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Pre- and Postnatal Development of Granulocytic Stem Cells in the Rat

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Summary

Bacterial sepsis is a relatively common problem in the neonatal period, particularly among prematurely delivered infants. The newborn rat has been widely used as a model for sepsis neonatorum, and in that model incomplete development of the neutrophil system has been postulated to be an important factor predisposing neonates to death from bacterial infection. In this study, that hypothesis was further tested by assessing neutrophil development in rats of various pre- and postnatal ages. Using standard soft agar colony techniques for detecting granulocyte-macrophage progenitor cells [CFU(c)], the number of CFU(c)/g of body weight was seen to increase from $0.5 + 0.1 \times 10^3$ at 19–20 days gestation to $10.5 \pm 0.2 \times 10^3$ at 4 weeks. The anatomic location of CFU(c) changed from totally hepatic at 16 days gestation to almost totally myeloid at 4 weeks. Lastly, the proportion of mature, stored neutrophils/CFU(c) decreased from 2440 ± 40 at 19–20 days gestation to 430 ± 75 at 4 weeks.

Abbreviations

CFU(c), colony-forming unit in culture
PMN, polymorphonuclear leukocyte
M5A, McCoy's 5A medium

Very early in gestation, it is unlikely that a fetus, existing in a sterile environment, requires the antimicrobial neutrophil system. Indeed, although red blood cells circulate in the human fetus before the 5th week of gestation (19), mature neutrophils generally do not appear until after the 12th to 14th week (13) and then exist only in very minute concentration until after birth (16). Because of this late appearance of neutrophils during human gestation, we postulated that infants born prematurely might possess an incompletely developed neutrophil system and that this deficiency might, in part, be responsible for the high incidence of bacterial infection in this group of patients (18). In

the present study, we examined a critical segment of neutrophil development in rats of various pre- and postnatal ages by quantifying the granulocyte-macrophage progenitor cells (11) [CFU(c)]. We determined: 1) the number of CFU(c)/g of body weight, 2) the anatomic location of the CFU(c); and 3) the ratio of mature stored neutrophils to CFU(c).

Experimental animals. Simonson albino Sprague-Dawley rats (Simonson Laboratories, Gilroy, CA) at various ages were tested. Rats at 10, 16, and 19–20 days gestation were delivered by hysterotomy from animals with timed gestations. Other rats were studied either late on the first day of life (12–14 h) or at 1, 2, or 4 weeks of age.

CFU(c) quantification. CFU(c) within the liver, spleen, bone marrow, and blood were quantified in animals anesthetized with methoxyfluorane (Abbott Laboratories, Chicago, IL). The liver and spleen were removed and, using sterile technique, separately subjected to sieving in M5A medium (Grand Island Biological, Grand Island, NY). The absolute number of nucleated cells per organ was calculated using the volume of cell suspension and concentration of nucleated cells measured by electronic cell counting (Coulter Electronics, Hialeah, FL). The cellularity of femoral marrow was determined by flushing the contents of both femurs into M5A, and counting the cells electronically. The cellularity of the entire skeletal marrow was calculated by utilizing the femoral marrow/total skeletal marrow relationship at various ages previously reported (8). The nucleated cell content of blood was determined by electronic counting and assuming a total blood volume of 0.01 ml/g of body weight (17). Sterile suspensions of cells from the spleen, liver, femoral marrow, and blood were added to 1 ml M5A with 15% fetal calf serum, 3% agar (Difco Laboratories, Detroit, MI), 200,000 units/liter penicillin, 200 mg/liter streptomycin, and 3% postendotoxin serum, as a source of colony-stimulating factor (15). As in our previous studies (3, 4), this concentration of postendotoxin serum (3%) promoted maximal colony growth. The postendotoxin serum was obtained 3 h after the intravenous injection of 10 μg *Salmonella typhosa* endotoxin (Difco)/g of body weight in adult rats and was frozen at $-70°C$ until use. Mixtures which contained 25,000, 50,000, and 100,000 cells/ml were plated with 6–10 replicates in 10 × 35 mm plastic dishes (Becton Dickinson and Co, Oxnard, CA) and incubated at 37°C in a 7% CO_2, high humidity atmosphere (Wedco, Inc., Silver Spring, MD) for 7 days, after which colonies of greater than 50 cells were counted using a stereomicroscope. In this way, the absolute number of CFU(c) within each site was determined and by summing the absolute number of CFU(c) from the liver, spleen, skeletal marrow, and blood, the quantity of CFU(c) within the entire animal was calculated. In premature and term newborn animals, the liver, spleen, and marrow from two individuals were combined. Therefore, 20 animals contributed cells for 10 experiments, each with 6–10 replicates. The liver, spleen, and marrow of 1-, 2-, and 4-week-old animals contained a sufficient quantity of cells to make combining organs from two animals unnecessary. However, in order to obtain a sufficient quantity of circulating nucleated cells, the blood samples from 2–10 animals were combined for each blood CFU(c) data point. Again, 10 such points, each with 6–10 replicates were obtained.

