Effect of Blood Transfusions on Macrophage–Lymphocyte Interaction in an Animal Model

J. PAUL WAYMACK, M.D.
KAMALA BALAKRISHNAN, M.D.
NANCY McNEAL, B.S.
SARA GONCE, B.A., B.S.
PHIL MISKELL, B.S.
GLENN D. WARDEN, M.D.
J. WESLEY ALEXANDER, M.D., Sc.D.

Blood transfusions have been reported over the last 2 decades to decease allograft rejection, to increase the rate of tumor growth, and to increase susceptibility to infectious complications. The effect of transfusions on macrophages, specifically on their regulation of lymphocyte proliferation, was investigated. Both macrophages and their supernatants obtained from transfused rats impaired lymphocyte blastogenesis to a greater degree than those from nontransfused rats. This effect was greatest when the lymphocytes were subjected to mitogen stimulation. The immunosuppression was seen with macrophages from both allogeneically and syngeneically transfused rats. Blood transfusions exert their immunosuppressive effect at least in part by increasing macrophage suppression of lymphocyte response to stimuli.

From the Department of Research, Shriners Burns Institute, Cincinnati Unit, the Paul I. Hoxworth Blood Center, and the Department of Surgery, University of Cincinnati Medical Center, Cincinnati, Ohio

BEFORE THE 1970s blood transfusions were to a large extent immunostimulatory. The basis for this concept came primarily from two observations. First, transfusions of incompatible red blood cells generate a strong immunologic transfusion reaction,[1] and second, the injection of leukocytes from a specific donor resulted in immunization of the recipient that caused a more rapid and vigorous rejection of skin grafts taken from the same donor.[2,3]

Medawar's reports,[2,3] in particular, led pioneering transplant surgeons to avoid transfusions in patients who were awaiting organ transplantation. However, several different investigators in the early 1970s reported that pretransplant transfusions actually decreased the rate of immunologic rejection of the transplanted organs.[4–7] By 1985, more than 68 studies documented that pretransplant transfusions decrease the rate of rejection of solid organ transplants.[8]

In the last 5 years the effects of transfusions on the rate of tumor growth in patients with cancer have been studied. There have been 13 retrospective studies on the effect of perioperative transfusions on the rate of cancer recurrence and on long-term survival.[9–21] These reports dealt with cancer of the colon, lung, and breast, and sarcomas. In nine of these 13 reports, there was a higher incidence of tumor recurrence and a shorter long-term survival rate among those patients who received perioperative transfusions.[9–12,15–18,21] The four remaining studies did not demonstrate any significant differences between transfused and nontransfused patients.[13,14,19,20]

We have previously reported that transfusions with allogeneic blood in rats diminish cell-mediated immunity as measured by contact sensitization to the hapten dinitrofluorobenzene.[22] With the same rat model, allogeneic blood transfusions increased the mortality rates in burned rats challenged with *Pseudomonas aeruginosa* 1244.[23] This effect was not seen when transfusions with syngeneic blood were used.

More recently we have reported that transfusions with allogeneic blood, but not syngeneic blood, increase macrophage production of the arachidonic acid metabolites prostaglandin E, thromboxane, and prostacyclin. The transfusions did not alter macrophage production of leukotriene B or C.[24,25] Since prostaglandin E is a known immunosuppressive metabolite,[26,27] the effect of the

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Reprint requests and correspondence: Dr. J. Paul Waymack, Shriners Burns Institute, 202 Goodman Street, Cincinnati, OH 45219.

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transfusions on macrophage–lymphocyte interactions was studied.

Materials and Methods

Animals

Adult male Lewis rats weighing 200–250 g were used as transfusion recipients. Adult male A'Sogaloff Cancer Institute (ACI) rats and additional male Lewis rats were used as blood donors. All rats were kept in stainless steel hanging cages with three rats per cage, and were observed for 2 weeks before the study to exclude the presence of underlying diseases. The animals were given food and water *ad libitum* throughout the experiment.

Blood Transfusions

Blood was obtained from donor animals by cardiac puncture and was mixed with standard citrate–phosphate–dextrose anticoagulant solution at a 4:1 volume ratio. The blood was stored for 24 hours at 4 C before infusion. Ten Lewis rats received a 1-mL transfusion with ACI blood, ten were transfused with 1 mL of blood from Lewis rats, and ten were transfused with 3 mL of lactated Ringer's solution. A threefold increase in the volume of crystalloid was made to closely approximate the intravascular volume change achieved with a 1-mL blood transfusion. This sequence of three groups of transfusion recipients was performed for each of the three phases of this study.

