Use of 8-Methoxypsoralen and Ultraviolet-A Pretreated Platelet Concentrates to Prevent Alloimmunization Against Class I Major Histocompatibility Antigens

By Nanette H. Grana and K.J. Kao

The use of 8-methoxypsoralen (8-MOP) and UV-A irradiation to inactivate contaminating donor leukocytes in platelet concentrates and to prevent primary alloimmunization against donor class I major histocompatibility (MHC) antigens in mice was investigated. CBA/CaH-TGJ mice with the H2k haplotype and BALB/cByJ mice with the H2d haplotype were used as donors and recipients, respectively. The mixed leukocyte reaction between these two strains of mice showed that treatment of spleen cells with 500 ng/mL 8-MOP and 5J/cm² UV-A inhibited 99% of responder and 92% of stimulator function. There was no measurable loss of platelet aggregating activity after the treatment. After two weekly transfusions of platelets without any treatment, 93% of control mice (n = 15) developed anti-H2k antibody. In contrast, only 33% of mice (n = 15) receiving platelets treated with 8-MOP and UV-A became alloimmunized. After six weekly platelet transfusions, all mice became alloimmunized. Nevertheless, the mean titers of anti-H2k antibody in sera of the treated groups were significantly lower than the control groups. One hour posttransfusion recoveries of 51Cr-labeled donor platelets were also higher in mice transfused with the treated platelets. Thus, the pretreatment of platelet concentrates with 8-MOP and UV-A irradiation effectively reduced the alloantigenicity of class I MHC molecules. The implication of this finding in relation to the mechanism by which donor leukocytes alloimmunize recipients is discussed.

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Materials and Methods

RPMI 1640 and Eagle's minimum essential tissue culture media, and antibiotic-antimycotic solution were purchased from GIBCO Laboratory (Grand Island, NY). Protein A- Sepharose, pepsin, bovine serum albumin (BSA), fluorescein-labeled F(ab)₂ fragment of sheep antimouse IgG antibody, alkaline phosphatase conjugates of goat antimouse IgG or IgG-Fc specific antibody, histopaque-1077, and p-nitrophenyl phosphate disodium were obtained from Sigma Chemical Company (St Louis, MO). Methyl-3H-thymidine and ¹⁵Cr-sodium chromate were the products of Amersham Corp (Arlington, IL). The HB-20 anti-H2k hybridoma was obtained from the American Type Culture Collection (Rockville, MD). All other chemical reagents were analytical grade.

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Animals

Eight-week-old syngeneic CBA/CaH-761 (CBA) mice with the H2<sup>α</sup> haplotype and BALB/cByJ (Balb/c) mice with the H2<sup>α</sup> haplotype were purchased from Jackson Laboratory. CBA mice with the H2<sup>α</sup> haplotype were used as recipients. Donor blood was collected by retro-orbital venous plexus bleeding after being anesthetized with methoxyflurane. Glass Pasteur pipettes used for collecting blood were washed with 5 mL PBS twice and resuspended in Eagle's high amino acid medium containing 2.5% mouse serum, 30 mmol/L mercaptopoethanol and 2 mmol/L L-glutamine. Cells were incubated with responding cells for 5 days before addition of H-thymidine (5 μCi). Each incubation was performed in triplicate.

Platelet Aggregation Study

Platelet aggregation was studied by using a Chronolog model 560 whole blood lumiaggregometer (Chrono-Log Co, Havertown, PA). Mouse platelet-rich plasma (PRP) was diluted to 3 × 10<sup>5</sup> cells/μL with platelet-poor plasma. Each aggregation mixture consisted of 450 μL of PRP and 50 μL of aggregating agents. Secretions of adenosine triphosphate (ATP) during platelet aggregation were also measured by using luciferin and firefly luciferase (Chrono-Log Co) according to the manufacturer’s instruction.

Platelet Transfusion Study

CBA mice with the H2<sup>α</sup> haplotype for both class I and II MHC antigens were used as blood donors and Balb/c mice with the H2<sup>α</sup> haplotype were used as recipients. Donor blood was collected by retro-orbital venous plexus bleeding after being anesthetized with methoxyflurane. Glass pasteur pipettes used for collecting blood were washed with 5 mL PBS twice and resuspended in 2 mL phosphate-buffered saline (PBS) after lysis of red cells as described. To treat cells with 8-MOP (Aldrich Chemical Co, Milwaukee, WI) and UV-A irradiation, the cells were washed with 5 mL PBS twice and resuspended in Eagle’s high amino acid medium containing 2.5% mouse serum, 30 mmol/L mercaptopoethanol and 2 mmol/L L-glutamine. The procedure of MLR essentially was the same as described previously. Stimulator cells were incubated with responding cells for 5 days before addition of H-thymidine (5 μCi). Each incubation was performed in triplicate.