Quantification of mature storage neutrophils. The number of storage neutrophils (PMNs, band neutrophils, and metamyelocytes) within the bone marrow, liver, and spleen of animals at various ages was determined as previously described (8). Briefly, 1000–2000 cell differential counts were performed on Wright-stained coverslip smears made from cell suspensions of femoral marrow, liver, and spleen. The absolute number of mature storage neutrophils within these organs was calculated by multiplying the per cent PMNs, bands, and metamyelocytes by the absolute nucleated cells counts.

RESULTS

The number of CFU(c)/g of body weight in rats of various ages is shown in Figure 1. At 19–20 days gestation (term = 21

days), animals had $0.5 \pm 0.1 \times 10^3$ CFU(c)/g of body weight (mean ± SEM), (mean CFUc/animal, 2580; mean weight, 5.1 g). One day following birth, CFU(c) had increased to $1.6 \pm 0.1 \times 10^3$/g (mean CFUc/animal, 9840; mean weight, 6.0 g) and by 1 week there were $2.0 \pm 0.1 \times 10^3$/g (mean CFUc/animal, 20.4 × 10^3; mean weight, 10.2 g). At 2 weeks of age, stem cell number had increased further to $2.8 \pm 0.2 \times 10^3$ (mean CFUc/animal, 68 × 10^3; mean weight, 24.3 g) and 4-week-old rats had $10.5 \pm 0.2 \times 10^3$ CFU(c)/g (mean CFUc/animal, 855 × 10^3; mean weight 81.4 g). At 10 days gestation, no CFU(c) could be detected. Thus, over the period from 1–2 days before birth to 4 weeks of age, the number of granulocyte-macrophage progenitors/g of body weight increased by over 20-fold.

In Figure 2, the anatomic location of the CFU(c) at these various ages is displayed. Prior to the 17th day of gestation, we could not macroscopically identify the spleen [the rat spleen is formed at 15 days gestation (2)] nor could we obtain peripheral blood; therefore, only hepatic and myeloid CFU(c) were measured on day 16 and all CFU(c) were seen to be located within the liver. By 19–20 days gestation, the liver was the predominant site of CFU(c), with smaller numbers in the spleen and marrow. Thereafter, the site of CFU(c) location gradually shifted to the bone marrow and by 4 weeks of age and the marrow contained essentially all of the CFU(c). The blood never contained more than 1% of total body CFU(c) and consequently is not shown in the figure.

