Age-dependent Change in Size Distribution of Blood Cells in Fetal and Young Mice.

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(Received 22 October 1986/Accepted 23 December 1986)

The mean corpuscular volume (MCV) and the size distribution of circulating blood cells were determined in the fetal mice of C3H/He strain by using a new electric cell size analyser, Coulter Channelyzer C-256. On the 12th day of gestation, the volume of circulating blood cells was distributed between approximately 240–820 fl (mode at 470 fl), and the MCV was 534.9 ± 30.1 fl, i. e., ten to eleven times that of adult one. On the 14th day, two types of cell population were observed; one with smaller cell volume and another with larger one corresponded to that of the blood cells on day 12. Therefore, two peaks were observed to be at 140 and 501 fl in the size distribution curve. The cell population with large volume observed on day 12–14 had been almost disappeared by the 16th day of gestation, and the small blood cells became dominant. The MCV of blood cells was then decreased with the development of fetus, from 188.2 ± 19.3 fl on day 16, to 135.1 ± 7.3 fl on day 18, and 117.5 ± 7.2 fl on day 20. The size of blood cells continued to decrease gradually after birth, and became adult range by 8 weeks after birth. The MCV values of the blood cells were 120.9 ± 8.6 fl, 87.5 ± 6.2 fl, and 48.7 ± 0.8 fl for the newborns of 1 day and 7 days old, and the adult mice, respectively. White blood cells were not separated from the blood samples in this study. However, the size distribution and MCV presented above were appeared to be related essentially to the red blood cells, since the number of white blood cells are negligible small compared with that of the red cells.

The mean corpuscular volume (MCV) and the size distribution of erythrocytes are frequently used in evaluating the hematological condition of animals, and recognized as one of the important and valuable indices in toxicological studies.

The normal values for the size distribution of red blood cells (RBC) have been reported in mice of different ages after birth [1, 13, 14]. Regarding the size of RBC in fetal mice, Russell and her coworkers [2, 14] have reported the diameter of fetal RBC which was measured on blood smears. Kuharcik and Forsthoefel [9] have determined the MCV in the fetal mice of the 18–20th gestational day.

Although it is apparent from their studies that the fetal RBC are more larger in size than those of adult mice, we are not aware of any previous studies illustrating the actual size distribution of RBC in the fetal or embryonic mice throughout the gestation. The reason may be that the amount of blood samples which can be collected from the fetal mice is too small to determine the size distribution of RBC by conventional methods.

Recently a new electric cell counter connected to a size analyser (Coulter Counter Model ZM with Channelyzer C-256) enable us to measure the size distribution of RBC in a tiny volume of blood. The volume of blood

samples which can be collected from the fetal mice is only 2–5 μl per fetus on day 12–14 of gestation, but it is still enough for this analyser to provide the size distribution curve of RBC accurately. The present study was therefore undertaken to establish the normal values for the size distribution of RBC in the circulating blood of fetal mice throughout the gestation.

Materials and Methods

Animals: The animals used were C3H/He mice of 12–17 weeks old, housed in a controlled environment and given food and water *ad libitum*. Vaginal smears were taken daily in order to determine the onset of oestrus at which time the females were paired with males. The appearance of a vaginal plug or the detection of sperm in the smear was taken to mark day one of gestation.

Cell Counter and size analyser: The blood cell counts were performed on the Coulter Counter, Model ZM, with the correction of coincidence. The setting of the counter was as follows: aperture size; 100 μm , aperture current; 0.5mA, attenuation; 2 to 4. The mean of 3 counts was taken as the final count. The size distribution of RBC, expressed in volume (fl), was measured on the Coulter Channelyzer C-256 connected to the above counter. This analyser can provide a cell size distribution curve in volume with 256 channels. The pulse edit facility of Channelyzer was used in this study to edit out mis-shapen pulses due to non-axial cell passage or to coincidence of more than one cell in the aperture at the same time. The calibration of Channelyzer was carried out with RBC of adult male mice, of which MCV had been calculated from the blood cell counts and the microhematocrit values. The MCV calculated was 49.8 fl for ten blood samples of the adult mice. The cell size distribution curve, were also obtained for these samples using the Channelyzer. The mean channel number of the size distribution curve was calculated to be in 26th to 27th channel, and then the Channelyzer was calibrated to give the value of 48.6–50.6 fl in the

26th channel. The accuracy and linearity of the measurement was checked according to the previous report [3] with the standard latex particles with 2–19 μm diameter and the blood cells of rabbits, rat, dogs and human subjects. The accuracy of measurement was also checked routinely with Coulter 4C cell control.