Platelet transfusion study was performed 9 days after the last platelet transfusion.
chromate at 25°C for 30 minutes. Thereafter, platelets were washed with 3 mL PBS buffer twice and resuspended in platelet poor plasma. Each Balb/c mouse was injected with 0.1 mL of ⁶⁷Cr-labeled platelets containing about 6 × 10⁶ cpm. Blood samples were collected from the retro-orbital venous plexus by using a heparinized glass capillary tube with an inner diameter of 1.2 mm (Fisher Scientific, Pittsburgh, PA) at 1 minute and 1 hour after transfusion. The volume of blood collected in each capillary tube was 240 µL. The radioactivity of each blood sample was counted in a LKB gamma counter with substraction of background. The percentage of platelet recovery at 1 hour posttransfusion was calculated by dividing the radioactivity in a blood sample collected at 1 hour posttransfusion by the radioactivity in the same volume of blood collected at 1 minute posttransfusion. The use of this approach avoided the significant error that might occur from extravasation of labeled platelets during tail vein injection and obviated the need of knowing precise total blood volume in each mouse for calculating percentage recovery of transfused platelets.

RESULTS

In Vitro Studies

MLR. The effect of treating mouse leukocytes with 500 ng/mL 8-MOP and 5 J/cm² UV-A on the MLR was studied. The results shown in Table 1 indicate that treatment with 8-MOP and UV-A irradiation effectively abrogated two-way MLR, one-way stimulator function, and one-way responder function by 98%, 90%, and 99%, respectively. Thus, the same doses of 8-MOP and UV-A irradiation were used for the subsequent platelet transfusion studies. The doses of 8-MOP and UV-A selected for the MLR study were based on the earlier experiments performed on human peripheral blood leukocytes. The concentration of 8-MOP (500 ng/mL) used in our study was about the same as the peak serum concentration in patients taking 8-MOP for phototherapy.

Platelet aggregation and secretion. To know whether the pretreatment of platelet concentrates with 8-MOP (500 ng/mL) and UV-A (5 J/cm²) adversely affect platelet function, platelet aggregation, and ATP secretion induced by adenosine diphosphate (ADP; 10 µmol/L) or collagen (100 µg/mL; Sigma) were studied. The concentrations of both aggregating agents used were less than those required to induce a maximal degree of platelet aggregation. Our results showed that platelet aggregation and ATP secretion did not change after the treatment of platelets with 8-MOP and UV-A.

Transfusion Studies

Platelet concentrates. Two platelet transfusion experiments were performed. The number of recipient mice, and the average numbers of platelets and leukocytes transfused each time for both experiments are summarized in Table 2. The data showed that there was no significant difference in the numbers of platelets and leukocytes transfused each time between the control group and the treatment groups. The higher variation of leukocytes in platelet concentrates of experiment II was due to a significantly low number of leukocytes (4.39 × 10⁷ cells/100 µL) in platelet preparation for one of the transfusions. Because the average total blood volume for an adult mouse was about 2 mL, the number of platelets and leukocytes transfused into a mouse each time were equivalent to the transfusion of 1 × 10¹⁵ platelets and 5.5 × 10⁶ leukocytes (2 units of random donor platelets) into an adult patient with 5,000 mL of total blood volume.

Alloimmunization. To detect the emergence of low concentrations of anti-H2k antibodies in recipient Balb/c mice, a sensitive immunofluorescent flow cytometry technique was used. Positive detection of antibody was defined as when the mean fluorescence intensity was greater than the mean + 2 SD of the pooled pre-immune serum. The incidences of alloimmunization for the control groups and the treatment groups were shown in Fig 2. The results demonstrated a delay in the development of anti-H2k antibodies among mice that received transfusions of platelets pretreated with 8-MOP and UV-A irradiation. In contrast, nearly all mice that received untreated control platelets were alloimmunized after two platelet transfusions.

