Recipient Humoral Immunity Against Leukoreduced Allogeneic Platelets Is Suppressed by Aminoguanidine, a Selective Inhibitor of Inducible Nitric Oxide Synthase

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Leukoreduced allogeneic platelet transfusions have been previously shown to initially stimulate an in vitro cellular cytotoxicity and subsequently induce the formation of immunoglobulin G (IgG) antidonor alloantibodies. To further characterize these responses and determine if they are mediated, recipient BALB/c H-2b mice were treated with aminoguanidine (AMG), a selective inhibitor of inducible nitric oxide synthase (iNOS), and transfused weekly with 2 × 10^10 C57BL/6 H2b platelets. In control, non-AMG-treated mice, transfusion significantly increased serum levels of interferon-γ (IFN-γ) by day 1 posttransfusion (PT). IFN-γ returned to pretransfusion levels by day 3 PT, and its production was not affected by AMG treatment. Serum interleukin-2 (IL-2), on the other hand, was undetectable before and during the transfusion protocol. By day 3 PT, recipient spleen cells could mediate in vitro anti-PL815 (auto), anti-EL4 (allo), and anti-R1.1 (third-party MHC) cytotoxicity, and these responses were maximal by day 7 PT. Concurrently, a significant reduction in the in vitro ability of recipient splenocytes to respond to Concanavalin A (ConA) was observed; this was not seen with lipopolysaccharide (LPS) stimulation. Elevated levels of NO_2 were found in the ConA culture supernatants from transfused mice at day 3 PT. Serum antidonor alloantibodies were detected by the fifth platelet transfusion. AMG treatment of recipient mice significantly inhibited the transfusion-induced cytotoxicity and ConA-stimulated NO_2 production, and restored ConA-induced proliferation to normal levels. AMG appeared to selectively inhibit platelet-induced alloantibody production in that it did not affect antibody production induced by transfusions with 10^8 allogeneic leukocytes. These results indicate that an in vivo AMG-sensitive mechanism is essential for recipients to initiate a humoral IgG immune response against allogeneic platelets.

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required for the induction of humoral antiplatelet alloimmunity.

**MATERIALS AND METHODS**

**Animals and cell lines.** Inbred female BALB/c (H-2b) mice, 8 to 12 weeks of age, were used as the transfusion recipients and female C57BL/6 (H-2b) mice, 6 to 10 weeks of age, were used as platelet donors; the mice were purchased from Harlan-Sprague Dawley (Indianapolis, IN). The cell lines, P815 (H-2b) mastocytoma, EL-4 (H-2a) thymoma, and R1.1 (H-2b) lymphoma, were used as cytokine targets and/or typing cells and were purchased from ATCC (Rockville, MD). All of the cell lines and in vitro assays were maintained in RPMI-1640 with 5% fetal calf serum (FCS), 100 µg/mL penicillin/streptomycin/fungizone, 100 mM/L L-glutamine, and 5 x 10^{-3} M 2-mercaptoethanol.

**Cell preparation and transfusion protocol.** For spleen cells, mice were killed, and the spleens removed and teased into single-cell suspensions. The cells were layered on a 1.077 g/mL Percoll cushion and white blood cells (WBC) enriched by centrifugation at 1,800g for 30 minutes at 20°C. Marine platelets were prepared as previously described. Briefly, mice were bled via the tail vein into EDTA-microvessels (Starstedt, St. Laurent, Canada), the blood pooled and centrifuged at 120g, and the platelet-rich plasma (PRP) aspirated off; care was taken not to disturb or aspirate the buffy coat. The platelets were washed three times in 1% EDTA-saline and contaminating leukocytes were enumerated by a Nageotte hemocytometer (Baxter, Chicago, IL) and by flow cytometry as previously described. Using these methods, we can detect ≤ 0.5 WBC/mL. For these studies, the mean leukocyte contamination in the platelets transfused was 1.9 ± 1.7 WBC/mL.