Experimental procedure: At 12, 14, 16, 18 and 20 days of gestation, the dams were killed by exsanguination after the anaesthesia with pentobarbital, and their uteri were removed and washed with physiological saline. Each fetus with an attached placenta was dissected from the uterus and placed on a piece of absorbant paper. The jugular vein of each fetus was cut under a dissection microscope, and 1 or 2 μl of blood was collected in a heparinized disposable pipet. In addition, blood samples were collected by cutting the jugular vein from newborns of 1 day and 7 days after birth and adult mice of 8–14 weeks old.

The blood samples, of which volume was 2 μl per fetus on the 12–18th day of gestation and 1 μl thereafter, were diluted with 50mI Isoton-II solution (Coulter Ltd.), and the blood cell counts were determined by Counter. The samples were then diluted again with Isonton-II to give the cell concentration of 0.5 to 2×10^4 cells per mL, and used to analyse the cell size distribution with the Channelyzer. The size of not less than 0.5×10^4 cells was sorted out into 256 channels. The original data given by the Channelyzer were a total number of cells examined as well as a cumulative number of cells in each channel. After converting the cumulative number of cells in each channel to the percent of the total cell number, the mean and standard deviation in each channel were computed for 10 to 15 individuals in the same experimental group.

Results

Fig. 1 shows the size distribution of circulating blood cells in the fetal mice on day 12 of gestation. In the figure, the solid line and shadowed area denote the mean $\pm$ one standard deviation. The blood cells were large at this gestational stage, and their MCV

Fig. 1. Size distribution curve in fetal RBC on the 12th day of gestation.

Fig. 2. Size distribution curves in fetal RBC on the 14th and 16th day of gestation.

Fig. 3. Size distribution curves in fetal RBC on the 18th and 20th day of gestation.

was calculated to be 534.9 ± 30.1 fl for 13 fetuses of three litters. On the 14th day of gestation, two types of cell population were

observed; one with smaller MCV and another with larger one corresponded to the blood cells of the fetuses on day 12 (Fig. 2). The

Fig. 4. Size distribution curves in mice of different ages after birth.

Table 1. MCV and Blood Cell Counts in Fetal Mice

Gestational age (days)	No. of* fetuses	No. of litters	Blood cell counts ($\times 10^9$ per ml)	MCV (fl)
12	13	3	0.25 ± 0.11	534.9 ± 30
14	11	3	0.48 ± 0.12	412.8 ± 45.9
16	15	3	1.09 ± 0.31	188.2 ± 19.3
18	13	3	2.77 ± 0.86	135.1 ± 7.3
20	15	3	2.98 ± 1.70	117.5 ± 7.2

Values given as mean $\pm$ s. d.

* 3 to 5 fetuses were used in one litter.

Table 2. MCV and Blood Cell Counts in Mice after Birth

Age (days)	No. of mice	Blood cell counts ($\times 10^9$ per ml)	Ht*	MCV** (fl)	MCV (Conv.)*** (fl)
0 (newborns)	4	3.29 ± 0.28	45.2 ± 1.6	120.9 ± 8.64	138.3 ± 12.2
7	10	3.50 ± 0.43	33.5 ± 1.4	87.47 ± 6.23	97.0 ± 12.4
60-90 (adults)	10	7.31 ± 0.85	36.2 ± 1.7	48.72 ± 0.81	49.9 ± 3.8

Values given as mean $\pm$ s. d.

* Ht means microhematocrit values

** MCV determined from the size distribution curves.

*** MCV calculated from the blood cell counts and Ht by the conventional method.

ratio of cell number between these two cell populations varied among the individual fetuses, resulting the size distribution curve with relatively large standard deviation. The cell sizes at the peak of the distribution curve were observed to be 140 and 501 fl. The MCV was calculated to be 412.8 ± 45.9 fl for all the cells, but this value is, of course, meaningless because of the exist of the two cell populations. The large cells which were observed on day 12 had been almost disap-

peared by the 16th day of gestation, and small cells became dominant (Fig. 2). At this gestational stage, the cell size at the peak point of the distribution curve was 143 fl, and the MCV was calculated to be 188.2 ± 19.3 fl for all the cells analysed. Fig. 3 shows the size distribution of blood cells on the 18th and 20th day of gestation. The size of cells became smaller steadily from 135.1 ± 7.3 fl on the 18th day to 117.5 ± 7.2 fl on the 20th day of gestation. The MCV values and blood cell counts

for the fetal mice were summarized in Table 1.