To further substantiate that the antibodies detected by immunofluorescence flow cytometry indeed had a specificity against H2k antigens, the serum samples collected at the end of the first experiment were tested against splenic lymphocytes from four other syngeneic strains of mice with different H2 haplotypes. As shown in Fig 3, the antibodies of all serum samples reacted positively only with splenic lymphocytes from B10.BR mice with H2k haplotype. The results confirmed that the antibodies developed among recipient mice indeed had a specificity against H2k MHC antigens. Furthermore, it was noted that the measured fluorescence intensities appeared to be lower for serum samples from mice transfused with 8-MOP and UV-A pretreated platelet concentrates. This finding suggested

Table 1. Inhibition of Mouse MLR by 8-MOP and UV-A Treatment

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Two-Way MLR</th>
<th>One-Way Stimulator Function</th>
<th>One-Way Responder Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>8-MOP + UV-A</td>
<td>Control</td>
</tr>
<tr>
<td>I</td>
<td>57,536 ± 3,888†</td>
<td>690 ± 40</td>
<td>12,382 ± 1,898</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>1.2%</td>
<td>100%</td>
</tr>
<tr>
<td>II</td>
<td>49,602 ± 3,211</td>
<td>697 ± 21</td>
<td>12,593 ± 3,261</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>1.4%</td>
<td>100%</td>
</tr>
</tbody>
</table>

*BALB/cByJ and CBA/CaH-T6J mice were used for both experiments. In experiment I, splenic leukocytes from BALB/cByJ mice were treated with or without 8-MOP (500 ng/mL) and UV-A irradiation (5 J/cm²). In experiment II, splenic leukocytes from CBA/CaH-T6J mice were treated with or without 8-MOP and UV-A.

†Each value is counts per minute of H-thymidine incorporated and is mean ± SD of triplicate incubations.
Table 2. Average Numbers of Donor Platelets and Leukocytes Transfused Into Recipient Mouse

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Untreated</th>
<th>8-MOP + UV-A</th>
<th>Untreated</th>
<th>8-MOP + UV-A</th>
<th>8-MOP</th>
<th>UV-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of recipient mice</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Platelet transfused*</td>
<td>4.14 ± 1.28 ± 10^7</td>
<td>4.24 ± 1.17 ± 10^7</td>
<td>5.34 ± 1.30 ± 10^7</td>
<td>5.36 ± 1.34 ± 10^7</td>
<td>5.20 ± 1.34 ± 10^7</td>
<td>5.43 ± 1.47 ± 10^7</td>
</tr>
<tr>
<td>Leukocytes transfused*</td>
<td>22.20 ± 6.83 ± 10^3</td>
<td>23.86 ± 6.87 ± 10^3</td>
<td>20.82 ± 18.5 ± 10^3</td>
<td>18.53 ± 16.93 ± 10^3</td>
<td>21.88 ± 21.78 ± 10^3</td>
<td>22.65 ± 22.64 ± 10^3</td>
</tr>
</tbody>
</table>

*Average numbers of CBA (H-2^d) donor platelets and leukocytes in 100 μL of platelet concentrates that were transfused into a Balb/c (H-2^d) recipient mouse each time.

† Mean ± SD (n = 6).

that antibody titers might be lower in mice transfused with 8-MOP and UV-A pretreated platelets.

We then measured the titers of anti-H2^k antibodies in serum samples collected after the last platelet transfusion. The titers were determined by using an EIA, as shown in Fig 4. The results showed that mice receiving 8-MOP and UV-A–treated platelets had lower titers of anti-H2^k antibod-

ies than those that received untreated platelets or platelets treated with 8-MOP or UV-A irradiation alone (Table 3).

Platelet recovery study. Two days after collection of the last serum sample, experiments were performed to measure the 1-hour posttransfusion recovery of 51Cr-labeled donor platelets. The results are summarized in Fig 5. Even though all mice had become alloimmunized after six consecutive weekly platelet transfusions (Fig 2), the mice transfused with platelets pretreated with 8-MOP and UV-A irradiation had significantly better 1-hour posttransfusion platelet recoveries than the control mice (Fig 5). There was no significant difference between the control group and the group that received platelets pretreated with 8-MOP or UV-A alone (Fig 5).

