Prevention of Platelet Alloimmunization in Dogs With Systemic Cyclosporine and by UV-Irradiation or Cyclosporine-Loading of Donor Platelets

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To study the immunosuppressive effects of three different treatments, 30 dogs received at weekly intervals eight platelet transfusions from a single random donor dog. The three experimental protocols were (1) daily oral cyclosporine (Cs) treatment of recipients; (2) in vitro ultraviolet (UV)-irradiation of donor platelets; and (3) Cs-loading of donor platelets. All nine recipients of Cs, 11/12 (92%) recipients of UV-irradiated platelets, and 5/9 (56%) recipients of Cs-loaded donor platelets remained nonimmunized to repeated transfusions of donor platelets. In contrast, only 3 of 21 untreated controls (14%) were not alloimmunized by donor platelets. Moreover, 44% to 67% of the nonimmunized recipients remained tolerant to continued platelet transfusions from their original donor even after experimental therapy was discontinued. Forty-three percent to 100% of transfusions from secondary donors were also accepted without causing alloimmunization, suggesting that tolerance induced by prior treatment was not specific for the primary donor. However, survival of both the original and secondary donor platelets was reduced to about half the starting level, suggesting that some immune response to platelets had occurred. Also, recipients immunized by their original donor's platelets frequently developed refractoriness to platelets from other donors.

To prevent or modify the immune response to transplanted tissue, a number of immunosuppressive treatments have been developed, such as irradiation, chemotherapeutic regimens, polyclonal or monoclonal antibodies, and the use of cyclosporine (Cs).

A more recent approach is the modification of donor cells or tissues. Certain antigen-presenting cells such as macrophages or dendritic cells are now known to be necessary for the donor's tissue to be recognized by the recipient. Thus, several attempts have been made to eliminate or inactivate these cells. Reportedly, successful inactivation has been achieved by maintaining long-term cell or tissue cultures in a high concentration of oxygen, by using monoclonal antibodies (MoAbs) directed at Class II antigens (expressed on antigen-presenting cells), and by exposing donor cells or tissue to ultraviolet (UV) light in the intermediate wavelength spectrum.

Although there is substantial information on the value of these and other agents in organ transplantation, little is known about their ability to prevent immune recognition of repeatedly transfused cells. In particular, we are concerned about alloimmunization to transfused platelets, since it represents a major problem in the management of thrombocytopenic patients. Studies suggest that 40% to 100% of repeatedly transfused patients become alloimmunized, often after only a few transfusions.

We have previously used a dog platelet transfusion model to evaluate methods of preventing platelet alloimmunization (and unpublished data). This report describes further studies using this model to evaluate recipient treatment with Cs and UV-exposure or Cs-loading of donor platelets as methods of preventing platelet alloimmunization.

METHODS

Selection of Donor and Recipient Dogs

The dogs used for these experiments were of various breeds and of either sex. Dogs were vaccinated (DHL and parvo virus) and quarantined for one to two months to exclude any diseases. In addition, two or three autologous 51Cr-labeled platelet survival measurements (see below) were performed on each recipient dog to ensure baseline normal platelet kinetics before donor transfusions were initiated. During the course of the study, eight different dogs were used as donors for the 30 platelet transfusion recipients. All of the donors and most of the recipients were typed for serologically determined dog histocompatibility antigens (DLA-A and B). In all instances tested, donors and recipients were incompatible for one or more DLA-antigens. In addition, for some studies mixed lymphocyte cultures were performed with cells obtained from donors and from recipients both pre- and posttransfusion. In all tested donor-recipient pairs, cells showed reciprocal stimulation both before and after the donor transfusions and experimental therapy.

Survival of Radiochromium (51Cr)-Labeled Platelets

Platelets were prepared from the donor dog and labeled with radiochromium as previously described. Every week 50 mL of blood was drawn from each donor for each recipient into 7.5 mL of acid-citrate-dextrose (ACD). A platelet concentrate was prepared by differential centrifugation and labeled with 300 μCi of 51Cr. After infusion of these platelets, blood samples (3 mL blood drawn into 0.1 mL potassium EDTA) were obtained at one hour, twice on the second day, and once daily during the third and fourth days. Survival was calculated by least squares computerization-fitting of the radioactivity in the blood samples. Autologous platelet survival in 56 normal dogs averaged 5.1 ± 0.9 days (mean ± 1 SD).

