet counts were 20,000 per
\( \mu L \) or less. The patients received an average of 31 transfusions (from 5 to 134 units), of ran-
domly obtained, untyped platelet concentrates\textsuperscript{7} over a period of 5-32 wk. The concentrates con-
tained an average of 29 ml of cell suspension containing \( 0.7 \times 10^{11} \) platelets. The average
number of contaminating leukocytes was \( 0.41 \times 10^8 \) per unit of concentrate. The patients’ plate-
let counts were measured\textsuperscript{8} just prior to transfusion as well as 1 and 20 hr afterward. The incre-
ment (Inc.) per \( \mu L \) in circulating platelets in the recipients was expressed as a function of the
total number of platelets transfused, adjusted to square meter (sq m) of
body surface area (B.S.A.).\textsuperscript{9}

\[
\text{Inc.} \times \text{sq m} = \frac{\text{post-transfusion count per } \mu L - \text{pretransfusion count per } \mu L}{\text{Total number of platelets transfused}} \times \text{B.S.A.}
\]

Samples of blood were collected from each patient prior to transfusion, at weekly intervals for
3 wk, and then every 2-3 wk until the patient died or the study was terminated. The blood was al-
lowed to clot in glass tubes for 2 hr at 37°C, and the serum separated by centrifugation at
4700 \( g \) for 30 min and stored at \(-70°C\). The presence of lymphocytotoxic activity in the serum
was evaluated by the use of a panel of cells from 40 donors of known HL-A type, selected to in-
clude all known HL-A groups as well as those without recognizable HL-A antigens (”blanks")
using the lymphocyte microcytotoxicity method.\textsuperscript{10} The number of cells killed in this system di-
vided by the total number of cells in the panel is called the per cent reactive antibody, a term
used subsequently in this paper as an expression of lymphocytotoxicity. Since HL-A antigens were
shared by some of the cells of the panel, the per cent reactive antibody allows only a very im-
precise estimate of the number of antibodies present.

The ability of serum to release serotonin from platelets was evaluated in the following way.
Blood was drawn daily from two normal human volunteers as previously described;\textsuperscript{11} platelets
were obtained by differential centrifugation of the blood\textsuperscript{12} and were washed twice in 2 ml of
0.07 \( M \) sodium potassium phosphate buffer, \( \text{pH 7.4} \) containing 0.5 mg/ml of disodium EDTA
and resuspended in 2.25 ml of this buffer without EDTA. The final number of platelets in each
tube was approximately \( 2.5 \times 10^8 \). The various samples of serum were preincubated at 36°C for
30 min, 0.25-ml portions of serum or buffer were then added to the platelet suspensions, and the
mixtures were incubated at 37°C for 30 min. Preliminary experiments established that maximum
release of serotonin was produced in most cases by final dilutions of serum of 1:40. That dilution
was used in all experiments unless otherwise stated. After incubation, the platelets were sedi-
mented\textsuperscript{12} and lysed in 2 ml of 0.02 \( M \) hydrochloric acid. The lysates, as well as appropriate
standard solutions, were deproteinized with 0.2 ml of 10\( \% \) zinc sulfate and 0.1 ml of 1 \( N \) sodium
hydroxide; the resultant precipitate was removed by centrifugation; and 1 ml of the supernatant
was added to 0.3 ml of concentrated hydrochloric acid. Serotonin in the lysates was then mea-
sured by spectrophotofluorometry.\textsuperscript{14} In some experiments rabbit and dog platelets were used in
this system instead of human ones.

Statistical analysis of the data was done with the aid of a 9100B Hewlett-Packard Calculator.