When antibody titers were correlated with 1-hour posttransfusion recoveries of donor platelets, a significant linear reverse correlation was observed (Fig 6). This finding suggests that the better posttransfusion platelet recovery for mice transfused with 8-MOP and UV-A–treated platelets was partially due to low titers of anti-H2^k antibodies. To
There is a possibility that different titers of pre-existing anti-H2\textsuperscript{k} antibody might variably reduce 1-minute posttransfusion platelet recoveries and affected the reliability of 1-hour posttransfusion platelet recovery results, the platelet recovery study was also performed in five untransfused Balb/c mice. The average 1-minute posttransfusion platelet recoveries were 80.5\% ± 5.6\% (±SD), 81.0\% ± 4.3\%, and 78.9\% ± 9.3\% for previously untransfused mice, mice transfused with untreated platelets, and mice transfused with 8-MOP and UV-A-treated platelets in experiment I, respectively. Similar values were also observed for all mice in experiment II. Therefore, the development of anti-H2\textsuperscript{k} antibody did not significantly affect the 1-minute posttransfusion platelet recovery in all groups of mice. The average 1-hour posttransfusion platelet recovery for previously untransfused Balb/c mice was found to be 67.4\% ± 10.2\%.

**DISCUSSION**

In this study we investigated the use of 8-MOP and UV-A irradiation to reduce the alloantigenicity of class I MHC molecules in murine platelet concentrates. Our results showed that transfusions of 8-MOP and UV-A-pretreated platelets delayed the development of primary alloimmunization in recipient mice (Fig 2) and that the titers of anti-H2\textsuperscript{k} antibodies developed in those mice were significantly lower than in mice of the control group (Table 3). Furthermore, mice transfused with 8-MOP and UV-A-treated platelets had better 1-hour posttransfusion recoveries of \(^{11}\text{C}\)-labeled donor platelets (Fig 5), even though they had become alloimmunized. All these findings indicate that inactivation of nucleated white blood cells in platelet concentrates by 8-MOP and UV-A is an effective means of reducing the alloantigenicity of class I MHC molecules.

The results of our study reaffirm earlier observations\textsuperscript{1,8} that contaminating donor leukocytes in platelet concentrates or allografts play a significant role in alloimmunization of recipients to class I MHC antigens. In addition, our results provide further insight into the mechanism by which donor leukocytes in platelet concentrates initiate the primary immune response against class I MHC antigens. Because the treatment of 8-MOP and UV-A irradiation induces covalent crosslinkage of DNA\textsuperscript{9} and leads to inhibition of gene transcription and cell proliferation,\textsuperscript{10,30} the success of using 8-MOP and UV-A treatment to reduce

**Table 3. Anti-H2\textsuperscript{k} Antibody Titers in Mice Receiving Platelet Concentrates Treated With 8-MOP and/or UV-A Irradiation**

<table>
<thead>
<tr>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated (control)</td>
</tr>
<tr>
<td>Antibody titer (mean ± SD)</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td>P &lt; .0002\textsuperscript{t}</td>
<td>P &lt; .015</td>
</tr>
<tr>
<td>No. of recipient mice</td>
<td>10</td>
</tr>
</tbody>
</table>

\*Antibody titer = \log (maximal dilution of serum with positive reaction)^{-1}.

\textsuperscript{t}Each group of mice receiving transfusions of treated platelets was compared with the respective control group receiving untreated platelets by using the Student's \textit{t}-test.
class I MHC alloantigenicity suggests that certain co-stimulatory factor(s) is required for the initiation of alloimmunization and needs to be synthesized through gene transcription.

As reported previously, interleukin-1 (IL-1) secreted from antigen-presenting cells plays a critical role in the initial stage of the primary immune response. It is also known that IL-1 is not constitutively expressed until antigen-presenting cells are activated. Among the leukocytes in donor peripheral blood, only monocytes and dendritic cells are known as major antigen-presenting cells that are capable of producing sufficient quantities of IL-1 after activation. Thus, it is reasonable to speculate that the sequence of events in primary allo sensitization to class I MHC antigens through transfusions of blood components may take place as follows (Fig 7).

