Pediatr. Res. *16:* 57–59 (1982)

Granulocyte Transfusion in Septic Canine Neonates

ROBERT D. CHRISTENSEN,[24] PETER P. BRADLEY, DENNIS A. PRIEBAT, HAROLD ANSTALL AND GERALD ROTHSTEIN

Departments of Pediatrics, Medicine and Pathology, Primary Children's Medical Center and the University of Utah College of Medicine, Salt Lake City, Utah, USA

Summary

Newborn dogs were inoculated intratracheally with 0.5–1.0 x 10^8 *Staphylococcus aureus*/g body weight. Neutropenia (490 ± 280 neutrophils/mm^3 *versus* 8,390 ± 490 control, $\bar{x}$ ± S.E., $P < 0.001$), and depletion of the marrow neutrophil storage pool (3 ± 1% *versus* 27 ± 2% storage neutrophils, $P < 0.001$) occurred 5–6 h following the inoculation. All animals died at 6–10 h. Additional inoculated pups were selected at random to receive transfusions of either granulocytes, plasma or red blood cells. Granulocyte transfusions (3×10^9 neutrophils/kg) improved survival ($P < 0.005$), but plasma and red blood cells did not.

Speculation

Previous reports have shown that certain neonates with bacterial sepsis utilize neutrophils more rapidly than they are replaced by the marrow mitotic neutrophil pool. The quantitative neutrophil deficiency thus produced, results in blood neutropenia, reduced neutrophil availability to affected tissues and high mortality. In this study, neonatal dogs were infected experimentally and found to develop neutropenia, depletion of neutrophil reserves, and death. Granulocyte transfusions improved the survival of these animals while plasma or packed red blood cell transfusions did not. Thus, granulocyte transfusion may present a useful therapeutic modality in those septic neonates who are found to have neutropenia and a depleted neutrophil supply.

Despite many advances in antimicrobial therapy and in neonatal intensive care, bacterial infection still accounts for up to 30% of all newborn deaths (23). Furthermore, approximately 40% of neonates who develop bacterial sepsis die (21). In order to understand why the neonate is unusually susceptible to death from bacterial infection, investigators have looked for and found deficiencies in several aspects of the neonates' host defense mechanism (21). The neutrophil plays a central role in host defense. Our studies, as well as those of Armstrong *et al.* (2), have demonstrated that lethally infected neonatal animals tend to develop neutropenia rather than neutrophilia. In addition, we have shown that in many instances, the neutropenia is due to depletion of the mature marrow neutrophil reserve (9) resulting in a compromised ability to supply neutrophils to infected tissues. Neutrophil replacement therapy by administering granulocyte transfusions would seem a logical treatment for subjects with sepsis and a depleted neutrophil supply. Indeed, as early as 1966, Morse demonstrated the ability of transfused granulocytes to increase the survival of septic adult patients with a compromised neutrophil supply (22), and subsequent animal (11) and human (1, 14) studies have confirmed the efficacy of granulocyte transfusions in this setting. In accordance with these facts, we have postulated that depletion of the neutrophil supply in septic neonates constitutes a physiologic disadvantage which likely contributes to their death. In the present study we tested this hypothesis in an animal model. First, we induced bacterial pneumonia and sepsis in neonatal dogs; neutropenia, marrow neutrophil supply depletion and death resulted. Then in other inoculated pups, the effect of granulocyte transfusion upon survival was investigated.

MATERIALS AND METHODS

Animals. Mongrel dog pups, born at the University of Utah vivarium, were used in all studies. The animals were taken from their mothers at 24–36 h of age and thereafter caged separately and warmed with infrared lamps. Simulated bitch milk (Bordon Co.) was offered by syringe every 4–6 h. The animals' state of activity, response to the offered feeding, respiratory rate and breath sounds were monitored every 4–6 h.

Preparation and Inoculation of Bacteria. Staphylococcus aureus (ATCC #29523) was grown overnight in tryptic soy broth (Difco Lab., Detroit, MI) at 37°C. McFarland standards (17) were used to standardize the bacterial suspensions which were then concentrated by centrifugation, and washed twice with phosphate buffered saline (PBS) to produce final concentrations of 0.5–1.0 x 10^8 colony forming units/g body weight of the animal to be inoculated. This organism was selected for these experiments because of its proven effectiveness and reproductivity in inducing lethal pneumonia in neonatal dogs (8). Other organisms were not tested. Methoxyflurane (Abbott Laboratories, North Chicago, IL) was administered to the pups by inhalation. Then, under direct vision laryngoscopy, a No. 8 French feeding tube was inserted until the tip was about 2 cm below the vocal cords. Either 0.5 ml of sterile PBS or 0.5 ml bacterial suspension was instilled, followed by 2 cc of air to flush the tubing. All animals received intramuscularly methicillin 50 mg/kg twice daily; the first dose being given 2 hr following inoculation.

