Efficiency of Neutrophil Migration in the Neonate

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Summary

To assess release of neutrophils from the neutrophil storage pool (NSP) and subsequent neutrophil migration into tissue sites, polyvinyl sponge discs were placed into subcutaneous pockets in either newborn or 4-wk-old rats. After 20 hr, the sponges were removed, and the animals' NSP size and the number of neutrophils which had migrated into the sponges were measured. In response to the sponge stimulus, neonatal rats released $83.6 \pm 1.2\%$ (mean $\pm$ S.E.) of their NSP ($106.3 \pm 15.3 \times 10^5$ neutrophils), whereas the older animals released $28.4 \pm 5.0\%$ ($628.5 \pm 124.4 \times 10^5$ neutrophils) of their NSP ($P < 0.001$). Neonatal neutrophils displayed a decreased ability to successfully migrate from the NSP to the sponges. The proportion of released neutrophils accumulating in the sponges was $9.2 \pm 0.6\%$ in the neonates, compared with $58.5 \pm 7.8\%$ ($P < 0.001$) in the 4-wk-old rats.

Speculation

When we applied a weight standardized NSP release stimulus, the neonatal rat released a much greater percentage of its stored neutrophils than did older animals. This fact coincides with recent clinical reports of neutrophil storage pool exhaustion in neonatal septicemia. In addition, the neonatal neutrophil, once released from the neutrophil storage pool, displayed a decreased ability to migrate *in vivo* to a tissue "target" site. These abnormalities may be of importance in understanding the neonates' unusual susceptibility to overwhelming bacterial infection.

It has been postulated that the unusually high morbidity and mortality observed with neonatal sepsis is due to defective host defenses; however, the nature of the defect(s) remains unclear (10). The studies of Miller (9) show that the chemotactic movement of human neonatal neutrophils in modified Boyden chambers is retarded, and it is tempting to postulate that this *in vitro* abnormality also occurs *in vivo*. To evaluate this hypothesis, we have developed an *in vivo* model with which we can quantify neutrophil release from the neutrophil storage pool (NSP) (which in the neonate exists in the bone marrow, liver, and spleen) and the ability of released neutrophils to migrate to a "target" site. Data obtained from this model indicate that the neonate is able to release neutrophils from the hepatic and splenic as well as from the myeloid storage pools. However, once released from the NSP, the neonatal neutrophils displayed a decreased ability to efficiently migrate to a tissue target.

MATERIALS AND METHODS

ANIMALS

Four- to 12-hr-old (5.5 to 6.5 g) or 4-wk-old (55 to 65 gm) albino Sprague Dawley rats (13) were used in these experiments with animals from the same litters serving as controls. The neo-

nates were housed with their mothers. The 4 wk olds were kept 4 to 6 per cage and allowed Simonsen laboratory chow (13) and water *ad libitum*.

QUANTIFICATION OF STORAGE NEUTROPHILS

The NSP was defined as all mature neutrophils [polymorphonuclears, bands, and metamyelocytes] within the bone marrow, and in neonatal rats, also within the spleen and liver. Hepatic and splenic compartments of the NSP were measured after sacrificing the animal (cervical dislocation), by removing the liver and spleen and sieving them through a steel cloth mesh (700 pores/cm²) with 1.0 ml Hanks' balanced salt solution (HBSS). The resulting cell suspensions, after being flushed twice through no. 23 gauge needles were counted electronically (14), and a 1000-cell differential count was performed on Wright's stained smears. The absolute nucleated cell count multiplied by the percentage mature neutrophils (PMN, band, and metamyelocytes) was taken as the NSP cell number for these organs. The bone marrow NSP compartment was measured as follows. The marrow content of both femurs was flushed into HBSS according to the method of Chervenick *et al.* (3). The resulting cell suspension was counted electronically, and a 1000-cell differential count was performed. The relationship of the combined femoral marrow to the total skeletal marrow was determined as follows. All of the bones of six neonatal rats were dissected from their soft tissue attachments and were subjected to sieving in HBSS to produce cell suspensions on which electronic and differential cell counting was performed. Utilizing this technique, the combined femoral marrow was found to constitute $11.1 \pm 1.1\%$ (mean $\pm$ S.E.) of the total skeletal marrow. Therefore, the total body NSP was calculated in individual neonatal rats by summing the absolute neutrophil count of the animal's liver + spleen + nine times the neutrophil content of the combined femurs. In the 4-wk-old rats, the liver and spleen contained no NSP. The combined femoral marrow was calculated to constitute $11.5 \pm 1.6\%$ of the total skeletal marrow, a figure compatible with one obtained utilizing radioactive iron (⁵⁹Fe) distribution in the adult rodent bone marrow of 11.6% (3). Therefore, in the 4-wk-old rats, the total body NSP was calculated by multiplying the combined femoral neutrophil count by 9.