After functionally active donor monocytes and/or dendritic cells in platelet concentrates or red cell units are transfused into a recipient, alloreactive helper T lymphocytes of the recipient recognize nonself class II MHC molecules on donor cells through their T-cell receptors. The interaction between T-cell receptors and foreign class II MHC molecules then leads to the activation of transfused donor monocytes and/or dendritic cells. Consequently, the key interleukins such as IL-1 and IL-6 are synthesized and secreted. Secreted and/or membrane-bound IL-1 then stimulates recipient helper T cells to proliferate and to release IL-2 and other interleukins. Concomitantly, IL-1 enhances recipient’s B-cell response to antigen-specific or nonspecific T-cell help. All these interactions eventually result in allo sensitization with production of antibodies against donor class I MHC antigens (Fig 7).

At present, it is not known whether self class I MHC peptides need to be present in the antigen-binding groove of the donor’s class II MHC molecules to be recognized by a recipient’s alloreactive helper T cells. It is also unclear whether T-cell receptors on the recipient’s alloreactive helper T cells will crossreact with self class II MHC molecules bound with foreign donor MHC peptides. All of these important questions need further investigation. Recently, Lagaaij et al reported that transfusion with HLA-DR antigen-matched red cells significantly reduced the incidence of primary HLA alloimmunization in patients awaiting kidney or heart transplants. This finding supports the importance of DR disparity for interaction between alloreactive T-helper cells of recipients and DR-positive leukocytes of donors during the initial stage of HLA alloimmunization.

The in vivo survival of platelets after pretreatment with 8-MOP and UV-A irradiation was not performed in our study due to the technical difficulty of non-traumatic sampling of blood from mice at multiple time points. Nevertheless, it is unlikely that the treatment of 8-MOP and UV-A irradiation would have resulted in any significant reduction of platelet survival because reduction of platelet counts has not been reported as an adverse side effect in patients with cutaneous T-cell lymphoma receiving repeated photopheresis therapy. In addition, platelet structural integrity and function have been shown to be fully preserved after treatment with 8-MOP and UV-A irradiation. The reduced alloantigenicity of 8-MOP and UV-A-treated platelets, therefore, could not be readily attributed to a shortened in vivo platelet survival.

Although the results of our study show that treatment with 8-MOP and UV-A irradiation can successfully reduce the alloantigenicity of platelet concentrates, this treatment...
did not prevent recipient mice from developing anti-H2k antibodies after platelet transfusions (Fig 2). This finding was not totally unexpected. As shown in Table 1, there was measurable residual MLR stimulatory activity in donor leukocytes after 8-MOP and UV-A treatment. It is possible that exposure to this residual stimulatory activity might be sufficient to induce the development of anti-H2k antibodies. Moreover, there are high concentrations of class I MHC antigens on platelets and in plasma.4,41 Repeated transfusions of platelets prepared from the same donors might provide sufficient class I MHC antigens to alloimmunize recipients through the classical pathway of immune response. Previously, Pellegrino et al42 reported that class I MHC antigens in plasma are able to elicit primary alloimmunization in recipients of plasma transfusions. Their finding suggests that soluble class I MHC antigens by themselves can be processed and presented by the recipient's antigen presenting cells to the immune system and can trigger the primary immune response. Thus, deletion or inactivation of donor leukocytes in platelet concentrates may not be able to prevent the primary alloimmunization to class I MHC antigens. Nevertheless, other investigators have shown that class I MHC antigens in plasma or on platelets are not very immunogenic.7,10-12 Consequently, deletion or inactivation of donor leukocytes reduces the alloantigenicity of platelet concentrates and may lead to the development of lower titers of antibodies, as observed in our study (Table 3 and Fig 6), that may allow adequate posttransfusion platelet recovery.

For the 1-hour posttransfusion platelet recovery studies, a considerable difference was noted between two sets of experiments (Fig 5). The exact reason(s) for the observed difference is not clear. The possible contributing factors include different litters of mice used for the experiments, varied quality and numbers of leukocytes transfused in the two experiments, and a qualitative difference of 51Cr-labeled platelets used in each recovery study. Alternatively, a combination of these factors might have caused the observed variation. Nevertheless, both experiments consistently showed that mice transfused with 8-MOP and UV-A-treated platelets had lower antibody titers and better posttransfusion platelet recovery than the control mice. Thus, it is reasonable to conclude that the treatment of donor platelet concentrates with 8-MOP and UV-A irradiation is indeed effective to reduce the alloantigenicity of platelet concentrates.

ACKNOWLEDGMENT

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Use of 8-methoxypsoralen and ultraviolet-A pretreated platelet concentrates to prevent alloimmunization against class I major histocompatibility antigens

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