Experimental Evidence That Granulocyte Transfusions Are Efficacious in Treatment of Neutropenic Hosts with Pulmonary Aspergillosis

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Although polymorphonuclear leukocytes (PMNs) are powerfully anti-*Aspergillus*, transfusion therapy remains controversial, with conflicting results, and experimental support has been lacking. We devised a pulmonary infection model in neutropenic BALB/c mice, used an antibacterial regimen to prevent confounding sepsis, and optimized PMN induction, purifications, and dose. Mice were given 150 mg/kg cyclophosphamide every 4 days and a gentamicin-vancomycin-clindamycin-imipenem regimen daily beginning 4 days before intranasal challenge with 5×10^5 *Aspergillus* conidia. This regimen produced leukopenia ($\sim 10\%$ of normal white blood cell [WBC] count; $\leq 10\%$ PMNs) for 10 days, without bacterial superinfection. PMN donors given 100 $\mu\text{g/kg}$ recombinant murine granulocyte colony-stimulating factor (G-CSF) for 10 days yielded 11×10^7 to 13.6×10^7 WBC/ml (81 to 87% PMNs). Infected mice were given PMN transfusions intravenously. In 2 experiments with up to 70% mortality of neutropenic controls, transfusion of 10^7 PMNs 1 and 4 days after challenge had negligible effects on peripheral WBC counts but improved survival ($P = 0.007, 0.02$), decreased lung CFU ($P = 0.03, 0.005$), and cleared infection in 28 to 50% of survivors. Transfusion of 5×10^6 PMNs showed partial protection. Transfusions given every other day did not improve protection. Our present results provide an experimental basis for enthusiasm for PMN transfusions in the therapy of aspergillosis in humans.

Prolonged neutropenia is a risk factor for the acquisition of invasive pulmonary aspergillosis, particularly in transplant and cancer patients (1), and the prognosis is much worse if there is not autologous recovery of neutrophils during aspergillosis (2). Although the introduction of new therapeutics has improved the response to treatment, failures, breakthrough infections, and ultimately mortality remain unacceptably high. One therapeutic modality that has been used periodically for over 40 years is that of white blood cell (WBC) or granulocyte transfusion (3–6). Differences in the number of cells that could be collected and transfused, as well as the cell collection techniques and donor matching, all have contributed to the difficulty in assessing the efficacy of a WBC transfusion. Improvements in techniques used in this modality were made with the use of granulocyte colony-stimulating factor (G-CSF) and steroids to improve granulocyte yield, and available data are suggestive that higher numbers of transfused granulocytes lead to improved outcome (5–9).

In spite of these improvements, WBC transfusions remain a controversial treatment, as their utility is difficult to discern since conflicting efficacy results have been reported (10, 11). A consensus on the value of this model of treatment has not been reached (12). The spectrum of infections in neutropenia following therapy of hematologic cancers or stem cell transplantation has shifted toward those caused by molds (3). It has been proposed that the numbers of patients needed in clinical trials to evaluate antifungals suggest that the trial size may be prohibitive for a trial of WBC transfusions in mycoses (13). If WBC transfusions could be proven beneficial in experimental models, this, then, would support the pursuit of WBC transfusions as a treatment option for humans.

Our goal in these studies was to create a satisfactory murine model for study of the treatment of invasive pulmonary aspergillosis by granulocyte transfusion. Several challenges arose in determining the combination of fungal inoculum and immunosuppression to produce lethality due to infection and not immunosuppression or

secondary bacterial infection; this required that a safe antibiotic regimen be developed. Lastly, we needed to address the issues of polymorphonuclear leukocyte (PMN) induction in donor animals, an optimal PMN separation method for mouse cells, and a transfusion schedule. Our results indicate that therapeutic transfusions of PMNs were beneficial in treating mice with invasive pulmonary aspergillosis.

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MATERIALS AND METHODS

Infection model. A series of preliminary studies were done to develop a useful model of pulmonary aspergillosis in neutropenic mice (14). In the course of these studies we examined different regimens and schedules of immunosuppression, different antibiotic dosages, and several inoculum numbers. We found that the regimens of immunosuppression and antibiotics and the inoculum numbers of conidia that are detailed below resulted in a murine model of progressive pulmonary aspergillosis in animals that were neutropenic for the duration of the infection study, 10 days.