RESULTS

Lymphocytotoxic Antibody Response (Table 1)

There was no relationship between the number of units of platelets (and/or
red cells) transfused, and the subsequent development of reactive antibody, the
Table 1. Lymphocytotoxic Antibody Response in Patients Following Transfusion of Platelets

<table>
<thead>
<tr>
<th>Number of Units Transfused</th>
<th>Maximal Per Cent Reactive Antibody</th>
<th>Time Initial Response (days)</th>
<th>Time Maximal Response (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>Prior to Initial Response</td>
<td>Total</td>
<td>12.5</td>
</tr>
<tr>
<td>A</td>
<td>3</td>
<td>5</td>
<td>15.0</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>16</td>
<td>32.5</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>18</td>
<td>12.5</td>
</tr>
<tr>
<td>D</td>
<td>74</td>
<td>134</td>
<td>10.0</td>
</tr>
<tr>
<td>E</td>
<td>11</td>
<td>11</td>
<td>7.5</td>
</tr>
<tr>
<td>F</td>
<td>0</td>
<td>33</td>
<td>10.0</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>10</td>
<td>70.0</td>
</tr>
<tr>
<td>H</td>
<td>21</td>
<td>31</td>
<td>7.5</td>
</tr>
<tr>
<td>I</td>
<td>15</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

Time when antibody activity was first detected, or the time at which a maximal response was observed.

Only two patients showed lymphocytotoxic activity prior to the first platelet transfusion; both had 7.5% reactive antibody; both had received blood transfusions in the remote past. Within 50 days after transfusions were begun, the frequency of positive reactions increased in the serum of all patients. The average maximum per cent reactive antibody was 14.8 (range 7.5–70.0). The frequency of positive reactions markedly diminished or disappeared in the serum of seven surviving patients over a period of 55–193 days from the date of the first transfusion despite continued administration of platelets. For example, patient D had a maximal per cent reactive antibody of 12.5% at 32 days; despite receiving 60 units of platelets and 10 units of packed red cells over the next 12 wk, per cent reactive antibody fell to 7.5% at 55 days and 5.0% at 106 days. Similarly, patient H had a maximal per cent reactive antibody of 70.0% at 39 days, falling to 4.5% at 66 days and 0.0% at 164 days despite transfusion of 10 U of platelets and 7 units of packed red cells over the next 4 wk.

Lymphocytotoxic Activity and Platelet Survival (Fig. 1)

The increment in circulating platelets at 1 and 20 hr after transfusion was significantly lower in the blood of those patients whose sera were lymphocytotoxic at the time of transfusion ($p < 0.10$ and $p < 0.005$, respectively).*

Release of Endogenous Serotonin (Table 2)

Washed human platelets from nine normal donors, incubated for 30 min in buffer at 37°C, had a mean content of serotonin of 3.64 ± 0.22 µg SEM per $2.5 \times 10^8$ platelets. Serum from nine healthy control subjects released no serotonin when incubated with these platelets; serum obtained from leukemic patients at a time when lymphocytotoxic activity was not present, released a relatively small, but significant, amount of serotonin; and serum obtained from these same patients at a time when lymphocytotoxic activity was present, released considerably more serotonin. Heparin, 25 U per reaction mixture, did not affect release of serotonin by these sera, indicating that they did not contain residual thrombin.¹⁵

*Analysis of response of the nine patients in 14 instances in which associated bleeding and/or infection were absent.
Table 2. Release of Serotonin from Washed Human Platelets by Serum

<table>
<thead>
<tr>
<th>Serum</th>
<th>Mean ± SEM</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls</td>
<td>0 ± 1%</td>
<td>—</td>
</tr>
<tr>
<td>Nonlymphocytotoxic</td>
<td>6 ± 2%</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>Lymphocytotoxic</td>
<td>26 ± 5%</td>
<td>&lt; 0.0005</td>
</tr>
</tbody>
</table>

*p test of the paired means.

Table 3. $\chi^2$ Contingency Table

<table>
<thead>
<tr>
<th></th>
<th>Lymphocytotoxic</th>
<th>Nonlymphocytotoxic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_{2+}$</td>
<td>28</td>
<td>13</td>
<td>39</td>
</tr>
<tr>
<td>$a_{20}$</td>
<td>8</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>27</td>
<td>61</td>
</tr>
</tbody>
</table>

*$\chi^2 = 4.079$.