Blood and Bone Marrow Studies. Capillary blood was obtained by puncture of a heel pad. Nucleated cell counts were done in triplicate (Coulter Electronics, Hialeah, FL) and the average determined. Coverslip smears were stained with Wright stain and a 100–200 cell differential count performed. After shaving the area and a 10% povidone-iodine (Perdue Frederick Co., Norwalk, CT) scrub, blood cultures were drawn from the external jugular vein.

Neutrophilic cell types were identified according to the criteria of Cartwright (5). A definite nuclear filament was required for a PMN (Seg); a metamyelocyte was distinguished from a band neutrophil when the smallest width of the nucleus was greater than ¼ the diameter of the cell. Bone marrow aspirates were obtained simultaneously with peripheral blood neutrophil counts. The distal thigh was shaved and prepared with 70% alcohol. Sterile #18 or #19 gauge Osgood marrow needles (Popper and Sons, Inc., New York, NY) were used. Aspirated samples were smeared on coverslips, Wright stained, and a 1,000 cell differential count performed. The neutrophil storage pool (NSP) % was defined as the proportion of nucleated marrow cells belonging to the postmitotic neutrophil category (metamyelocytes, band neutrophils, and segmented neutrophils).

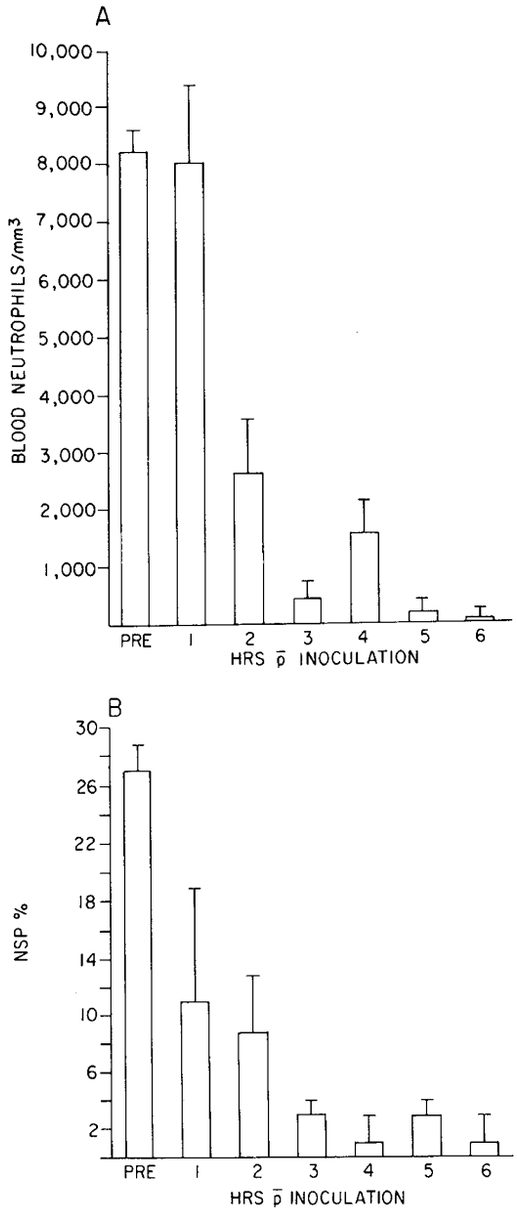

Fig. 1*a*. Blood neutrophil counts in 6 lethally infected neonatal dogs. The bar represents the mean, the bracket the S.E. of the mean.

Fig. 1*b*. Marrow NSP% in 6 lethally infected neonatal dogs. The bar represents the mean, the bracket the S.E. of the mean.