For transfusion, platelets were adjusted to 2 x 10^11 cells/mL and WBC to 10^11 cells/mL. In each transfusion protocol, all mice were bled 24 hours before the first transfusion and then injected (100 µL) with either platelets or WBC weekly via the tail vein; this dose of platelets is equivalent to a transfusion of 5 x 10^11 platelets in humans. At 1, 2, 3, 5, and 7 days PT and weekly thereafter, groups of 10 mice were bled, killed, and their spleens used in the in vitro assays. Three control nontransfused mice were also assayed at each time interval.

**AMG treatment.** Mice were injected intraperitoneally with 100 mg/kg of AMG hemisulfate (AMG; Sigma Chemcial, St Louis, MO) daily beginning on day -1 of the transfusion protocol, and their drinking water was dosed with AMG to a final concentration of 1.0% (wt/vol) to maintain more constant in vivo levels of AMG. Dosage schedules similar to this have been used and found to be nontoxic and effective for inhibiting iNOS activity. In our hands, this dosage schedule did not appear to cause adverse reactions in any mice.

**Cytokine determinations.** Blood was allowed to clot for 1 hour on ice and centrifuged at 3,000g at 4°C. The sera were collected and frozen at -80°C. All sera were analyzed for the presence of interferon-γ (IFN-γ) and interleukin-4 (IL-4) by commercial enzyme-linked immunosorbent assays (ELISAs; Immunocorp, Montreal, Canada). Standard curves were generated with titrations of recombinant cytokines.

**Cell culture.** Proliferative ability of recipient spleen cells was assessed in 48-hour mitogen assays using the indicated concentrations of either Concanavalin A (ConA; Sigma) or lipopolysaccharide (LPS; Sigma) as previously described. Briefly, 10^6 recipient WBC were incubated with titrations of mitogen in 200-µL triplicate cultures for 24 hours at 37°C. The cultures were pulsed with 1 µCi [3H]thymidine and incubated a further 24 hours. Wells were harvested onto filter paper and incorporated cellular radioactivity measured in a β-counter (Pharmacia-LKB, Mississauga, Canada).

Cell-mediated cytotoxicity was measured using recipient spleen cells as effector cells in 8-hour cytotoxicity assays using 51Cr-labeled target lines. Recipient WBC were titrated in 96-well V-bottom plates (Costar, Corning, Cambridge, MA) to make final effector to target (E:T) ratios of 100:1 to 0.75:1. Target cells were labeled (100 µCi sodium 51Cr/10^6 cells) for 1 hour at 37°C, washed five times, and used at 5,000 cells/well. Minimum and maximum cytolysis was determined for target cells and percent lysis was determined by the formula: cpm of cpm of cpm of cpm × 100. Spontaneous release of the target cells did not exceed 15%.

**NO_2 assay.** NO_2 production was measured in the ConA-stimulated culture supernatants using the Greiss reagent as previously described. Standard curves were generated with titrations of sodium nitrite. The sensitivity of this assay was greater than 8 µmol/L.

**Flow cytometric analyses of sera.** For detection and characterization of recipient IgG antidonor antibodies, 10^3 donor or recipient WBC were incubated with dilutions of recipient sera for 45 minutes at 20°C, washed one time, and labeled with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (FITC-GAM, Fc-specific; Cedarlane Laboratories, Hornby, Canada) for 30 minutes at 20°C in the dark. To further characterize the immune sera for Th1- or Th2-mediated Ig subclasses, cells were labeled with either FITC-GAM IgG_2a (Cedarlane) or FITC-GAM IgG_1 (Cedarlane), respectively. Cells were analyzed on a FACSort flow cytometer (Becton Dickinson, San Jose, CA) operating with an argon ion laser at 15 mW; 10,000 events were acquired using an electronic cellular (lymphocyte) gate based on forward and side scatter and were analyzed using LYSYS II software (Becton Dickinson). Matched prebleed serum was used as the negative control in all experiments. In sera positive for antidonor antibodies, donor specificity of the IgG was confirmed by positive reactivity with donor and EL-4 (H-2b) cells, but absence of reactivity with recipient or P815 (H-2b) or third-party R1.1 cells (H-2L).