Preliminary experiments established that maximum release of serotonin by a particular sample of serum was often achieved by diluting it beyond the 1:10 dilution of the original test system. This observation suggested a prozone phenomenon in these sera, a hypothesis that was tested by means of a nonlinear regression method using the formula $y = a_0 + a_1x + a_2x^2$ (wherein $y =$ percent serotonin released, $a_0 =$ y intercept, $a_1 =$ the linear coefficient, $x = \ln$ (dilution), and $a_2 =$ half the second derivative) and then by construction of a $\chi^2$ contingency table (Table 3). Positive values for $a_2$ indicate that the formula describes a concave parabola, consistent with a prozone effect. The table indicates that 39 samples of serum demonstrated activity consistent with a prozone phenomenon and 22 did not and that the phenomenon was significantly more common in lymphocytotoxic sera ($p < 0.025$ for 1* of freedom).
In order to determine whether or not the frequency of cytotoxic reactions in the various samples of serum correlated with the degree of release of serotonin from platelets by these sera, the data were analyzed by the Duncan Multiple Range Test (Table 4). The analysis indicated a significant difference in serotonin release between sera which were not lymphocytotoxic and those with 7.5%-12.5% reactive antibody. There was also a significant difference between the latter sera and those with 15% or more reactive antibody. The Pearson product moment (r) expressing the correlation between per cent reactive antibody and percent serotonin released was .4343 (p < 0.001 for 59 degrees of freedom).

Specificity of Serotonin Release

Sera (No. 3 of patient B and No. 9 of patient I) were used to check the specificity of serotonin release by lymphocytotoxic serum (Table 5). When incubated with human platelets these sera released 41 and 40% of endogenous serotonin but released no serotonin from rabbit or dog platelets. The two latter types of platelets appeared, in fact, to accumulate serotonin present in the two samples of serum.

Adsorption of Lymphocytotoxic Serum (Table 6)

Sera from three different patients (patient E No. 4, G No. 1, and H No. 4) were demonstrated to release serotonin from washed platelets. Then samples of these sera (1.5 ml) were incubated at 4°C for three successive 24-hr periods with $3 \times 10^6$ washed platelets from three different normal donors (the platelets were sedimented and the serum reincubated with fresh platelets at the end of each day). As a control, samples of the same sera were also incubated in a similar way without platelets.

When incubated with fresh platelets (obtained from the same donor from whose platelets preadsorbed sera released serotonin) the adsorbed sera now resulted in a net accumulation by platelets of serotonin. In contrast, the incubated
Table 6. Effect of Adsorption of Lymphocytotoxic Serum

<table>
<thead>
<tr>
<th>Serum</th>
<th>Per Cent Reactive Antibody</th>
<th>% Serotonin Release</th>
<th>Serum Diluted 1:10</th>
<th>Serum Diluted 1:80</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>G</td>
<td>H</td>
<td>E</td>
</tr>
<tr>
<td>Preadsorption</td>
<td>5.0</td>
<td>7.5</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>Incubated adsorbed</td>
<td>2.5</td>
<td>2.5</td>
<td>5</td>
<td>-10</td>
</tr>
<tr>
<td>Incubated unadsorbed</td>
<td>5.0</td>
<td>7.5</td>
<td>37</td>
<td>7</td>
</tr>
</tbody>
</table>

control sera released as much serotonin as they had prior to incubation. Similarly, lymphocytotoxic activity markedly diminished in the adsorbed, compared to the unadsorbed, sera.