Granulocyte Procurement and Transfusion. A 31 kg German shepherd, housed at the University of Utah vivarium was used as the granulocyte donor. Thiamylal Sodium (Parke Davis, Detroit, MI), 10 mg/kg was injected intravenously resulting in light anesthesia after which granulocytes were obtained by means of intermittant flow centrifugation (Haemonetics Model 30, Waltham, MA) using the 250 ml bowl. The animal was not premedicated with corticosteroids. Hydroxyethyl starch was used as the sedimenting agent. Following four 250 ml passes, the product was concentrated by centrifugation at 100 X *g* for 10 min, from which a sample was obtained for nucleated cell count and differential. The plasma recovered from this concentration step was administered to control animals in place of granulocytes. Other animals were transfused with packed red blood cells as a second control for the fact that the granulocyte packs contained red cells. Thus, four groups of animals were studied, those receiving methicillin, and those receiving methicillin plus either plasma, packed red blood cells, or granulocytes. Three h following the bacterial inoculation, the pups received a 10 cc/kg transfusion, and after 5 h they received a second transfusion of 5 cc/kg, both via a #23 gauge butterfly needle (Deseret Co., Sandy, UT) inserted into the external jugular vein. The test material was infused over 5 min and flushed with 1 cc of normal saline. In addition, all animals received 0.5 meq $NaHCO_3$ in 1 ml at the time of transfusion.

RESULTS

EXPERIMENTAL STAPHYLOCOCCAL SEPSIS

Pups inoculated with 0.5–1.0 x 10^8 *Staphylococcus aureus*/g body weight developed positive blood cultures within 2 h. Three h after inoculation, all animals ate poorly or not at all, had pulmonary rales, were unable to lift their heads from the cage floor and had no root or suck reflex. Seven of the 8 infected, nontransfused animals died 6–10 h following the inoculation and the remaining animal died on the fifth day. At autopsy examination, all pups had hemorrhagic pneumonia with positive blood and lung cultures. In addition, the animal who survived for 5 days had a positive post mortem cerebrospinal fluid culture. Blood neutrophil counts before and at intervals following bacterial inoculation are shown in Figure 1*a*. A drop in circulating neutrophils was present 2 h following inoculation and by 5–6 h all pups were profoundly neutropenic. The NSP %, measured from marrow aspirates is displayed in Figure 1*b*. NSP diminution occurred in all animals within 2–3 h and persisted or worsened until death. The single animal who survived 5 days exhibited neutrophilia (60,000/mm^3) 24 h after the inoculation and replenished its NSP to 25%.

GRANULOCYTE TRANSFUSIONS

Following the initial mortality studies, subsequent littermates were randomly assigned to receive 15 cc/kg of either adult canine plasma, packed red blood cells or granulocytes. As in the mortality experiments, all animals also received 50 mg/kg methicillin twice daily in order to parallel the clinical situation. The packed red cells contained 8.5 x 10^9 red cells/ml and 0.01 x 10^8 neutrophils/ml. The granulocyte transfusions contained 6.5 x 10^9 red cells and 2.0 x 10^8 neutrophils/ml. The plasma was acellular. In four instances the blood neutrophil count was measured immediately before and 10–15 min following the initial granulocyte transfusion. In two instances the recipient experienced little or no increase in blood neutrophil concentration: from 230/mm^3 to 230/mm^3, and from 330/mm^3 to 530/mm^3. An elevation in blood neutrophil count following transfusion was recorded in the other 2 pups: 280/mm^3 to 1540/mm^3 and 390/mm^3 to 1490/mm^3.

In Table 1, the survival of animals in relation to their transfusion treatment is given. All seven of the animals who received plasma transfusion died; 6 animals between 6–10 h following inoculation and the other at 120 h. Four of 5 packed red blood cell recipients died 8–10 h after inoculation; the other survived. All animals had positive blood cultures and those autopsied had histologic findings of hemorrhagic pneumonia. One of 6 animals who received granulocyte transfusions died ($P < 0.005$). In that all animals in this study received methicillin, the effect of granulocyte transfusion in the absence of antibiotics was not tested. Twenty-four and 48 h following the inoculation, blood neutrophil counts were obtained in the 5 granulocyte transfusion recipients who survived. The 24 h counts ranged from 9,570–31,790/mm^3 ($\bar{x}$, 21,540/mm^3), and the 48 h count from 7,970–28,120/mm^3 ($\bar{x}$, 15,800/mm^3).

Table 1. *Transfusion of septic, neutrophil depleted neonatal dogs*

	Control	Plasma	PRBC	Granulocytes
Number of animals tested	8	7	5	6
Number of survivors	0	0	1	5[1]

[1] $P < 0.005$ *vs* control.