**Human γ globulin immunization and ELISA.** Prebleed BALB/c mice (−/+ AMG treatment) were immunized intramuscularly with 50 µg of human γ globulin (IVlg; Miles, Etobicoke, Canada) emulsified in complete Freund’s adjuvant (CFA), rested 2 weeks, and boosted with 50 µg IVlg in CFA. Mice were bled weekly and serum antihuman γ globulin antibodies were detected by a solid-phase ELISA. ELISA plates were coated with 5 µg/mL of purified IVlg (50 µL/well) in carbonate/bicarbonate buffer, pH 9.6, for 18 hours at 4°C. The plates were washed three times with washing buffer (phosphate-buffered saline [PBS]-Tween, 0.05% Tween 20), blocked with PBS containing 0.3% Tween 20 (250 µL/well) for 2 hours at 37°C, and again washed three times with washing buffer. Sera from the transfused mice were diluted in washing buffer, added to the plates (50 µL/well), and incubated for 2 hours at 20°C. Similarly diluted normal mouse serum and murine antihuman IgG (Cedarlane) were used as baseline and positive controls, respectively. The plates were washed three times in washing buffer and 50 µL of alkaline phosphatase-conjugated Fab’ goat antimouse IgG (Cedarlane) was added. After incubation at 20°C for 2 hours, the plates were washed four times and 100 µL of substrate solution (p-Nitrophenyl phosphate; BioRad Laboratories, Mississauga, Canada) was added until color conversion occurred. The plates were read at 405 nm.

**Statistical methods.** Significant differences between means were determined by Student’s t-test.

**RESULTS**

**In vivo cytokine levels in transfused recipients.** Because IFN-γ and IL-4 cytokine levels may be critical in modulating the generation of secondary Th1- or Th2-cell responses, respectively, they were analyzed by ELISA before and after platelet transfusion. Prebleed serum IFN-γ concentrations
Fig 1. Serum IFN-γ cytokine levels in recipient mice at times after receiving 1 leukoreduced platelet transfusion. ELISA sensitivities were > 7.8 pg/mL for IFN-γ and > 7.5 pg/mL for IL-4. Data are presented as the mean ± SD of IFN-γ concentration (pg/mL) from 15 recipient mice. IL-4 was not detected in any mice.

were found to be 12 ± 5 pg/mL (n = 15, Fig 1), whereas IL-4 levels were undetectable (n = 10) throughout the transfusion protocol. By day 1 PT, a transient but significant (P < .01) increase in serum IFN-γ concentration (75 ± 38 pg/mL, n = 15) was observed, which subsequently declined to prebleed levels by day 3 PT (Fig 1). AMG treatment of the recipient mice did not significantly alter IFN-γ production.

Effects of AMG treatment on cytotoxicity in platelet transfusion recipients. To analyze cellular cytotoxicity within the spleens of recipient mice, 51Cr-release assays were performed. Figure 2A shows a PT daily time course analysis of splenic alloreactive anti-EL4 and autoreactive anti-P815 cytotoxicity in transfused recipient mice. Compared with pretransfusion cytotoxicity, both alloreactive and autoreactive cytotoxicity were significantly stimulated by day 3 PT (P < .025) and increased to maximal levels by day 7 PT (P < .001). Similar results were seen when a third-party R1.1 (H-2b) cell was used as a target.

When mice were treated with AMG and transfused with allogeneic platelets, a significant inhibition (P < .001) of day 7 PT splenic anti-EL4 alloreactive cytotoxicity was seen (Fig 2B). Results were similar for the splenic autoreactive (anti-P815) and anti-R1.1 cytotoxicity. Thus, an H-2b allogeneic platelet transfusion into H-2d recipients stimulates an early AMG-sensitive splenic cytotoxicity, which lyses NK-insensitive (EL-4, P815, and R1.1) cell targets of either donor, recipient, or third-party MHC haplotypes.