Macrophage Harvesting

Four days after transfusion the peritoneal cavity of each animal was infused with 3 mL of sterile brain–heart infusate. Four days later the animals were killed by cardiac air embolism. Their peritoneal cavities were lavaged with Hanks balanced salt solution (HBSS) without Mg^{++} or Ca^{++} and with 5mm ethylenediamine tetra-acetic acid (EDTA). The cell suspensions were hypotonically lysed of contaminating red blood cells and washed three times with HBSS with Mg^{++} and Ca^{++} and without EDTA to remove the red cell fragments. The number of macrophages in each suspension was determined and each cell suspension was recentrifuged and suspended in sufficient M-199 medium with penicillin, streptomycin, and 7.5% fetal calf serum, to achieve a final macrophage concentration of 1×10^6/mL. One-milliliter aliquots of these suspensions were cultured in plastic flat-bottom polystyrene tissue culture plates. They were incubated at 37 C in 5% CO_2 for 1 hour, at which time the culture medium was removed and replaced with fresh M-199 medium to remove any nonadherent cells. The suspensions were cultured for 24 hours, after which the acellular medium was removed and frozen at −70 C until the samples could be incubated with normal splenocytes.

For the second set of experiments, macrophages were harvested from the peritoneal cavity of three groups of rats that were identically transfused as those described before. These cells were also hypotonically lysed of contaminating red cells and washed three times to remove any cellular debris. After the final washing they were resuspended in sufficient RPMI medium with penicillin, streptomycin, and 7.5% fetal calf serum (complete RPMI) to achieve a final macrophage concentration of 5×10^4/50 μL.

Splenocyte Harvesting

Normal splenocytes were obtained from healthy untreated Lewis rats. The rats were killed by cardiac air embolism and their spleens were aseptically removed. Three spleens were used in the first two phases of the study. The spleens were bivalved under sterile conditions in a laminar flow hood and the splenocytes removed from each splenic half by lavage. The splenocytes from the three spleens were combined, hypotonically lysed of contaminating red cells, and washed as previously described. After the final wash they were resuspended in sufficient complete RPMI to achieve a final concentration of 1×10^5/100 μL.

For the first set of experiments, 100-μL aliquots of the normal rat splenocytes were combined with 50 μL of each of the macrophage supernatants. To this was added 100 μL of complete RPMI. These were cultured for 72 hours in round-bottom polystyrene culture plates. They were pulsed with 1.0 μCi of ^{3}H-thymidine and cultured for another 18 hours, after which they were harvested with an automated cell harvester. The filters were placed in scintillation vials with scintillation fluid and were counted on a standard beta counter.

For the second set of experiments, the above procedure was repeated except for the addition of 50 μL of the macrophage suspension containing 5×10^4 macrophages instead of the macrophage supernatant. The macrophage–splenocyte suspension was then cultured, pulsed, and counted.

In the final set of experiments, ten rats from each of the three transfusion groups had their spleens aseptically removed. Splenocytes were harvested from these spleens and after lysing contaminating red blood cells, the number of lymphocytes and macrophages per splenic lavage was determined. The splenocytes were resuspended after the final washing in sufficient complete RPMI to achieve a splenocyte concentration of 1×10^5/250 μL. A 250-μL aliquot of each splenocyte suspension was then cultured, pulsed, and counted as previously described.

All splenocyte suspensions were cultured both with 10 μg of phytohemagglutinin (PHA) per culture well and without PHA. Duplicate tests were run on all cultures in all phases of this study.

TABLE 1. *Per cent of lymphocyte blastogenic response obtained with the addition of macrophage supernatants from transfused rats compared with macrophage supernatants from nontransfused rats*

	Control Rats	Rats Transfused with Blood from Lewis Rats	Rats Transfused with Blood from ACI Rats
Supernatants without PHA stimulation	100.0 ± 5.0%	111.8 ± 5.5% p = .180	118.9 ± 7.6% p = .099
Supernatants plus PHA stimulation	100.0 ± 2.9%	86.0 ± 2.7% p = 0.003	90.4 ± 2.0% p = 0.041

TABLE 2. *Per cent of lymphocyte blastogenic response obtained with the addition of macrophages from transfused rats compared with macrophages from nontransfused rats*