Platelet recovery was calculated as follows by extending the survival curve to time zero (TO):

\[
\text{Recovery (\%) } = \frac{\text{Radioactivity at } T0 \times \text{blood vol}}{\text{Total radioactivity of injected platelets}} \times 100
\]

\[
\text{Recovery (\%) } = \frac{\text{Radioactivity at } T0 \times \text{blood vol}}{\text{Estimated at 80 mL/kg}} \times 100
\]

\[
\text{Recovery (\%) } = \frac{\text{Radioactivity at } T0 \times \text{blood vol}}{\text{Total radioactivity of injected platelets}} \times 100
\]
Autologous recovery in normal dogs was 51% ± 12% (mean ± 1 SD) of the injected platelets.

Alloimmunization was defined as a decrease in the recovery of labeled donor platelets to <5% at 24 hours posttransfusion. One additional donor transfusion was given after the development of alloimmunization to confirm the accuracy of the end point. Thus, additional donor transfusion was given after the development of labeled donor platelets to <5% at 24 hours posttransfusion. One of the injected platelets.

Twenty of 21 (95%) became immunized after an average of 3.1 ± 0.7 (range one to 12) transfusions (Fig 1). As 18/21 (86%) of these recipient controls were immunized by eight or fewer transfusions, an eight-week transfusion schedule was selected for the subsequent alloimmunization studies, ie, the treated dogs received platelets from their donor until alloimmunization developed or a maximum of eight transfusions had been given.

**Treatment Programs**

**Systemic cyclosporine.** Cyclosporine (Cs, Sandoz, Basel, Switzerland) was emulsified in corn oil17 and administered orally in doses of 15 mg/kg/d. Treatment was started on the day of the first platelet transfusion and continued for up to eight weeks or until alloimmunization occurred. To document absorption of drug and maintenance of adequate levels, Cs serum levels were monitored in two recipient dogs throughout their treatment using a radioimmunoassay (RIA), as previously described.18 Serum drug levels were obtained every three to five days as trough levels just before the next daily dose. For one dog the average trough levels were 105 ± 68 (range 22 to 255) ng/mL and for the second dog 139 ± 30 (range 108 to 192) ng/mL. Therapeutic Cs drug levels are considered to range between 200 to 400 ng/mL.

**UV-exposed platelets.** After radiochromium labeling, platelets were resuspended in a volume of 6 to 10 mL of Ringer's-citrate-dextrose (RCD) and placed (to a depth of 1.5 mm) in an open Petri dish and continuously agitated on a shaker platform during UV exposure. UV irradiation was delivered by a mineral light lamp (UVP, San Gabriel, CA) providing wavelengths between 220 to 290 nm. The dose delivered was determined by using a Black-Ray Ultra-violet Meter (Model J225, UVP) with a maximum sensitivity at 254 nm. After UV exposure the contents of the Petri dish were aspirated into a syringe for injection.

**Cyclosporine-loaded platelets.** Radiochromium-labeled donor platelets, resuspended in 4.6 mL of RCD, were incubated for 1 hour at 22 °C with 55 ng/mL of Cs prepared by adding 0.25 mg (5 μL of a 50 mg/mL solution) of Cs in ethanol: Cremophor (intravenous [IV] solution from Sandoz) to the platelets. Following incubation the platelets were injected IV, the recipients receiving only 0.25 mg of Cs per injection. Blood taken immediately and one hour after injection contained no measurable Cs.

Uptake of Cs was assessed by measuring the platelet Cs content by high-pressure liquid chromatography and by using radiolabeled 3H-cyclosporine provided by Dr Eric Wiskott of Sandoz. The amount of Cs incorporated was 6% to 12% of that added. There was no difference in Cs uptake in donor platelets between dogs who subsequently became immunized and those who did not.

**Further Transfusion Studies**

Any recipient not immunized by eight transfusions while receiving the experimental treatment was given an additional eight transfusions from the same donor while off treatment or until immunization developed, whichever occurred first. When the transfusion schedule from the original donor was completed, the recipient dogs then received transfusions from one to three additional donors (referred to as secondary donors) while off experimental treatment. Transfusions were provided from the randomly selected secondary DLA-incompatible donors until immunization developed or until a maximum of eight transfusions were given.

**Statistical Analysis**

Mean values of experimental and control groups were compared by Student's t test.19

**RESULTS**

**Autologous Platelet Transfusions**

**Recovery and survival measurements with experimental treatment.** To ensure that treatment did not adversely affect the viability of transfused platelets, autologous platelet recovery and survival measurements were performed for each experimental treatment. Four dogs were given oral Cs during the transfusion of radiolabeled autologous platelets. Recoveries and survivals with this treatment were not significantly different from those in 56 untreated controls (P > 0.10) (Table 1).