Immunosuppression. Male BALB/c mice (six weeks old; Charles River Laboratories, Hollister, CA) were used in these studies, in groups of 10. Neutropenia was induced by intraperitoneal injection of 150 mg/kg cyclophosphamide (Cytoxan; Baxter Health Care Corp., Deerfield, IL),

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TABLE 1 WBC counts and differentials of mice given recombinant murine G-CSF^a

Donor group	WBC count (cells/ml)	% PMNs
1	1.34×10^8	81%
2	1.10×10^8	87%

^a Blood was obtained via brachial artery and collected in tubes containing sodium heparin. WBC counts on donor blood were performed on the day of collection, which was 1 day after the cessation of treatment with recombinant murine G-CSF. WBC counts prior to treatment with recombinant murine G-CSF fell within a range of 6.4×10^6 to 1.6×10^7 WBC/ml of blood (average, 1.14×10^7 WBC/ml; PMN average was 10%).

and the mice were housed within microisolator cages. All mice were given the initial injection on day -4 prior to infection, and subsequent injections were given every 4 days thereafter.

Antibiotics regimen. Mice were given drinking water with 33.3 mg/kg gentamicin (APP Pharmaceuticals, Schaumburg, IL), 167 mg/kg clindamycin (Patheon, Inc., Ontario, Canada), and 167 mg/kg vancomycin (NOVAPLUS, Hospira, Inc., Lake Forest, IL) beginning 4 days prior to day 0 and every day thereafter. Fresh drinking water was prepared daily; mice were assumed to drink 5 ml of water per day. Mice were also given 167 mg/kg imipenem/cilastatin (Primaxin; Merck & Co., Inc., Whitehouse Station, NJ) subcutaneously beginning 4 days before infection and every day thereafter. Mice were weighed every 4 days, and doses of antibiotics were adjusted based on body weight.

Organism. *Aspergillus fumigatus* 10AF was thawed from -80°C storage and grown for 4 days at 37°C on potato dextrose agar (Difco PDA; Becton, Dickinson and Co., Sparks, MD) plates (15–18). Conidia were harvested, suspended in 0.05% Tween 80 saline, and washed three times by centrifugation. Conidial viability was determined by plating on Sabouraud dextrose agar (BBL SDA; Becton, Dickinson), and numbers of conidia were also determined by the use of the hemacytometer.

Infection. Similar to a pulmonary model described previously (15), mice were infected by intranasal challenge of *A. fumigatus* conidia given in 30 μl . The mice were anesthetized with isoflurane and conidia instilled. The inocula were 5.0×10^5 viable conidia in one study and 5.76×10^5 viable conidia, per mouse, in the second study described.

Mortality was tallied daily through 10 days of infection. All surviving mice were euthanized by CO_2 anoxia and the lungs removed for determination of fungal burden by quantitative plating for CFU as described previously (15, 19).

Collection of granulocytes. Age-matched male BALB/c mice were placed into groups of 20 and given 100 $\mu\text{g/kg}$ of recombinant murine G-CSF (donated by Amgen, Thousand Oaks, CA) via daily intraperitoneal injection for 12 days prior to the day of PMN collection. This regimen induces a maximal response of the donor mice (J. Andresen, Amgen, personal communication; data not shown) to produce sufficient numbers of PMNs for collection from peripheral blood and subsequent transfusion (Table 1). Donor mice were bled via brachial artery incision, and blood was placed in tubes containing heparin; approximately 1 ml of blood was obtained from each donor mouse. The heparinized whole blood was diluted 1:2 with 0.9% sodium chloride and then mixed with an equal volume of 6% dextran (molecular weight [MW], 200,000 to 250,000; Sigma D-7265). This suspension was incubated for 1 h at ambient temperature to sediment red blood cells (RBC). The upper portion (ca. three-fourths of the total volume) of the suspension was collected and centrifuged. The remaining RBC were lysed with 0.85% ammonium chloride for 10 to 15 min on ice and the cells pelleted by centrifugation. Cell pellets were suspended in saline. PMNs were separated from mononuclear cells using a Ficoll-Hypaque method, with 6 ml of cell suspension layered over 6 ml of Ficoll-Hypaque (Histopaque 1077; Sigma). Gradients were centrifuged at $365 \times g$ for 30 min at 4°C . Pelleted PMNs were recovered and suspended in RPMI 1640 medium with L-glutamine and NaHCO_3 (Sigma). These cells were washed once by centrifugation and suspended in 2 ml of RPMI

1640. This method produced a purified preparation of $>99\%$ PMNs, with viability of 93 to 99% and with $>29\%$ PMN recovery from donor blood.