	Control Rats	Rats Transfused with Blood from Lewis Rats	Rats Transfused with Blood from ACI Rats
Macrophages alone	100.0 ± 24.6%	47.7 ± 25.4% p = .110	46.6 ± 16.2% p = .048
Macrophages plus PHA stimulation	100.0 ± 38.9%	27.1 ± 9.4% p = .028	12.6 ± 3.0% p = .006

Statistical Analysis

The average blastogenic response for each transfusion group was divided by the average response of cells from the nontransfused control rats in that set of experiments. This number was expressed as the per cent of response compared to using cells or supernatants of cells obtained from nontransfused control rats. All data were analyzed on the CLINFO Computer System in the General Clinical Research Center of the University of Cincinnati Medical Center, Cincinnati, Ohio. Statistical significance was determined using the Student's t-test.

Results

Macrophage supernatants from both transfusion groups increased the nonstimulated uptake of ^{3}H-thymidine by splenic lymphocytes compared with the rats treated with lactated Ringer's solution when PHA was deleted, but this difference was not significant (Table 1). In those cultures in which PHA was present, there was an impairment in blastogenesis when macrophage supernatants from transfused rats were added (Table 1). The addition of supernatants from macrophages of rats transfused with blood from Lewis rats decreased the blastogenic response to 86.0 ± 2.7% of that seen with the addition of macrophage supernatants from rats treated with lactated Ringer's solution (p = 0.003). The addition of macrophage supernatants from rats transfused with blood from ACI rats also significantly decreased the blastogenic response to 90.4 ± 2.9% of control rats (p = 0.041).

The addition of macrophages from transfused rats decreased the blastogenic response to an even greater degree (Table 2). When cultured without PHA stimulation, macrophages from rats transfused with blood from Lewis rats decreased the blastogenic response to 47.7 ± 25.4% of that seen with the addition of macrophages from control rats. The use of macrophages from rats transfused with blood from ACI rats decreased the response to a slightly greater degree (46.6 ± 16.2% of control rats, p = 0.048).

The addition of PHA to the cultures increased the degree of blastogenic inhibition seen with the addition of macrophages from the transfused rats (Table 2). Macrophages from rats transfused with blood from Lewis rats decreased the blastogenic response to 27.1 ± 9.4% of that seen with the addition of macrophages from rats treated with lactated Ringer's solution (p = 0.028). The impairment in lymphocyte response was even greater with the addition of macrophages from rats transfused with blood from ACI rats (12.6 ± 3.0% of control rats, p = 0.006).

There were no statistically significant differences between the blastogenic responses of splenocytes from blood of ACI and Lewis rats and rats treated with lactated Ringer's solution (Table 3). Without PHA, the rats transfused with blood from Lewis rats had a splenocyte blastogenic response of 197.1 ± 51.6% compared with that of the control rats. The splenocyte blastogenic response of rats transfused with blood of ACI rats decreased to 72.1 ± 22.8% of that seen in control rats. Neither of these changes was statistically significant.

When PHA was added to the cultures, there was an increase in the response of the splenocytes from rats transfused with blood from Lewis rats (186.4 ± 44.8%) and rats transfused wth blood from ACI rats (133.7 ± 23.7%) compared with control rats.

There was a decrease in the number of lymphocytes obtained from the splenic lavage of the rats transfused with blood from Lewis and ACI rats. Control rats had 7.02 ± 1.26 × 10^7 lymphocytes per spleen. Rats transfused with blood from Lewis rats had 4.62 ± .86 × 10^7 lymphocytes per spleen (NS) and rats transfused with blood

TABLE 3. *Per cent of lymphocyte blastogenic response seen with splenocytes from transfused rats compared with splenocytes from nontransfused rats*

	Control Rats	Rats Transfused with Blood from Lewis Rats	Rats Transfused with Blood from ACI Rats
No PHA stimulation	100 ± 29.1%	197.1 ± 51.6% p = .119	72.1 ± 22.8% p = .461
PHA stimulation	100 ± 23.7%	186 ± 44.8% p = .105	133.7 ± 23.7% p = .328

from ACI rats had $1.50 \pm .37 \times 10^7$ lymphocytes per spleen ($p = 0.001$).