Doses of UV irradiation given to autologous platelets were 200, 400, or 600 μW/cm² for one minute (total doses of 12, 24, and 36 mL/cm², respectively). These doses did not affect autologous platelet survival (Table 1). However, in two dogs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Dogs Tested</th>
<th>Platelet Recovery (%)</th>
<th>Survival (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>56</td>
<td>51 ± 12</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td>Systemic Cs</td>
<td>4</td>
<td>58 ± 9</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>UV-exposed platelets</td>
<td>14</td>
<td>52 ± 6</td>
<td>4.3 ± 1.1</td>
</tr>
<tr>
<td>Cs-loaded platelets</td>
<td>5</td>
<td>57 ± 6</td>
<td>4.9 ± 1.3</td>
</tr>
</tbody>
</table>

*Given as the mean ± 1 SD.

By Student's t test, none of the treatment programs produced platelet recovery and survival measurements that were statistically different from untreated controls.
that received 600 μW/cm² for ten minutes (total dose of 360 mJ/cm²), there was no recovery or survival of autologous platelets (data not shown). Cyclosporine-loading in five dogs did not reduce platelet viability (Table 1).

**Allogeneic Platelet Transfusions**

**Recipient treatment with oral cyclosporine.** Of the nine recipients who received oral Cs therapy, none became immunized after eight transfusions from a single random donor (Fig 1). These results were significantly different from those in the 21 untreated control dogs (P < 0.001); only three of them (14%) remained unimmunized after eight transfusions. Furthermore, 6/9 recipients (67%) were still responsive to transfused platelets when Cs was stopped, even after eight additional transfusions from the same donor (P < 0.001, compared to controls). The other three recipients became immunized after four, five, and six additional donor transfusions given off treatment (Fig 1).

Although zero time-recovery values for the original donor's platelets in the nonimmunized recipients were within the normal range throughout the 16 transfusions (eight on and eight off treatment), donor platelet survivals were only 2.2 ± 0.5 days, ie, about half normal (Fig 2).

**UV exposure of donor platelets.** Overall, 11/12 recipients (92%) of UV-exposed donor platelets (12 to 36 mJ/cm²) were protected from alloimmunization. These UV-exposed donor platelets produced a significantly lower rate of alloimmunization than unmodified platelets given to control recipients (P < 0.001) (Fig 1).

To determine if an effective dose of UV irradiation could be achieved through a blood bag, platelets from two donors were individually irradiated through a piece of plastic cut from a Fenwall platelet storage bag (PL 732, Fenwall Laboratories, Deerfield, IL) that was laid over the Petri dish containing the platelets. (As the UV penetrance through the plastic bag was poor with the available UV source, a maximum dose of 100 μW/cm² could be obtained.) However, by irradiating at 100 μW/cm² for four minutes, the equivalent of our standard dose of 400 μW/cm² for one minute was achieved, ie, the total UV dose given to platelets by each of these exposures was 24 mJ/cm². Two recipients received donor platelets irradiated through the plastic and, as controls, two others received the same donor's platelets given the exact exposure (100 μW/cm² for four minutes) without interposition of plastic; all four recipients were protected from alloimmunization (data included above).

When transfusions from the original platelet donor were continued off treatment, three recipients became immunized after one, two, and five transfusions, respectively (Fig 1). Thus, 8/12 recipients (67%) were not immunized by a total of 16 transfusions, eight on and eight off treatment (P < 0.001 compared to controls).

The eight nonimmunized recipients were given platelets from two or three other donors until immunization occurred or a maximum of eight transfusions from each of these other donors had been given. Prior treatment prevented alloimmunization to platelets from 10/23 (43%) of the secondary donors tested (Table 2). However, compatible donors were found for only four (50%) of the nonimmunized recipients. After completion of these secondary donor transfusion studies, six of the nonimmunized recipients were transfused with platelets from their original donor and two (33%) were immunized.

Three of the four immunized recipients were each given platelets from one or two other donors; only 1/5 of the secondary donors' platelets (20%) remained compatible during eight transfusions (Table 2).

Recovery of UV-exposed donor platelets in the nonimmunized recipients was normal, but survival was reduced (Fig 3). However, the survival of donor platelets off treatment (3.1 ± 0.4 days) was somewhat better than on treatment (2.5 ± 0.3 days). Recoveries from the secondary donors in the nonimmunized recipients averaged 42% ± 10%, and survivals were 2.9 ± 1.3 days.

