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Sex-Related Changes in the Population of Lymphocytes in the Bone Marrow of the Mouse. An Electron Microscope Study*

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Summary. Lymphocytes in the bone marrow were qualitatively and quantitatively examined by electron microscopy in dd-mice of both sexes at various ages. In males, marrow lymphocytes represented 4% of all the nucleated blood cells in the marrow at 4 days of age, 14% at 20 days and 21% at 35 days. From 60 days to one year of age they remained about 8%. In females, marrow lymphocytes were almost similar in proportion to those in males in early life until 35 days, and the proportion of lymphocytes, unlike that in males, remained almost unchanged thereafter until one year of age. Thus marrow lymphocytes were significantly greater in proportion in females than in males after puberty.

The marrow lymphocytes were mainly small lymphocytes. The marrow small lymphocytes could be classified, by ultrastructural features, into two types: dark and light small lymphocytes. Dark small lymphocytes constituted about 90% of all the marrow small lymphocytes in early life in both sexes, but they then underwent a decrease especially in males. Light small lymphocytes were few in early life, but they increased with age in both sexes. The two types of small lymphocytes were discussed in relation to the lymphocyte subpopulations which have been generally accepted.

It has recently been confirmed that the bone marrow produces lymphocytes as well as myeloid elements and that the lymphocytes produced are seeded into peripheral lymphatic tissues as B-lymphocytes (OSMOND and EVERETT, 1964; EVERETT and CAFFREY, 1967; BRAHIM and OSMOND, 1970, 1973; ROSSE, 1971, 1976; GREAVIS et al., 1973) and also that the marrow provides the thymus with precursor cells for thymic lymphocytes (GOLDSTEIN and MACKAY, 1969). About the morphology of marrow lymphocytes, however, little detailed information has been available in the literature. This study was undertaken qualitatively and quantitatively to examine lymphocytes in the mouse marrow by electron microscopy.

Materials and Methods

Eighty-nine normal dd-mice of both sexes were used at 4, 20, 35, 60, 90, 150 days and one year of age. All the mice were maintained on standard pellets (NMF, Oriental Enz. Co., Japan) and water ad libitum under constant environmental conditions. At each age more than five mice were used of each sex. After the animals were killed with chloroform overdose, the femurs were rapidly removed, cleaned of the surrounding tissues and cut lengthwise with razor blades to expose the marrow. Then, pieces of the bone with the exposed marrow were placed in 5% formalin in 0.1M phosphate buffer, pH 7.5, at 4°C for 48 hrs. The marrow was then removed

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Fig. 1. Schematic diagram showing the way to select fields for photography. As seen in this figure, when six holes of specimen grid are covered by ultrathin section (dotted area), photographs are taken at 1, 2 and 3.

from the bone and postfixed in 2% OsO_4 in the same buffer for 2 hrs. After dehydration in a graded series of ethanol, pieces of the marrow tissue were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy.

Quantitative methods

Fields for photography were taken at random as shown in Figure 1. The differential counts of nucleated hemopoietic cells were made on micrographs taken at original magnification of 1,000 times and enlarged photographically to final magnification of 3,500 times. For each

case 900 or more nucleated blood cells were counted and the percentages of each hemopoietic cell series were determined. Macrophages, fat cells, reticular cells, endo-

Fig. 2. Part of the bone marrow. Various series of hemopoietic cells are seen throughout. Lymphocytes (L) are diffusely distributed. $\times 2,500$

thelial cells and non-nucleated blood cells, such as erythrocytes, reticulocytes and platelets were not included in the count. The nucleus:cell ratio of marrow small lymphocytes was determined by the point counting method. A transparent plastic sheet with a regular point lattice was superimposed on electron micrographs enlarged to a magnification of 6,000 times. The points were regularly distributed so that two neighboring points were $1\text{ }\mu\text{m}$ apart at the magnification. For each case the numbers of points lying on the nucleus and cytoplasm were counted on 60 small lymphocytes randomly selected. Statistical significance of differences between each group was evaluated by Student's *t*-test.

Results

A. General Observations

In the bone marrow, free nucleated cells packed within the stromal meshes of reticular cells are composed of various series of hemopoietic cells, such as erythroid cells, granuloid cells, lymphocytes, megakaryocytes and plasma cells. The majority of the free cells are erythroid and granuloid cells and lymphocytes. Erythroid and granuloid cells are generally distributed singly or in small groups, and lymphocytes are scattered diffusely throughout the marrow (Fig. 2). Neither lymphoid nodules nor germinal centers are observed in the mouse marrow. Marrow lymphocytes are

Fig. 3. Part of the bone marrow, showing two types of small lymphocytes. Three dark small lymphocytes (*d*) and one light small lymphocyte (*l*) are observed. $\times 6,000$

mainly small lymphocytes, and larger lymphocytes occur only in small numbers. By electron microscopy, marrow small lymphocytes can be divided into two types; dark and light small lymphocytes (Fig. 3).