Cell numbers were determined by hemacytometer count. Cell viability was determined by dye exclusion using 0.2% Trypan blue in phosphate-buffered saline (PBS) (pH 7.2). Dilutions to reach the final desired cell numbers were done in RPMI 1640. PMNs were held at 4°C until they were transfused into the infected mice on the same day as they were collected.

Transfusion. Neutropenic, infected recipient mice were given PMN transfusions via injection into a lateral tail vein. Transfusions were done on days 1 and 4 postinfection. Preliminary studies had shown this schedule to be optimal. Mice received 5×10^6 or 1×10^7 PMNs per mouse. One group of infected mice did not undergo PMN transfusion and was used as an untreated control group.

Statistics. Analyses of survival were done using a log rank test. Comparisons of CFU were done using a Mann-Whitney U test. Data points missing due to death from infection of the mice were assigned an arbitrary value of $\log_{10} 5$ CFU, a value close to that found just prior to death, and included in the statistical analyses (20, 21). Assignment of this value ensures that death is considered a worse outcome than is survival with any burden (20, 21).

RESULTS

Leukopenia and WBC counts for donor and infected recipient mice. Through empirical investigation, we devised a satisfactory system of producing and maintaining leukopenia in mice, with minimal numbers of deaths due to immunosuppression, secondary infection, or antibiotic toxicity. Using the dose of cyclophosphamide and antibiotic regimen described in Materials and Methods, we induced a steady leukopenia of approximately 10^6 WBC/ml for 10 days without bacterial superinfection. This is 10% of the normal numbers of murine WBC and $\leq 10\%$ PMNs. The baseline total WBC count of the mice 1 day prior to the first dose of cyclophosphamide averaged 1.28×10^7 WBC/ml. On day 0, just prior to infection, the average WBC count was 2.17×10^6 WBC/ml (Fig. 1). These kinetics, and the lack of intercurrent infection, are similar to what is seen in mice given this antibiotic and immunosuppressive regimen but not challenged with *Aspergillus* (data not shown).

Total WBC and differential counts were performed on days of

FIG 1 WBC counts of mice throughout the duration of the experiment. Arrows indicate days when cyclophosphamide was given. $n = 3$ or 4 samples per time point. Day 1 and 4 values are 3 h prior to transfusion with PMNs. Days 3 and 5 to 9 represent values 24 h or more posttransfusion. Day -5 represents the value for nonneutropenic mice, and day 0 represents the value at the time of infection.

FIG 2 WBC counts before and after PMN transfusion. The first transfusion is indicated at 0 h, and the second transfusion is shown at 72 h. Arrows indicate PMN transfusions. $n = 3$ or 4 samples per time point.

transfusion on pooled blood from donor mice (Table 1). Recipient mice were bled prior to transfusion and again 1 hour post-transfusion. Subsequent WBC counts were taken at 24 and 48 hours after the first transfusion and 24 hours after the second transfusion (Fig. 2). Infected mice remained pancytopenic throughout the experiment, with an average WBC range between 6.0×10^5 WBC/ml and 2.75×10^6 WBC/ml on any day.

One hour after the first transfusion, the total WBC counts were slightly lower than the pretransfusion counts. However, the percentage of PMNs in the differential counts increased almost 3-fold from 6.3% to 17.7%. Counts at 24 and 48 h posttransfusion declined further, and the percentage of PMN decreased to <9%. After the second PMN transfusion, there was a <2-fold increase in the total WBC count and a substantial increase in the percentage of PMNs to ~39%. By 24 h posttransfusion, the WBC counts declined slightly and the percentage of PMNs had dropped to about 20%, lower than at 1 h posttransfusion but higher than in the pretransfusion samples (Fig. 2).

Efficacy of WBC transfusion against pulmonary aspergillosis in neutropenic mice. Data on survival and CFU were gathered from two experiments. From the first experiment, one mouse from the untreated control group died immediately after infection. This mouse did not recover from anesthesia and was not included in the data on the assumption that the mouse died from the procedure and not from infection. There were no mice that died immediately after infection for the second experiment. At day 10 postinfection, there were 3 survivors in the untreated control group, 7 survivors in the group given 5×10^6 PMNs/mouse, and 9 survivors in the group given 1×10^7 PMNs/mouse. Similarly, at day 10 postinfection in the second experiment, there were 6 survivors in the untreated control group and 8 survivors in the group given 5×10^6 PMNs/mouse and all 10 mice survived in the group given 1×10^7 PMNs/mouse.