The effects of AMG on transfusion-induced splenic ConA unresponsiveness. Mitogen-stimulated spleen cell cultures from the recipients were examined at the various time intervals PT. Compared with pretransfusion levels, the ability of splenic T cells to respond to ConA in vitro was significantly inhibited beginning at day 3 PT (P < .01) and was maximally inhibited by day 7 PT (P < .001) (Fig 3A). In contrast, proliferation of spleen cells upon LPS stimulation was not significantly altered in transfusion recipients (Fig 3A). Table 1 shows that elevated levels of NO3− could be detected in the ConA-stimulated culture supernatants from transfused mice at day 3 PT compared with naive control mice. AMG treatment reduced the ConA-induced NO3− production to pretreatment levels (Table 1).

Figure 3B shows the dose-response curves of splenic ConA responsiveness in recipient mice at day 7 PT. Alloreactive platelet transfusions in the absence of AMG induced a significant inhibition of in vitro ConA responsiveness (P < .001), whereas transfused mice treated with AMG had similar ConA responses to control naive mice or mice treated only with AMG. Thus, AMG treatment normalized the reduced ConA proliferation seen in the platelet-transfused mice.

Fig 2. (A) Alloreactive anti-EL4 (●) and autoreactive anti-P815 (□) cell-mediated cytotoxicity of spleen cells from BALB/c recipient mice at daily intervals after a single platelet transfusion. Results are presented as the mean ± SD of percent lysis at the indicated E:T ratios. (●) Platelet transfused/non-AMG-treated mice (n = 25). (□) platelet transfused/AMG-treated mice (n = 20). (△) nontransfused/non-AMG-treated control mice (n = 20), and (■) nontransfused/AMG-treated control mice (n = 8).
transfusion. Results are presented as mean cpm treated control mice (n from BALB/c recipient mice at daily intervals after a single platelet 'H-thymidine in responding spleen cells at 1 pg/mL of mitogen from transfused AMG-treated mice in presence of IgG antidonor antibodies by flow cytometry. 

Cytotoxicity and ConA unresponsiveness may be related to antibody formation. AMG-treated control mice (n = 20), (B) nontransfused/AMG-treated control mice (n = 8).

The effects of AMG on platelet-induced antidonor alloantibody formation. To determine whether the AMG-sensitive cytotoxicity and ConA unresponsiveness may be related to the formation of platelet-induced alloantibodies, AMG-treated and nontreated mice were transfused weekly with 2 × 10⁸ platelets for 10 weeks and their sera tested for the presence of IgG antidonor antibodies by flow cytometry. Control transfusions with syngeneic platelets did not induce an antidonor IgG response in any mice tested (n = 20). Allogeneic platelet transfusions, on the other hand, induced IgG antidonor antibody formation in untreated recipient mice by the fifth transfusion (Fig 4A). Further characterization by flow cytometry showed that the immune sera contained primarily IgG₂α and IgG₁ antidonor antibodies. The immune sera showed reactivity with cells of donor origin (C57BL/6 leukocytes, platelets, or EL-4 cells), but not with P815 cells, R1.1 cells, or cells of recipient origin (BALB/c leukocytes or platelets). In addition, a 1-hour preadsorption of sera by EL-4 cells significantly reduced reactivity with donor platelets as measured by a decrease in mean fluorescent channel (from 163 ± 95 to 21 ± 12, n = 5, P < .001), indicating reactivity to donor MHC-derived cells and no residual anti-platelet reactivity. Thus, the antidonor alloantibodies are IgG, directed solely toward cells of the donor MHC haplotype, and do not appear to contain non-MHC platelet-antigen reactivity; the finding that the sera contain IgG₂α suggests a CD4+ Th1-mediated response.²⁸

AMG treatment of BALB/c recipient mice totally inhibited the platelet-induced antidonor alloantibody formation (Fig 4C). In contrast, antidonor alloantibody formation (beginning at week 2) in mice transfused with 10⁶ C57BL/6 allogeneic WBC was similar in AMG-treated and nontreated mice (Fig 4B and D). Thus, AMG inhibits platelet-induced antidonor alloantibody formation, but does not affect the alloantibody response stimulated by leukocyte transfusions. These results were observed in all of 20 mice tested.