The ratio of postmitotic storage neutrophils (PMN, band neutrophils, and metamyelocytes) within the bone marrow, liver, and spleen, to the number of CFU(c) was also determined and is shown in Figure 3. Animals 1–2 days before birth were seen to have a significantly greater ratio of stored neutrophils to stem
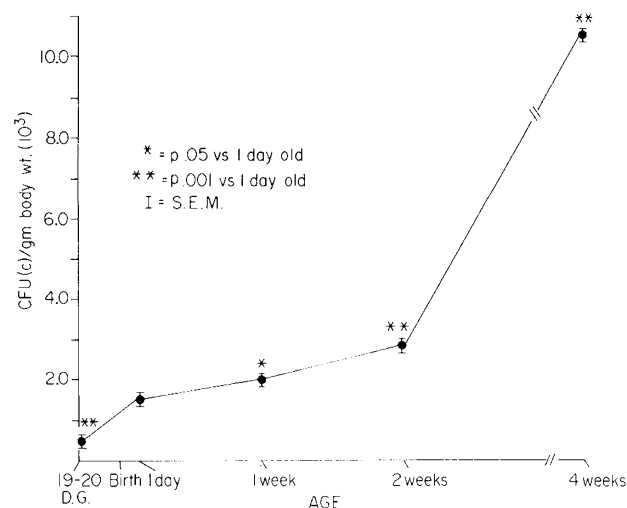

Fig. 1. The number of CFU(c)/g of body weight measured in groups of 10 or more rats at various pre- and postnatal ages: 19–20 days gestation (DG), 1 day after birth, 1 week, 2 weeks, and 4 weeks of age.
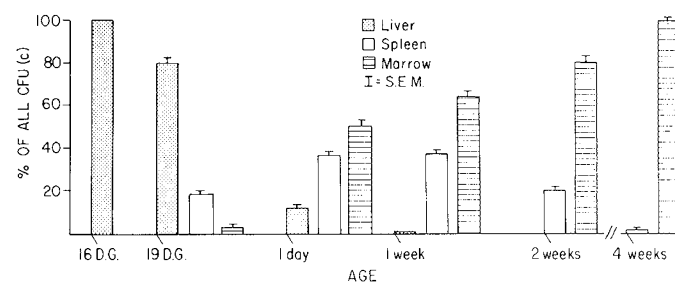

Fig. 2. The anatomic location of CFU(c) in groups of 10 or more rats of various pre- and postnatal ages: 16 days gestation (D.G.), 19 days gestation, 1 day, 1 weeks, 2 weeks, and 4 weeks old.
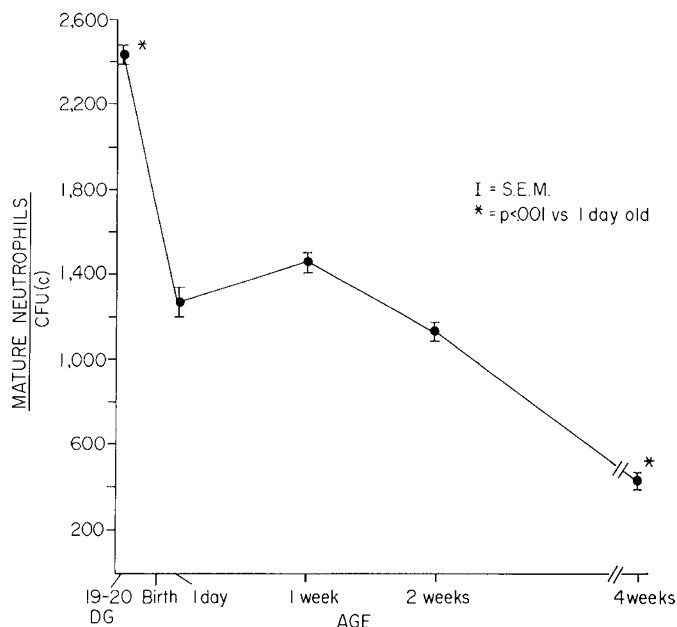
Fig. 3. The ratio of mature, stored neutrophils (PMNs + band neutrophils + metamyelocytes) to CFU(c) in rats of various pre- and postnatal ages: 19–20 days gestation (D.G.), 1 day, 1 weeks, 2 weeks, and 4 weeks of age.

cells, [2440 ± 40 stored neutrophils/CFU(c), than animals at 4 weeks of age (430 ± 75 stored neutrophils/CFU(c)].