Figure 3 shows the survival for the groups in the first experiment. There was a significant difference between the untreated control group and the group given 1×10^7 PMN/mouse for both

survival ($P = 0.007$) and CFU ($P = 0.04$). The group given 5×10^6 PMNs/mouse showed a trend toward prolonged survival, but the difference was not significant compared to results for the untreated control group ($P = 0.1$), nor was there any difference from the group given 1×10^7 PMNs/mouse ($P = 0.2$). This lower PMN dose of 5×10^6 per mouse also was intermediate between the effective 1×10^7 dose and the controls in lowering lung CFU, with no significant difference in lung CFU for the group given 5×10^6 PMNs/mouse and the untreated control group ($P = 0.32$) or the group given 1×10^7 PMNs/mouse ($P = 0.17$) (Fig. 4). However, 4 mice in the 1×10^7 PMNs/mouse group were free of detectable pulmonary infection with *A. fumigatus*, compared to 2 given the lesser number of cells.

Figure 5 shows the survival for the groups in the second experiment. Again, there was a significant difference between the untreated control group and the group given 1×10^7 PMN/mouse for both survival ($P = 0.03$) and CFU ($P = 0.005$). The group given 5×10^6 PMNs/mouse again showed a trend toward prolonged survival, but the difference was not significant compared to results for the untreated control group ($P = 0.3$), nor was there

FIG 3 Survival curve of mice from experiment 1. Arrows indicate days of PMN transfusion of either 1×10^7 or 5×10^6 PMNs per mouse. Control mice received no treatment. There was a significant difference in survival between the control group and the group given 1×10^7 PMNs/mouse ($P = 0.007$).

FIG 4 CFU count for experiment 1. There was significant difference between the control group and the group given 1×10^7 PMNs/mouse ($P = 0.03$). Horizontal bars represent the median values.

a difference from the group given 1×10^7 PMNs/mouse ($P = 0.2$). There was no significant difference in lung CFU for the group given 5×10^6 PMNs/mouse and the untreated control group ($P = 0.2$) or the group given 1×10^7 PMNs/mouse ($P = 0.2$) (Fig. 6). Similar to the first study, 5 mice given 1×10^7 PMNs/mouse were free of detectable pulmonary infection.

We also assessed the number of transfusions needed to obtain an optimal result. We found that giving 5×10^6 or 1×10^7 WBC every other day (i.e., on days 1, 3, 5, and 7 postinfection) did not improve protection. In other experiments with a more lethal outcome (80% lethal dose [LD_{80}] or LD_{90} in controls), a trend for improved survival and reduction of lung CFU with transfusion of 10^7 WBC on days 1 and 4 remained but did not reach statistical significance (data not shown).

In addition to the survival and CFU, at the time of organ removal, the lungs of the surviving control mice on average weighed more than those from PMN-treated mice. The control mouse lungs looked hemorrhagic compared to those of the PMN-treated mice, which appeared more normal (pink).

DISCUSSION

Our aims in these studies were 2-fold and included the development of a persistently neutropenic model of pulmonary aspergillosis and the assessment of the utility of PMN transfusion as a therapeutic intervention. Through a series of empirical studies, we

FIG 5 Survival curve of mice from experiment 2. Arrows indicate days of PMN transfusion. There was a significant difference in survival between the control group and the group given 1×10^7 PMNs/mouse ($P = 0.02$).

FIG 6 CFU count for experiment 2. There was significant difference between the control group and the group given 1×10^7 PMNs/mouse. ($P = 0.005$). Horizontal bars represent the median values.

developed a reproducible model of invasive pulmonary aspergillosis in persistently neutropenic BALB/c mice. As a result, we found that an inoculum of 5×10^5 conidia, which is an inoculum up to 10-fold or more lower than those used in other models of pulmonary aspergillosis initiated by intratracheal or intranasal inoculation of conidia, induced lethal disease (22, 23). We found that the model required the administration of a multiple-drug antibiotic regimen to prevent secondary bacterial infection. Furthermore, the dose and schedule of cyclophosphamide were critical to induce neutropenia but not cause drug-related lethal toxicity, with 150 mg/kg given every 4 days satisfying this requirement.