To control for the possibility that AMG treatment may nonspecifically inhibit recipient antigen-presenting cell (APC) function, AMG-treated mice were immunized with a foreign protein antigen that requires recipient APC for immunity, ie, human γ globulin. AMG-treated mice generated an IgG antihuman γ globulin response similar to that of nontreated mice (Fig 5). Thus, in this system, AMG treatment of recipient mice appears to selectively inhibit platelet-induced IgG antidonor alloantibody production.

**DISCUSSION**

Blood transfusions can lead to immunomodulation within the recipient, either immunosuppression and/or immunostimulation. With respect to immunostimulation in humans, the development of human leukocyte antigen (HLA) alloimmunization can lead to a state of clinical refractoriness to subsequent platelet transfusions.²⁰⁻³³ We have previously shown, in a murine model of platelet-induced alloimmunization, that leukoreduced allogeneic platelet transfusions initially stimulate in vitro cellular cytotoxicity and subsequently stimulate the formation of IgG antidonor alloantibodies.¹⁸ It was found that the cytotoxicity could be inhibited in vitro by the general NOS inhibitor NMMA.¹⁸ In addition, we observed that the initial cytotoxicity and subsequent alloantibody production

**Table 1. Effects of AMG on NO** Levels (µmol/L, mean ± SD) in Splenic Cell Cultures From Control and Transfused BALB/c Mice (day 3 PT, n = 5 in each group)

<table>
<thead>
<tr>
<th>Transfused</th>
<th>AMG</th>
<th>None</th>
<th>ConA</th>
<th>IFN-γ/LPS</th>
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<td>9 ± 6</td>
<td>19 ± 5</td>
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* Mice were transfused with 2 × 10⁸ platelets.
† Forty-eight-hour cultures with ConA (0.2 µg/mL) or IFN-γ (100 U) and LPS (1 µg/mL).
in BALB/c mice appeared to always be associated, i.e., we never observed the cytotoxicity without the subsequent formation of antidonor antibodies. Thus, the current study was initiated to further characterize these responses and determine if the initial cytotoxicity and alloantibody formation are related. The results indicate that the platelet-induced in vitro cytotoxicity and IgG immune responses are both dependent on an AMG-sensitive mechanism.

Compared with NMMA, AMG is a rather selective inhibitor of iNOS. Unlike NMMA, which inhibits all NOS isoforms, AMG is selective for the iNOS isoform. Therefore, the selective inhibition of iNOS by AMG allows for a more precise examination of the role of NO in the immune response induced by allogeneic platelets.

When recipient mice received a leukoreduced allogeneic platelet transfusion, one of the earliest detectible events was a transient but significant increase in serum IFN-γ at day 1 PT, which was not affected by AMG treatment. On the other hand, IL-4 was not detected in any of the transfused mice. IFN-γ has potent inflammatory functions, such as stimulation of MHC class II expression and macrophage activation. Although the mechanism of the platelet-induced IFN-γ production is unknown, this cytokine is only produced by T cells and NK cells, and its presence indicates that allogeneic platelets may initially affect one of these cell types. Furthermore, the transient nature of the IFN-γ response may be related to observations showing that platelets, being MHC class I−positive and class II−negative, are incapable of costimulating naive allogeneic T cells in vitro. We are currently studying the source of IFN-γ in the recipient mice. In addition, IFN-γ in the absence of IL-4 has...
been shown to be associated with primary T-cell immune responses in mice, which develop into secondary Th1-cell-mediated responses; it may suggest that IFN-γ is part of a primary immune response against allogeneic platelets that subsequently leads to a Th1-mediated IgG2a alloantibody response at 5 weeks (Fig 4A).