DISCUSSION

Analogous to the pulmonary system, the neutrophil system develops late in gestation, yet must be functionally operational by the time of birth if extrauterine existence is to be successful. The extent to which a prematurely born individual is compromised because of an incompletely developed neutrophil system, and the degree of neutrophil development at the time of normal term birth have been the subjects of several reports from this and other laboratories. In those studies, it has been observed that: 1) newborn animals are much more susceptible to death from bacterial infection than are older animals (5, 20); 2) during lethal bacterial sepsis, profound neutropenia and marked depletion of storage neutrophils occurs (5–7, 20); 3) the quantity of stored, mature neutrophils is much smaller in prematurely born animals $(1 \times 10^6/g$ of body weight) than in those at term $(2 \times 10^6/g)$ or at 1, 2, or 4 weeks of life $(5 \times 10^6/g$ at 4 weeks);[8] 4) during bacterial infection in adult rats, CFU(c) rapidly increase in number and in proliferative rate; however, neither the CFU(c) number nor the proliferative rate increases in infected newborn rats (3, 4). The explanation for this latter finding appears to be that the CFU(c) cell cycle in fetal and neonatal rats is already maximally shortened in the noninfected state; therefore, an additional increase in proliferative activity is not possible (4).

In the present studies, we extend these observations by quantifying: 1) CFU(c)/g of body weight; 2) the anatomic location of CFU(c); and 3) the ratio of mature, stored neutrophils/CFU(c) in developing rats of various pre- and postnatal ages. Metcalf and Moore (11) observed that the first CFU(c) to appear in fetal mouse liver do so at 10–11 days gestation (term = 19 days). We could not demonstrate CFU(c) at 10 days gestation in the fetal rat liver (term = 21 days), but at 16 days gestation, $0.5 \pm 0.1 \times 10^3$ CFU(c)/g of body weight were seen. Thereafter, the quantity of CFU(c)/g increased markedly with increasing age, reaching $10.5 \pm 0.2 \times 10^3/g$ at 4 weeks of age, a value comparable to 8.5 $\times 10^3$ CFU(c)/g reported by Lipshitz and Udupa (10) in adult mice and $14 \times 10^3/g$ in adult humans.

We observed that the anatomic location of CFU(c) in the developing rat basically parallels the pattern of maturation de-

scribed by Metcalf and Moore (11) in fetal mice, with the liver containing the first visceral CFU(c), the spleen assuming a myelopoietic role only a few days before term, and skeletal marrow containing very few CFU(c) until following birth. However, one qualitative difference was seen: in the mouse, the spleen continues as a significant source of CFU(c) in adult life (11), yet in the developing rat, only very small numbers of splenic CFU(c) remain after 3 weeks of age. One can see that the change from extramyeloid to myeloid production of neutrophilic stem cells is not complete by the time of term birth, and in fact, myeloid neutrophil production has not even commenced in rats delivered only a few days prematurely. Whether neutrophils produced in nonmyeloid tissue differ from those produced within the marrow, and whether this difference confers any disadvantage or advantage to the host remains to be determined. Similarly, the mechanisms which induce this change in the site of neutrophil production during gestation and postnatal life await description.

Finally, we measured the ratio of mature, stored neutrophils to CFU(c) at various ages and detected marked differences. At 19–20 days gestation, the fetal rat possesses 2440 ± 40 PMN, band neutrophils, and metamyelocytes within the liver, spleen, and skeletal marrow for every CFU(c). This proportion falls after birth, and continues to fall to 430 ± 75 mature neutrophils/ CFU(c) at 4 weeks of age. We speculate that this change may be due to at least two factors. First, the fetal rat, while producing neutrophils, may not yet be utilizing them, because it exists in a sterile environment. Rather, fetal neutrophil production may largely be for the purpose of accumulating a neutrophil reserve in anticipation of the requirement for neutrophils following birth. It can be speculated that a fetus, with a small mass of CFU(c)/g, a very rapid CFU(c) proliferative rate (4), and yet decreased neutrophil utilization, would likely maintain a relatively large proportion of stored mature neutrophils to CFU(c), as we have observed. This is also consistent with observation of Miller et al. (12) that the circulating neutrophil concentration in fetal sheep is very low, frequently with no measurable circulating neutrophils at all until birth, when neutrophilia occurs. Secondly, perhaps the proliferative neutrophils of a fetus and neonate undergo one or two more cell divisions than do neutrophils of older animals. This is supported by our observation that in newborn rats the concentration of myeloperoxidase [a granulocyte enzyme produced only at the promyelocyte stage (1)] is only 27% of that found in the neutrophils of older animals (14). One possible explanation for this finding is that additional cell division(s) in the fetal and neonatal myelocyte divide a fixed quantity of myeloperoxidase between larger numbers of mature neutrophil progeny. Further support for this idea is derived from the work of Grouls and Helpap (9) who injected newborn rats with tritiated thymidine and based on labeling index in the marrow concluded tha an additional cell division likely occurs at the neonate's myelocyte stage.