DISCUSSION

The neutrophil storage pool (NSP) is the supply of mature neutrophils held in reserve for release into the circulation in times of increased neutrophil need, such as during bacterial infection (6). In the adult dog (18), as well as in the adult human (4), the NSP contains more than 12 times the number of neutrophils in the total blood neutrophil pool (TBNP) (circulating plus marginated). NSP exhaustion during bacterial infection results in a diminished neutrophil supply to the infected tissues and therefore diminishes the host's ability to survive the infection (18). NSP exhaustion during bacterial sepsis is distinctly uncommon in adults, although it is occasionally seen in immune suppressed or alcoholic patients (20). In contrast, NSP depletion is common in neonates with sepsis. Neonatal rats inoculated with group B *streptococcus* developed neutropenia and depletion of the storage neutrophils (7). In other studies, septic neonatal dogs developed neutropenia, depletion of the storage neutrophils and death (8). Depletion of mature marrow neutrophils in human neonates with sepsis has also been reported (9) and a profound depletion can be correlated with a high likelihood of early death (8).

The reason for this propensity among septic neonates to exhaust their neutrophil reserve is unknown, although one observation has been made which affords a partial explanation. The size of the neutrophil reserve per g body weight is much smaller in the neonate than in the adult (12). For instance, in the adult rat the neutrophil reserve contains about 14 times the number of cells in the total blood neutrophil pool (TBNP); however, in the one–day–old rat the storage neutrophils equal only 2 times the TBNP. When neutrophils rapidly exit the marrow, as occurs during bacterial infection, a small neutrophil storage pool could become depleted more quickly than a large one.

Neutropenia accompanying NSP depletion in septic adults has been treated with granulocyte transfusion, and recently Laurenti *et al.* (16) have suggested that this may also be an efficacious treatment for septic human neonates. Workers have demonstrated that transfused neutrophils can migrate into sites of infection (13), can phagocytose bacteria (19) and can reduce the mortality from sepsis (1, 14). The optimal number of neutrophils for a granulocyte transfusion has not been defined, though a quantity equal to the recipient's total blood neutrophil pool (TBNP) has been suggested as a reasonable goal (3). In the adult dog, the TBNP has been measured at $1.0 \pm 0.3 \times 10^9$ cells/kg ($\bar{x} \pm$ S.E.) (18) and in the adult human at $0.6 \pm 0.2 \times 10^9$ cells/kg (4). In actual practice, one is seldom able to deliver this quantity of neutrophils to adult patients since the TBNP of the donor and recipeint are approximately the same size (15). However, when an adult is the granulocyte donor and a neonate is the recipient, a quantity of cells exceeding one TBNP can be delivered. This was the case in the present study where 3.0×10^9 neutrophils/kg were transfused in only 15 cc/kg. In addition, we have administered quantities of neutrophils exceeding one TBNP to septic human neonates in volumes of 10–15 cc/kg (10). Finally, when we tested the effect of granulocyte transfusion in the present study, the survival of septic, NSP depleted pups was improved.

In conclusion, many neonates with bacterial sepsis experience the phenomenon of exhaustion of the mature neutrophil reserves, and subjects with this disorder are unlikely to survive. Supplying adult neutrophils to these neonates by administering granulocyte transfusion improved the survival in this study. This fact supports the contention that the neutrophil depletion produced in these animals was a factor which contributed to their death and suggests the possibility that granulocyte transfusion might be a useful therapeutic modality in septic, neutropenic, NSP depleted human neonates.