POLYVINYL SPONGE IMPLANTATION

Polyvinyl sponge discs (15) were boiled for 1 hr in water which had been subjected to charcoal filtration and deionization by a Milli Q Filter System (16). Three mg of sponge per g body weight, saturated with sterile HBSS, was placed into surgically created subcutaneous pockets on the dorsum of groups of either newborn or 4-wk-old rats in a fashion similar to that described by Isenberg *et al.* (8). To determine the effect of the surgical procedure alone on NSP release, litters were divided into 3 groups: nonoperated controls, sham-operated control (who were subjected to pocket-creating and closing procedures), and sponge recipients. All animals including nonoperated controls were anesthetized by inhalation of methoxyflurane (17). The entrance of the subcutaneous

pocket was closed with a 4/0 silk suture and sealed with Flexible Collodion, U.S.P. (18).

BLOOD NEUTROPHIL COUNTS

At the time of sponge removal, blood was obtained for nucleated cell count and differential. The external jugular vein was lacerated in the anesthetized neonates, and 10 μ l of blood were drawn into an EDTA coated microcapillary tube. Blood was drawn from the inferior vena cava into an EDTA coated syringe in the 4-wk-old animals. Cover slip smears were made on each animal and stained with Wright's stain and subjected to a 200-cell differential count. Nucleated cell counts were performed electronically (14) in triplicate, and the average was determined.

QUANTIFICATION OF THE NEUTROPHILS MIGRATING TO THE SPONGE

Twenty hr after implantation, the sponges were removed from the subcutaneous pockets by incising the original collodion sealed entrance. This 20-hr incubation interval was selected to correlate with the time of maximal neutrophil appearance in the sponges placed in adult rats by Wiener *et al.* (12). The sponges were then placed into a weighed beaker containing 0.5 ml HBSS, wrung in the solution 10 times, removed, and wrung once more against the beaker wall. The increase in weight of the beaker plus solution was recorded, after which the cell suspension was counted electronically, and a 200-cell differential count performed. In addition, the sponge fluid was centrifuged at $600 \times g$ for 2 min, and soluble DNA was measured in the supernatant according to the colorimetric indole method of Ceriotti (2) to estimate cell lysis within the sponge. Thus, the absolute number of cells, lysed and intact, contained in the sponge was estimated.

RESULTS

NSP SIZE

The total NSP, consisting of all PMN's, band neutrophils, and metamyelocytes in the bone marrow, liver, and spleen of nonoperated control, sham, and sponge-recipient neonatal rats is listed in Table 1. It can be seen that the control neonatal rat NSP was composed of 57.2×10^5 cells contained within the bone marrow, 10.5×10^5 cells in the spleen, and 59.5×10^5 in the liver, totaling 127.2×10^5 neutrophils in the total body NSP. Ten animals subjected to the sham operation experienced diminution of the total body NSP to 90.9×10^5 cells ($P < 0.005$). The sponge recipients' total body NSP was diminished to 20.9×10^5 cells with profound diminution of myeloid, splenic, and hepatic NSP compartments, indicating release of cells from all storage sites in response to sponge implantation. In Table 2, the data for the 4-wk-old group are shown. In these animals, the liver and spleen were no longer neutrophil storage sites and therefore the myeloid NSP was the total body NSP. In the 4-wk-olds, the sham operation resulted in an insignificant diminution of the NSP. Those receiving 3 mg sponge per g body weight, however, experienced a NSP reduction from 2216.3×10^5 (control) to 1587.8×10^5 ($P < 0.001$).