We conclude that the neutrophil system is not fully developed in either prematurely delivered, or term rats. One to two days before birth, the number of CFU(c)/g of body weight is very small, but growing rapidly, the site at which neutrophils are produced is changing daily, and the relationship of the quantity of mature neutrophils/CFU(c) falls significantly during this fetal to adult transition. In prematurely born subjects, the limitations on neutrophil supply imposed by these and the other previously discussed factors may in part be responsible for the rapid depletion of the neutrophil reserves and the high mortality rate during bacterial infection.

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Increased Regional Myocardial Blood Flows and Oxygen Deliveries during Hypoxemia in Lambs

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Summary

Newborn humans may develop myocardial dysfunction in con-junction with severe birth asphyxia. Subendocardial or papillary muscle ischemia appears to be the basis for the myocardial dysfunction, yet patent coronary arteries have been demonstrated in the nonsurvivors. The asphyxiated newborns also have expe-rienced hypoxemia, which has been suggested as the cause of myocardial ischemia. This study was designed to determine the relationship between reduced aortic blood oxygen content and blood flow as well as oxygen delivery within different regions of the heart. Three days after surgery, we measured blood flow and oxygen delivery to the free walls and papillary muscles of the right and left ventricles, the ventricular septum, and the atria in 10 lambs, during 20-min periods in 8–10% and 5–6% environ-mental oxygen concentrations. In each region of myocardium, blood flow increased linearly as the aortic blood oxygen content decreased. The slopes of the regression lines were similar for each region of ventricular myocardium. Atrial myocardial blood flow also increased as a linear function of the reductions in aortic blood oxygen content, but at a slower rate than in the ventricular regions. Hypoxemia was associated with increased oxygen deliv-ery in each region of myocardium, but the two variables were not related in a linear or quadratic fashion. The results demonstrate that isolated hypoxemia is associated with increased blood flow and oxygen delivery in the free walls and papillary muscles of the right and left ventricles, the ventricular septum, and the atria.

Several reports have demonstrated that newborn humans may develop regional or global myocardial dysfunction in conjunc-tion with severe birth asphyxia (6, 7, 33). Subendocardial and/ or papillary muscle ischemia appears to be the basis for the myocardial dysfunction (4, 12, 30, 33). While coronary stenoses are present in most adults who develop myocardial ischemia, there has been no evidence of coronary stenoses in asphyxiated newborns who have expired after developing severe myocardial dysfunction (4, 12, 30, 33). Consequently, the factor(s) that produce myocardial ischemia during birth asphyxia remain to be determined (33).

Several investigators have suggested that the myocardial ische-mia of birth asphyxia may be the result of the simultaneous occurrence of hypoxemia, with or without additional stresses such as hypoglycemia (4, 21, 30). In contrast, our recent work in unanesthetized fetal and newborn lambs has demonstrated that blood flow to the left ventricular free wall increased during hypoxemia (13, 14). Since right and/or left ventricular ischemia and dysfunction may occur in asphyxiated human newborns, the present investigation was designed to determine the relation-ship between hypoxemia and blood flow as well as oxygen delivery within the papillary muscles and free walls of the right and left ventricles, the ventricular septum, and the atria.

MATERIALS AND METHODS

Surgical technique. Using techniques previously described, we operated on 10 Western newborn lambs 1 to 11 days (5 ± 2,