**Cyclosporine-loaded donor platelets.** Undesirable side effects of systemic Cs are sometimes observed in immuno-suppressed patients. We therefore evaluated the use of in vitro Cs-loaded donor platelets for preventing alloimmunization. Five of 9 (56%) of the recipients of Cs-loaded platelets did not become alloimmunized (Fig 1) (P < 0.05 compared to untreated controls). Furthermore, 4/9 or 44% remained nonimmunized to the original donor's platelets even off treatment (P < 0.001). In addition, none of the three nonimmunized recipients tested were alloimmunized after receiving untreated platelets from either of two secondary donors (Table 2).

In contrast to the data in the nonimmunized recipients, all three tested recipients who had become immunized to their original donors' platelets also were alloimmunized after receiving fewer than eight transfusions of unmodified platelets from five secondary donors (Table 2).

Recoveries of the original donor's platelets in the recipients were within the normal range for both the treated and untreated transfusions while survivals were reduced (Fig 4). Recovery of the secondary donor's platelets averaged 51% ± 14% in the nonimmunized recipients and survival was 3.0 ± 1.0 days.
specific attempts to prevent platelet alloimmunization in chronically transfused thrombocytopenic patients, other than by using single donor platelets or leukocyte-free blood products, have not been reported. Our studies have shown that both immunosuppression with systemic Cs and UV irradiation of donor platelets were almost uniformly successful (100% and 92%, respectively) in preventing alloimmunization to eight weekly platelet transfusions from a single random donor. These results are much better than the nonimmunized rate of only 14% in untreated controls ($P < 0.001$ for both treatments). Furthermore, almost two thirds of the nonimmunized previously treated recipients remained unimmunized after eight additional transfusions from the same donor, suggesting the induction of a tolerant state. To determine if this tolerance was specific for the original donor’s platelets, platelets from other random donors were transfused without treatment of the recipient or the donor’s platelets. The number of Cs-treated recipients evaluated for such specificity was too small to permit a firm conclusion (data not given). However, among the recipients tolerant to eight UV-treated and eight nontreated platelet transfusions from their original donor, platelets from 43% of the secondary donors tested were also compatible. In addition, there was some evidence that maintenance of this quasitolerant state required continuous transfusions of the presumed tolerizing antigen; interruption of the transfusion program for a period of months resulted in immunization by untreated transfusions from the primary donor in at least one third of the recipients tested. Similar observations have been made in other models of tolerance.

We were concerned about the substantial immunosuppressive effects of Cs on human subjects as well as its toxicity to liver and kidneys. Therefore, in analogy to the use of vinblastine-loaded platelets in patients with autoimmune thrombocytopenic purpura, we evaluated the ability of Cs-loaded donor platelets to prevent immunization. Although 56% of the recipients were apparently unable to recognize Cs-loaded donor platelets, this treatment was inferior to that obtained with either systemic Cs or UV-irradiated donor platelets ($P < 0.001$). However, treatment with Cs-loaded donor platelets was very successful in inducing a state of tolerance not only to untreated platelets of the original donor but also to secondary donors.

In summary, our studies show that some treatment programs that prevent the rejection of organ allografts were successful in reducing the recognition of repeatedly transfused donor platelets in a canine transfusion model. Furthermore, to some extent, these treatments induced a state of “nonspecific tolerance” that persisted even after discontinuation of experimental therapy. However, maintenance of the tolerant state apparently required continuous exposure to the tolerizing antigen. Conversely, in those recipients who
became immunized by the original donor’s platelets either on or off therapy, alloimmunization usually also developed in response to untreated platelets from other donors. We also found that the survival of allogeneic donor platelets in every treatment program was consistently reduced as compared to similarly treated autologous platelets. Therefore, we conclude that some degree of alloimmunization to donor platelets probably occurred in recipients who were by definition considered to be nonimmunized. However, as autologous platelet survivals on treatment were only performed on one occasion, we cannot exclude the possibility that there was some cumulative effect of the treatment on donor platelet survival measurements. The recovery of platelets from both the original and secondary donors in the nonimmunized recipients was not compromised, and platelet survival remained about half normal; therefore, useful transfusion support was achieved by this approach. Furthermore, previous studies using in vitro platelet aggregation tests, have shown that human platelets maintain normal function after UV exposure or Cs loading. Although the applicability of our results to man has not been tested, we believe these studies provide a sufficient basis to consider human experiments.

ACKNOWLEDGMENT
The authors acknowledge the technical assistance of Michael Wilson and the secretarial support of Ginny Knight.

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