EFFICIENCY OF NEUTROPHIL MIGRATION

To determine the efficiency with which neutrophils released from the NSP migrated to the sponge, the sponge content of neutrophils was measured.

Table 1. NSP alteration with sham operation or sponge placement for neonatal rats (10^5)

Group	N	Bone marrow NSP	Splenic NSP	Hepatic NSP	Total NSP
Control	11	57.2 ± 5.4^1	10.5 ± 1.0	59.5 ± 5.3	127.2 ± 7.1
Sham	10	40.5 ± 5.0	9.6 ± 0.7	40.8 ± 3.3	90.9 ± 5.4
Sponge	21	7.6 ± 0.8	3.2 ± 0.3	10.1 ± 1.0	20.9 ± 1.3
P control vs. sham ²		<0.05	N.S. ³	<0.02	<0.005

¹ Mean $\pm$ S.E.

² For all other comparisons, $P < 0.001$.

³ N.S., not significant.

It seems likely that some of the cells accumulating in the sponge lyse before the sponges are harvested and therefore are not recognized by electronic cell counting. Soluble DNA in the sponge fluid supernate was measured to account for these cells, calculating 6 pg of soluble DNA per lysed cell (2). After the intact cells had been extracted from the sponges, nearly identical quantities of residual DNA were identified in the newborns' (31.8×10^5 pg) and 4-wk-olds' (41.4×10^5 pg) sponges ($P = \text{NS}$). This corresponds to 5.3×10^5 cells which presumably lysed within the sponge in the neonates and 6.9×10^5 cells in the 4-wk-old rats. Thus, by adding the calculated number of cells reaching and lysing within the sponge to the number of intact cells retrieved from the sponge, the total number of PMN's accumulating in the sponge was determined.

The number of neutrophils, both intact and lysed, accumulating in the sponge in the neonates and the 4-wk-old groups is shown in Table 3. Also shown is the number of neutrophils missing (mobilized) from the NSP after sponge placement in the neonates and 4-wk-olds. This figure was calculated by subtracting the NSP in sponge-bearing animals from that obtained in littermate controls (see Tables 1 and 2). The sponge stimulus (3 mg/g body wt) elicited an 83.6% diminution of the NSP in the neonates and a 28.4% diminution in the 4-wk-olds ($P < 0.001$). Efficiency of neutrophil migration to the target sponges was defined as the number of cells reaching the sponges divided by the number of cells missing from the NSP. A neutrophil migratory efficiency of $9.2 \pm 0.6\%$ in 20 neonatal animals is contrasted with an efficiency of $58.5 \pm 7.8\%$ in 11 4-wk-olds ($P < 0.001$).

Examination of the blood of these animals did not disclose the presence of the neutrophils not reaching the lesion. In fact, the absolute blood neutrophil count in neonates diminished from $1830 \pm 220/\text{mm}^3$ (control) to 1120 ± 240 in the sponge recipients ($P < 0.05$), whereas no blood neutrophil count change was observed in the 4-wk-old animals (1170 ± 130 control versus 1230 ± 185 sponge).

Table 2. NSP alteration with sham operation or sponge placement for 4-wk-old rats (10^5)

Group	N	Total NSP
Control	37	2216.3 ± 109.6^1
Sham	19	2137.0 ± 188.0
Sponge	11	1587.8 ± 93.4
	Control vs. sham	$P = \text{N.S.}^2$
	Sham vs. sponge	$P < 0.001$
	Control vs. sponge	$P < 0.001$

¹ Mean $\pm$ S.E.

² N.S., not significant.

Table 3. Efficiency of neutrophil migration in newborn and 4-wk-old rats

Animals	N	Neuts (lysed + intact) migrating to the sponge (10^5)	Neuts mobilized from NSP (10^5)	Efficiency of neut migration (%)
Newborn	21	9.8 ± 0.5^1	106.3 ± 15.3	9.2 ± 0.6
4-wk-old	11	367.6 ± 83.0^2	628.5 ± 124.4^2	58.5 ± 7.8^2

¹ Mean $\pm$ S.E.