There was a slight decrease in the number of macrophages from the spleens of transfused rats. Control rats had $2.14 \pm .68 \times 10^6$ macrophages per splenic lavage and rats transfused with blood from Lewis rats had $1.68 \pm .53 \times 10^6$ macrophages per spleen (NS). The decrease to $6.8 \pm 2.9 \times 10^5$ macrophages per splenic lavage in the rats transfused with blood from ACI rats did not reach statistical significance ($p = 0.065$).

Discussion

Blood transfusions have become a common treatment for correction of severe anemias and for the replacement of significant losses resulting from traumatic injuries and surgical operations. Such transfusions are possible because of the discovery of the ABO blood groups by Landsteiner in 1900.[28]

The immunologic alterations resulting from blood transfusions have only recently been recognized. The possibility that transfusions cause a broad-spectrum immunosuppression is supported by the decreased rejection rate of allografts[5-8] and the increased rate of recurrence of cancer in transfused patients.[9-21] Our work has also documented an increased susceptibility of transfused rats to lethal infections.[23]

We have concentrated our initial efforts on determining the mechanism of this broad-spectrum immunosuppression by investigating its effect on the monocyte–macrophage cell line. Our earlier work in rats documented an increased production of the immunosuppressive metabolite prostaglandin E by macrophages from transfused rats and burned transfused rats.[24,25] Since prostaglandin E inhibits lymphocyte function,[26,27] we evaluated whether the macrophages themselves suppress lymphocyte function. Macrophages also produce a variety of other lymphokines including the lymphocyte stimulator interleukin 1 (IL1).[29] If the transfusions stimulated macrophage production of such immunostimulatory compounds to a greater degree, the net effect of transfusion on macrophage–lymphocyte interaction might be to increase lymphocyte blastogenesis.

Our study tested the effect of both macrophages and their supernatants on lymphocyte blastogenesis. By isolating these cells from the transfused rats and mixing them with lymphocytes from normal untreated rats, we eliminated the possible interference of suppressor T-lymphocytes generated directly by the transfusions. Such suppressor T cells have been found after transfusions.[30,31]

We found that macrophages from rats transfused with both syngeneic and allogeneic blood impair lymphocyte blastogenesis. The use of the supernatant from the macrophage culture also decreased the blastogenic response compared with supernatant from nontransfused rat cells. The inhibition with the supernatant was roughly 10% of that of the corresponding macrophage from both syngeneic and allogeneically transfused rats. Thus it appears that a direct macrophage–lymphocyte interaction is required for maximal inhibition. An alternative explanation is that there is a positive feedback loop in the release of immunosuppressive metabolites between the macrophage and lymphocyte. That is, the macrophage's initial secretion of such immunosuppressive metabolites as prostaglandin E results in the lymphocyte secretion of compounds that stimulate the macrophage to produce more immunosuppressive metabolites.

Our failure to demonstrate an impairment in lymphocyte blastogenesis using splenocytes from transfused rats indicates the complex nature of the PHA response. There are several possible explanations for this failure to demonstrate impaired blastogenesis after immunosuppressive blood transfusions. One explanation is that the blastogenic response measured was from the cellular division of suppressor T cells. If this were the case, then the blastogenic assay would not document the immunosuppression resulting from the increased number of suppressor T-lymphocytes. Another explanation is that there was an insufficient number of macrophages in the splenocyte cultures. The ratio of macrophages to lymphocytes in the second phase of this study was 1:2. There was a marked impairment in the lymphocyte blastogenic rate at this ratio. In the third set of experiments there was, on average, a ratio of only 1:30. It is possible that at this low concentration the macrophages were not able to inhibit the function of a larger number of lymphocytes.

The decrease in the number of lymphocytes and macrophages from the splenic lavages of the transfused rats is an interesting finding. Decreased recovery could be due to an increased adhesiveness of the cells to the splenic pulp that prevented them from being washed out with the lavage. A second possibility is that the transfusions altered the distribution of the cells to regions other than the spleen. In a previous study, we found that transfusions diminish migration of macrophages to the peritoneal cavity in response to inflammatory stimuli.[22,25]

This study demonstrated a statistically significant impairment in lymphocyte blastogenesis by macrophages taken from rats after transfusions with either allogeneic or syngeneic blood. This finding is different from our earlier reports that demonstrated a greatly increased susceptibility to infections after allogeneic transfusions but only a slight increase after syngeneic transfusions.[23,25] These findings suggest an important contribution of factors not related to histocompatibility. These factors could include hemolysis and lysis of platelets and/or neutrophils. Additional investigations in this area are needed to study this clinically important problem.

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