REFERENCES AND NOTES

1. Alavi, J. B., Root, R. K., Djerassi, I., Evans, A. E., Gluckman, S. J., MacGregar, R. R., Guerry, D., Schreibner, A. D., Shaw, J. M., Koch, P. and Cooper, R. A.: A randomized clinical trial of granulocyte transfusions for infection in acute leukemia. New Eng. J. Med., *296:* 206 (1977).
2. Armstrong, D., Zeligs, B. and Bellanti, J. A.: The inflammatory response of the neonatal rat to streptoccal infection. Pediatr. Res., *14:* 554 A (1980).
3. Athens, J. W., Boggs, D. R., Cartwright, G. E. and Wintrobe, M. M.: An evaluation of granulocyte transfusions. Haemat. Latina, *10:* 109 (1967).
4. Bishop, C. R., Rothstein, G., Ashenbrucker, H. E. and Athens, J. W.: Leukokinetic studies, XIV. Blood granulocyte kinetics in chronic, steady-state neutropenia. J. Clin. Invest., *50:* 1678 (1971).
5. Cartwright, G. E.: Diagnostic Laboratory Hematology. Third edition. p. 11 (Grune and Stratton, Inc., New York, 1963).
6. Cartwright, G. E., Athens, J. W. and Wintrobe, M. M.: The Kinetics of granulopoiesis in normal man. Blood, *24:* 780 (1964).
7. Christensen, R. D., Shigeoka, A. O., Hill, H. R. and Rothstein, G.: Circulating and storage neutrophil changes in experimental type II group B streptococcal sepsis. Pediatr. Res., *14:* 806 (1980).
8. Christensen, R. D., Bradley, P. P. and Rothstein, G.: The leukocyte left shift in clinical and experimental sepsis. J. Pediatr., *98:* 101 (1981).
9. Christensen, R. D. and Rothstein, G.: Exhaustion of marrow neotrophils in neonates with sepsis. J. Pediatr., *96:* 316 (1980).
10. Christensen, R. D., Anstall, H. and Rothstein, G.: Neutrophil transfusion in septic, neutropenic neonates. Transfusion, In press (1981).
11. Djerassi, R., Roy, A. Franklin, A., Brivkalns, A.: Filtration leukophoresis in the rat. Exp. Hemat., *2:* 336 (1974).
12. Erdman, S. H., Christensen, R. D., Bradley, P. P. and Rothstein, G.: The supply and release of storage neutrophils: a developmental study. Biol. Neo., in press, (1981).
13. Graw, R. G., Herzig, G., Perry, S. and Henderson, E. S.: Normal granulocyte transfusion therapy: treatment of septicemia due to gram-negative bacteria. New Eng. J. Med., *287:* 367 (1972).
14. Herzig, R. H., Herzig, G. P., Graw, R. G., Bull, M. I. and Ray, K. K.: Successful granulocyte transfusion therapy for gram-negative septicemia. New Eng. J. Med., *296:* 701 (1977).
15. Higby, D. J. and Burnett, D.: Granulocyte transfusions: current status. Blood, *55:* 2 (1980).
16. Laurenti, F., Ferro, R., Isacchi, G., Panero, A., Savignoni, P. G., Malagnino, F., Palermo, D., Mandelli, F. and Bucci, G.: Polymorphonuclear leukocyte transfusion for the treatment of sepsis in the newborn infant. J. Pediatr., *98:* 118 (1981).
17. Lennette, E. H., Spaulding, E. H. and Truant, S. P., Editors: Manual of clinical microbiology. p. 934 (American Society for Microbiology, Washington, D.C., 1974).
18. Marsh, J. C., Boggs, D. R., Cartwright, G. E. and Wintrobe, M. M.: Neutrophil kinetics in acute infection. J. Clin. Invest. *46:* 1943 (1967).
19. McCullough, J., Weiblen, B. J., Deinard, A. R., Boen, J., Fortuny, I. E., and Quie, P. G.: *In vitro* function and post-transfusion survival of granulocytes collected by continuous-flow centrifugation and filtration leukopheresis. Blood, *48:* 315 (1976).
20. McFarland, W. and Libre, E. P.: Abnormal leukocyte response in alcoholism. Ann. Intern. Med., *59:* 865 (1963).
21. Miller, M. E.: Host defenses in human neonate. Monographs in Neonatology. pp 1–2 (Grune and Stratton, Inc., New York, 1978).
22. Morse, E. E., Freireich, E. J., Carbone, P. P., Bronson, W. and Frei, E.: The transfusion of leukocytes from donors with chronic myelocytic leukemia to patients with leukopenia. Transfusion, *6:* 183 (1966).
23. Naeye, R. L.: Underlying disorders responsible for the neonatal deaths associated with low apgar scores. Biol. Neo., *35:* 150 (1979).

24. Requests for reprints should be addressed to: Dr. Robert D. Christensen, Division of Hematology, University of Utah College of Medicine, 50 North Medical Dr., Salt Lake City, UT 84132.
25. This research was supported by a grant from the Thrasher Research Fund and in part by a National Institute of Health Research Training Grant AM-07115.
26. Received for publication March 16, 1981.
27. Accepted for publication June 3, 1981.

0031-3998/82/1601-0057$2.00/0

Printed in U.S.A.