Following the IFN-γ response, significant in vitro splenic MHC nonrestricted cytotoxicity was seen at day 3 PT and this was inhibited by AMG treatment. This cytotoxicity suggests that allogeneic platelets cause an early activation of an effector-cell population. In addition to the in vitro NMMA sensitivity and in vivo AMG sensitivity of this cytotoxic response, several lines of evidence suggest that macrophages are primarily responsible for mediating the cytotoxicity. For example, we have previously shown that the cytotoxicity is not mediated by CD8+ T cells, since T-cell depletion could not inhibit the response. Furthermore, P815, EL-4, and R1.I cells are generally insensitive to NK-mediated lysis, and we have shown that cytotoxicity against the NK-sensitive YAC-1 target is suppressed at day 7 PT. Thus, taken together, the data suggest that allogeneic platelets initially induce in vivo IFN-γ production, which stimulates macrophage activation; this activation is responsible for the observed cytotoxicity and may be required for enhanced antigen processing and presentation for subsequent alloantibody production.

The in vitro splenic cytotoxicity at day 3 PT also correlated with ConA unresponsiveness. Albina et al have shown that ConA T-cell responses could be suppressed by the addition of excess macrophages; ConA-stimulated T-cell-derived IFN-γ in the cultures stimulated macrophage NO production, which was cytotoxic for the responding T cells. They also found that LPS could not stimulate iNOS. Our results are in support of these observations, since we detected increased levels of NO in the ConA cultures from transfused recipient mice at day 3 PT, which were inhibited by AMG (Table 1), whereas allogeneic platelet transfusions did not affect in vitro LPS-stimulated B-cell proliferation (Fig 3A). Thus, it is possible that the reduced response to ConA stimulation in platelet transfusion recipients is an in vitro phenomenon due to macrophage iNOS activation. However, whether leukoreduced platelet transfusions induce T-cell suppression in vivo is unknown.

Two studies using different NOS inhibitors have recently demonstrated that iNOS has a role in modulating in vivo rat cardiac allograft rejection in vivo. Winlaw et al...
strated that NMMA caused a small increase in graft survival and Worral et al. showed that AMG treatment of recipient rats prevented increased NO production and attenuated the pathogenesis of organ rejection. It was speculated that inhibition of iNOS may provide a novel therapeutic modality in the management of acute transplant rejection. We found that AMG treatment appeared to selectively inhibit alloantibody formation induced by platelet transfusions; AMG did not affect alloantibody production induced by leucocyte transfusions or by immunization with a foreign protein antigen, human γ globulin. These observations suggest that recipient cells that mediate an AMG-sensitive activity appear to be critical for the development of platelet-induced antidonor antibody formation. Since our previous data have shown that antiplatelet alloantibody formation is associated with in vitro indirect platelet antigen presentation to Th cells, and since Oh et al. have recently shown that in vitro platelet-pulsed recipient macrophages are capable of inducing platelet immunity within inbred rats, we conclude that recipient macrophages are the primary cell type responsible for inducing immunity to allogeneic platelets. The probable reason why AMG treatment does not affect the WBC-induced alloimmunization is because of direct allore cognition of donor APC by recipient T-helper cells, therefore bypassing the requirement of recipient APC. While it is not clear how AMG treatment of recipients inhibits platelet-induced humoral immunity, iNOS has been shown to affect the activities of several enzyme systems such as FeS enzymes, aconitase, and mitochondrial respiratory enzymes. It is possible that AMG may affect the processing and/or presentation of platelet-derived alloantigens by inhibiting iNOS activity. We are currently studying this possibility.

In summary, we have demonstrated that recipients of allogeneic platelet transfusions require an early iNOS activation in macrophages to stimulate an IgG antidonor antibody response. These results lend support to the concept that recipient antidonor responses against leukoreduced allogeneic platelets can be inhibited by selectively modulating recipient macrophage activity.

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