**DISCUSSION**

Platelets contain isoantigens, first demonstrated by the relatively insensitive technique of platelet agglutination21,22 and later defined precisely by Shulman and his associates2,23 by use of complement fixation. That some of these antigens are also common to other cells was suggested by the adsorption by leukocytes, from serum, of antibody directed against platelet antigens.24 It is now clear that these shared antigens are tissue histocompatibility (HL-A) antigens and that platelets are convenient cells with which to establish HL-A genotype.3

Transfusion of nonmatched allogeneic platelets elicits an immune response in the recipient, the appearance of which has been said to be dependent on the number of platelets transfused.2,24 Shulman using complement fixation reported for example, that less than 5% of patients receiving less than ten platelet transfusions were immunized, as opposed to 80% of patients receiving 100 or more transfusions.2 In this regard it is generally believed that immunosuppressed patients with acute leukemia are less likely to produce antibody in response to an antigenic stimulus than are normal people.6 However, Silver et al.25 showed that leukemic patients, when challenged with specific antigens, do develop antibodies, although at a somewhat lower titer than do normal controls. The current study, similarly, shows invariable development of lymphocytotoxic activity in the serum of immunosuppressed patients with acute leukemia. It is likely, although unproved without further characterization of these antibodies, that they were directed against HL-A antigens. The failure of others to demonstrate this high an incidence of allosensitization in leukemic patients may be explained by the kinds of donors used4 or by the frequency at which serum was tested. (In our studies antibodies appeared within 20-50 days and then diminished or disappeared within 88-193 days following the first of, on the average, 31 transfusions per patient.) Furthermore, the method of lymphocytotoxicity, employed in the present studies, is even more sensitive in detecting HL-A antibodies in serum than is complement fixation26 so that antibodies conceivably may have been present but not identified in previous reports.2
The explanation of the disappearance of antibodies in the serum of our patients, despite continued transfusions, is not clear although similar observations have been made by others. It is possible, but unproved, that the patients, as the result of continued immunosuppressive chemotherapy developed tolerance to the repeated antigenic stimuli or that their immune response, in general, became impaired.

Alloimmunization of recipients clearly results in shortened survival of transfused platelets, and Dausset et al. showed that some of the antibodies in the serum of alloimmunized patients were adsorbed by both platelets and leukocytes, suggesting HL-A specificity. Yankee et al. showed that survival of platelets after repeated transfusions was considerably longer in patients with aplastic anemia if HL-A compatible donors were used. In the current studies, also, the survival of transfused platelets was significantly prolonged in those patients who had no detectable lymphocytotoxicity in their serum compared to those who had.

Since the number of platelets transfused in these studies was consistently far in excess of the number of contaminating leukocytes, we believe that platelets were the primary antigenic stimuli. In any case, we demonstrated that lymphocytotoxic sera released serotonin from washed platelets, and that the sera toxic against the most cells of the panel released the most serotonin. It seems likely, therefore, that antibodies mediated the release of serotonin in these experiments. The small but significant release of serotonin caused by nontoxic sera may reflect the presence of alloantibodies, at a low concentration, that are not lymphocytotoxic. It is also possible that serotonin release from platelets is a more sensitive way to detect antibodies in serum than is lymphocytotoxicity. The species specificity of the release reaction and the parallel diminution in both lymphocytotoxicity and the ability of sera to release serotonin following adsorption by human platelets are further indications that antibodies caused serotonin release. Hirschman and Shulman showed recently that platelet isoantibodies, as well as sera from patients with immune thrombocytopenic purpura, released 14C-labeled exogenous serotonin that had been accumulated by washed human platelets. It may be that serotonin release from platelets by serum of immunized patients will prove to be a sensitive index of HL-A incompatibility.

The observation that maximum release of serotonin often required dilution of serum beyond the 1:10 dilution of the standard assay is consistent with a prozone effect. It seems unlikely in these experiments, however, that this phenomenon indicates antibody excess in the relatively undiluted sera since, on theoretical grounds, there is no reason why such an excess would inhibit release. A more attractive hypothesis is that sera requiring dilution to cause maximum release contain inhibitors to the reaction present in an effective concentration lower than that of the releasing antibody. Since so-called blocking antibodies to platelets have been demonstrated by their inhibition of complement fixation in the serum of alloimmunized patients, it seems possible that many of the samples of serum from our patients contained blocking antibodies. The mechanics of the assay for lymphocytotoxicity prevented us from testing this hypothesis further by use of that system.
REFERENCES

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