As shown in Fig. 4, the size of blood cells continued to decrease gradually after birth, and became adult range by 8 weeks after birth. The mode of cell sizes were 93, 58, and 47 fl, and the MCV values for all the cells analysed were 120.9 ± 8.6 , 87.5 ± 6.2 and 48.7 ± 0.8 fl, for the newborns of 1 day and 7 days old, and the adult mice, respectively. The size distribution of blood cells was essentially the same between the fetus on day 20 of gestation and the newborn on the 1st day after birth. The MCV values and blood cell counts for the adults and newborns were summarized in Table 2. The range of cell size also became smaller with the development of mice. Ninety percents of total cells were distributed between 38–75 fl in the adult mice, while they were between 240–820 fl in the fetuses on the day 12 of gestation.

Total blood cell counts (cell number/ml blood) are shown in Table 1 and 2. The total blood cell counts were increased with the development of the fetus from 0.25×10^9 per ml on the day 12 of gestation, to 2.98×10^9 per ml on the day 20 of gestation, and 7.31 ± 10^9 in the adult mice. Microhematocrit values in the newborns and adults are also shown in Table 2. In the mice of 7 days after birth, the microhematocrit values were lower than those of the newborns, but recovered gradually to the adult level thereafter. The total blood cell counts and hematocrit values allowed us to estimate the MCV by the conventional method, which are shown as MCV (conv.) in Table 2. Although the MCV (conv.) was a little larger than the MCV calculated from the size distribution curve, there were no significant differences statistically between the two types of MCV at any developmental stages after birth.

Discussion

The origin of fetal red blood cells and their morphology and biochemistry in mice have been the subject of a number of investigations. A few excellent reviews have been published with respect to the mice hematology [1, 14]. Up to the present, however,

the actual size distribution of blood cells has not been determined in fetal mice throughout the gestational period. By using a new electric cell counter with size analyzer, we were able to determine the normal values for the size distribution of fetal blood cells. We also examined the gross morphology of the blood cells by the smear method. However, the results were not presented in this paper, since our morphological observations fully agree with the previous findings [1, 2, 10, 13, 14], and would not be original in themselves.

Circulating white blood cells (WBC) were not separated from the blood samples before the size analysis in the present study. Conceivably the size distribution and the MCV values presented in this study might have been affected by the coexistence of WBC and did not represent those of the RBC. However, it is generally recognized that the white cell production does not start until the 15th day of gestation [1, 12]. This suggests that the WBC neither exists in the blood samples nor responsible for the size distribution and MCV of the blood cells on the 12–14th gestational days. After the 16th day, the WBC becomes discernible in the circulating blood [12]. It has been reported that the number of WBC is 3.4×10^6 /ml on the 17th day of gestation [12], 8.0×10^6 /ml in the newborns [10] and 5.1 – 11.4×10^6 /ml in the adults [1, 14]. These findings indicate that the number of WBC is negligible small compared with that of RBC, i. e., 400–800 times less than that of RBC. Therefore it is reasonable to conclude that the coexistence of the WBC is not significantly affect the results, and that the size distribution and MCV presented in this paper are related essentially to those of RBC.

Three stages of erythropoiesis can be recognized in the developing mouse. The earliest red cells are derived from the blood islands of the yolk sac from the 9th through 12th day of gestation [7, 8]. Russell and Bernstein [14] mentioned that the MCV of the blood cells derived from the yolk sac was four to five times that of the adult erythrocytes. In the present study, however, the MCV of the blood cells in fetal mice on day 12 was determined to be 534.9 fl, which is

ten to eleven times that of the adult erythrocytes. Although the reason for the difference in the size of yolk sac cells between these two studies is not readily apparent, the difference in the methods of size measurements may be responsible, at least in part, since Russell and Bernstein [14] estimated it on the smeared sample.