Performance of granulocyte transfusions using cells isolated from peripheral blood would not have been possible without the induction of the donor mice with G-CSF; induction was required to obtain sufficient numbers of PMNs, with one donor mouse providing enough cells for transfusion of two recipient mice. Despite infusing up to 1×10^7 cells/mouse, the peripheral leukocyte count in recipient mice did not increase significantly after transfusion. However, most importantly, we demonstrated that the percentage of PMNs in the blood of the recipient mice increased substantially. We presume that the transfused WBC largely traffic to the site of infection rather than remaining in the peripheral circulation, which would be consistent with what is known about the physiology of WBC during infection. The neutrophil concentration determined in venous blood represents about half of intravascular neutrophils, with the remainder adherent to vascular walls (24). Studies in humans have shown that transfused granulocytes go to areas of infection in neutropenic patients, including sites of *Aspergillus* infection (25, 26). The homing of infused neutrophils to infection is consistent with our efficacy results. Thus, a transfusion of 1×10^7 PMNs to each infected mouse resulted in significant prolongations of survival and reduction of pulmonary burden of aspergillosis. Also, we found that two doses given 3 days apart were sufficient to result in significant survival advantage and reduction of fungal burden in the lungs.

The utility of PMN transfusion as a treatment against infectious disease in the neutropenic patient has been a controversial topic for many years. Although there is a lack of convincing controlled trial data (6, 12), reviews of case studies and small trials would speak to a role for granulocyte transfusions in addition to antifungal or antibacterial therapy, with positive results in up to

70% of patients. Open trials in mycoses in neutropenia (2) and favorable results in fungal infections in chronic granulomatous disease patients are particularly relevant (27–29). Treatment protocols and recommendations vary among investigators (6, 12). Our study likely addresses the most difficult hurdle for WBC transfusion, as most (4, 30–34) but not all (35–37) studies have suggested that fungal infections, particularly those caused by molds, are more refractory than bacterial infections to the potential benefits of these transfusions.

Our data, using a model of pulmonary aspergillosis in neutropenic mice, indicate that PMN transfusion appears to be beneficial and is dependent on the number of cells given. This is well illustrated by our results showing that only the highest number of transfused cells resulted in significantly prolonged survival and reduction of fungal burden in the lungs; up to 50% of the treated mice were free of detectable pulmonary infection. The relation of success to number of transfused cells is emphasized throughout the clinical literature.

Our experimental model has several parallels to clinical experience. We used G-CSF to stimulate our donors; G-CSF has been shown to prime the oxidative burst in donor cells and to increase cell survival, phagocytosis, microbicidal activity, recruitment to sites of infection, and proteins involved in cell adhesion (3, 38, 39). Repeated G-CSF administrations have been shown to be superior to single administrations in improving these functional properties (40). It is recommended that the granulocytes be transfused as soon as possible after they are obtained, without storage (41, 42), as we did, although it has been shown that neutrophil functions can be preserved for 24 h *ex vivo* (43), and viability after G-CSF mobilization is preserved up to 72 h after collection, while interleukin 1 (IL-1) or IL-8, implicated in transfusion reactions, remains low (44). Between collection and transfusion the cells were refrigerated, as is optimal with human cells (41). Our WBC dose calculates as 4×10^8 granulocytes/kg, which is close to the dose of $\geq 3 \times 10^8$ granulocytes/kg recommended in humans (34, 45). Our recipient WBC kinetics after transfusion are similar to those described in humans (3, 5, 39, 42), and survival has not correlated with a measurable increase in neutrophils (34). Daily WBC transfusions do not appear to be necessary for success (40).

Our model, however, may be favored over the situation in humans, as our donors and recipients are isogenic, a situation rarely obtained in humans. The genetic identity removes the possible complications of graft-versus-host disease (necessitating, in humans, radiation of the WBC) and alloimmunization (44). We are less aware of infusion-related side effects (none were noted) and unaware of any as subjectively reported in humans (3, 27).

It will be of interest in future studies to determine whether PMN transfusion in combination with conventional antifungal therapy may lessen the number of PMNs needed to induce a clinical benefit as well as determine whether regimens that will result in cure of all mice can be found. Thus, our results provide an experimental basis for continued interest in granulocyte transfusion as a beneficial therapeutic option, particularly against pulmonary aspergillosis.

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We have no conflicts of interest to declare.

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