² $P < 0.001$ compared with newborn.

DISCUSSION

The mortality rate in human neonates with systemic bacterial infection approaches 50%, a figure which has not been significantly altered in recent years despite major advances in the supportive care and antimicrobial treatment of infected neonates (10). Numerous clinical observations and laboratory investigations support the concept that this susceptibility to overwhelming infection is due to the fact that the neonate is a compromised host (10). One cause of this compromise probably includes the neonate's lack of type-specific bacterial opsonin to group B streptococcus (7) and other organisms (6). In addition, the studies of Miller (9) show that human neonatal neutrophil movement is defective *in vitro*. Although the chemotaxis of neonatal rat neutrophils has not yet been studied *in vitro*, this same predilection toward overwhelming bacterial infection has been observed among the neonates of that species (5). In the present studies, we utilize an *in vivo* neonatal rat model to further explore the developing neutrophil system. The NSP (the mature, nonmitotic neutrophils contained within the bone marrow, spleen, and liver) was measured in groups of nonoperated control, sham operated, and sponge-bearing littermate rats. Mobilization of neutrophils from the NSP and migration of those neutrophils to the implanted sponges were compared in newborn and 4-wk-old animals. It was found that in response to the sponge placement, the neonate can release its NSP including hepatic and splenic fractions. In fact, with an implanted stimulus of 3 mg sponge per g body weight, the neonate released a much greater proportion of its NSP than did the 4-wk-old animal (83.6 versus 28.4% NSP diminution). This exuberant NSP release among the neonatal group resulting in depleted neutrophil stores is reminiscent of the marked NSP depletion reported in fatal neonatal septicemia (4). These observations may cast doubt on the neonate's ability to discriminately release its NSP, producing, under certain conditions, an exuberant neutrophil release resulting in NSP depletion. On the other hand, the difference in NSP size between the neonatal and 4-wk-old rat needs to be considered. Actually, the 4-wk-olds released more than 6 times the stored neutrophils released by the neonate in response to 3 mg sponge per g body weight. However, because the NSP of the 4-wk-old is some 17-fold larger than that of the neonate, the neonates' NSP was depleted but the 4-wk-olds' was not. Obviously, the mechanisms governing NSP release require further definition before it can be determined whether or not the neonate's NSP release system operates appropriately.

For the neutrophil to perform its phagocytic and bactericidal functions, it must be able to reach the inflammatory site, as illustrated by the fact that individuals with deficient neutrophil migration are compromised hosts (1, 11). The neonatal human neutrophil displays an *in vitro* deficiency in chemotaxis (9), and in these studies, we investigated an *in vivo* correlation of chemotaxis, the ability of neutrophils released from the NSP to migrate to subcutaneously implanted sterile polyvinyl sponges. We found that only $9.2 \pm 0.6\%$ of the neutrophils mobilized from the newborns NSP reached the inflammatory stimulus compared with $58.5 \pm 7.8\%$ in 4-wk-old animals. Thus, we postulate that the deficiency in neonatal neutrophil migration observed *in vitro* is

reflected in the *in vivo* state and that this deficiency may constitute a significant factor in the neonates' reduced host defenses. The polyvinyl sponge method of Wiener *et al.* used in this study has proven to be a useful and reliable cell attractant in several studies (8, 12) although its method of action has not been defined. How the sponge releases the NSP cells, why the sponge acts as an attractant, and how it interacts with complement and other serum and cellular factors remain interesting topics for further exploration.

From this investigation, we conclude that the neonate is able to release neutrophils from the hepatic and splenic as well as from the myeloid NSP, but neonatal neutrophils display a markedly decreased ability to migrate from the NSP through the tissues to a target site; they seem to become "lost" en route.

The mechanism of decreased host resistance in neonates is of immediate interest to both the neutrophil physiologist and clinical neonatologist. The present studies represent an approach to the possible identification and understanding of defects in NSP release and neutrophil migration that may prove important as host resistance factors.

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- Received for publication July 11, 1979.
- Accepted for publication February 5, 1980.