Craig and Russell [2] found that at 13 to 14 day of gestation two distinct cell populations were circulating in fetal blood. According to their data, the large nucleated cells in fetal blood were 94-100% on the 12th gestational day, 23-37% on the 14th day, and none on the 16th day. These figures are well consistent with our present findings (Fig. 1, 2). The rapid fall in the number of the yolk sac cells coincides with commencement, about 12th gestational day, of hemopoiesis in the liver [6]. The liver is the sole source of RBC from the 12th through the 16th days of development, and continued to be a important site of erythropoiesis up to the end of gestation [7, 11]. Therefore, it is reasonable to assume that the population of the small cells which were observed after 14 gestational days had been originated from the fetal liver.

Kuharcik and Forsthoefel [9] reported that the MCV values of fetal mice on day 18 to 20 of gestation were 123.0 fl in the DP strain mice and 115.0 fl in the C57BL strain. These values are similar to ours of 135.0 fl on day 18 of gestation, and 117.5 fl on day 20. The spleen begins hemopoiesis at the 15th to 16th day of gestation, and marrow hemopoiesis appears near parturition [1]. These sites produce erythrocytes with adult characteristics. On the other hand, the liver continues to produce the intermediate type of RBC which are larger than the adult ones. Therefore, two populations of RBC, i. e., one derived from the liver and another from the spleen and bone marrow, should be circulating in the fetal blood on day 16 to 20 of gestation. However, the two types of cell populations were not observed on the size distribution curves as shown in Fig. 3. The reason may be that the size of RBC derived from the liver is not enough large to be distinguished from the erythrocytes produced in the spleen and

marrow.

The blood cell size, including the MCV values, of mice at early postnatal stages have been investigated with relation to the neonatal anemia. Grunberg [5] used CBA mice and determined their MCV to be 103 fl on the 1st day after birth and 72 fl on the 7th day. A similar result was presented by Grewal [4] in which the MCV values of 109.7 and 76.8 fl were obtained in C3H mice on the 1th and 7th days after birth. Both sets of results are in good agreement with those of the present study. The actual size distribution of RBC in the newborn and adult mice have been obtained by Bannerman [1], while no available data have been reported in the fetal mice. In his study, the peak cell size on the distribution curve were 90.6, 55.7 and 46.7 fl for the newborns of 1 day and 7 days, and the adult mice, respectively. These figures are again in good agreement with those of the present study.

The blood cell counts increased with the development of mice as is shown in Table 1 and 2. Although the mean values of the blood cell counts were slightly lower than those of previous studies [13, 15], there were no significant differences statistically. Microhematocrit values were decreased after birth from 45.2 ± 1.6 in the newborns of 1 day after birth to 33.5 ± 1.4 in the mice of 7 days, then increased to 36.2 ± 1.7 in the adults. This transient dip in the hematocrit values may be attributable to the neonatal anemia as reported by other authors [4, 10, 12].

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胎仔および幼若マウスにおける血球容積の加齢に伴う変化

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胎仔期および幼若期の C3H/He マウスにおける循環血球の平均容積 (MCV) と容積分布を電気的な細胞容積分析装置 (Coulter Channelyzer C-256) を用いて決定した。妊娠12日における胎仔循環血中の血球容積はほぼ 240fl から 820fl の間にあり、最頻値は 470fl, MCV は 543.9 ± 30.1 fl となり、成熟マウスの 10-11 倍の MCV であった。妊娠14日目には、12日目に観察された血球群に加え、比較的小さな血球群が出現し、全体として 140fl と 501fl にピークを有する容積分布曲線が認められた。妊娠16日目になると妊娠 12-14 日目で観察された大きな容積を有する血球群はほとんど消失し、血球は再

び、単一のピークを有する容積分布を示した。その後、血球容積は胎仔の発育に伴い全体として低下し、MCV は16日で 188.2 ± 19.3 fl, 18日で 135.1 ± 7.3 fl, 20日で 117.5 ± 7.2 fl となった。生後1日目の MCV は 120.9 ± 8.6 fl であり、以後、血球容積は成長に伴いさらに低下し、生後7日目では 87.5 ± 6.2 fl となり、生後60-90日目で 48.7 ± 0.8 fl と成体の MCV レベルに達した。本研究では、白血球を血液試料から分離していないが、白血球数は赤血球系細胞の数に比較し無視できるほど少ないことから、得られた容積分布曲線や MCV は主として赤血球系細胞に関するものであると推察される。