Dark small lymphocytes

Dark small lymphocytes have a rounded nucleus, 4–5 μm in diameter, with a smooth outline. The chromatin materials are condensed in clumps, especially immediately inside the nuclear envelope and around the nucleolus. One or two nucleoli show a ring-like form with a diameter of about 1 μm . By the nuclear characteristics, small lymphocytes can easily be distinguished from the other hemopoietic cells in the marrow. The cytoplasm appears as a thin rim around the nucleus. It contains relatively large amounts of free ribosomes which are distributed throughout the cytoplasm. The Golgi apparatus is poorly developed and round or elliptical mitochondria are relatively few in number. The smooth endoplasmic reticulum is generally sparse. Multivesicular bodies and small lipid droplets, though few in number, are observed. The nucleus:cell ratio of dark small lymphocytes is 0.71 ± 0.09 .

Light small lymphocytes

The nucleus of light small lymphocytes is usually rounded with a diameter of 4–5 μm , but the nuclear membrane is occasionally indented. The chromatin materials are aggregated in clumps around the nucleolus and along the nuclear membrane. The cytoplasm of light small lymphocytes is paler and more abundant than that of dark small lymphocytes. Free ribosomes are scattered sparsely throughout the cytoplasm. The Golgi apparatus and mitochondria are generally more prominent than those in dark small lymphocytes. The endoplasmic reticulum, lysosomes and multivesicular bodies, though poorly developed, are also seen in the cytoplasm. Tubular complexes, an accumulation of tubules measuring about 50 nm in diameter, sometimes occur in the cytoplasm of light small lymphocytes. The nucleus:cell ratio of light small lymphocytes is 0.56 ± 0.1 , and it is significantly smaller than that of dark

Fig. 4. A larger lymphocyte. The nucleus with dispersed chromatin is larger than that of a small lymphocyte and the relatively abundant cytoplasm appears dark. $\times 8,000$

small lymphocytes. As the numbers of counting points lying on the nucleus are almost equal in both dark and light small lymphocytes, the difference of the nucleus:cell ratio between dark and light small lymphocytes indicates that the cytoplasm is significantly more abundant in light lymphocytes than in dark ones.

Larger marrow lymphocytes

Larger lymphocytes number only about 3% of marrow lymphocytes. The nucleus is usually rounded in outline, measuring 5–7 μm in diameter. The nuclear chromatin is not so dense as that of small lymphocytes. The cytoplasm appears relatively dark and

is packed with abundant ribosomes (Fig. 4). Ribosomes occur not only as monoribosomes but also as polyribosomes. Other organelles, such as the Golgi apparatus, mitochondria and endoplasmic reticulum, are poorly developed.

B. Quantitative Observations

Frequency of lymphocytes

The nucleated hemopoietic cells in the marrow are classified into five groups: erythroid, granuloid, megakaryocytes, plasma cells and lymphocytes. The proportions of each group of the nucleated cells in both sexes at various ages until one year are presented in Figures 5 and 6. In males, as seen in Figure 5, lymphocytes represent 4.2% of all the nucleated hemopoietic cells at 4 days and 14.4% at 20 days. The percentage attains its maximum (21.4%) at 35 days, but it is decreased to 8.2% at 60 days. Lymphocytes remain almost unchanged thereafter. In females, lymphocytes constitute 5.8% of the nucleated cells at 4 days, 17.6% at 20 days and 15.3% at 35 days, respectively (Fig. 6). After 35 days they remain almost constant in percentage (14.9–16.6%).

Thus, the proportion of marrow lymphocytes increases equally in both sexes early in life until 20–35 days. Thereafter, however, the difference becomes apparent in proportion of lymphocytes between the two sexes. The proportion of lymphocytes decreases until 60 days and then remains at a relatively low level in males, whereas in females the percentage of lymphocytes remains almost unchanged until one year. Thus, from 60 days to one year the proportion of lymphocytes is significantly higher in females than in males ($p < 0.01$) (Fig. 7).

Erythroid cells account for the majority of marrow cells during early life in both sexes, but then show a progressive decrease with age. In both sexes from 35

Fig. 5. Frequencies of hemopoietic cells in the bone marrow in males at various ages. Bars represent standard deviations. *d* days, *y* year.

days to one year, granuloid cells are higher in proportion than erythroid cells. Megakaryocytes comprise only 0.2–0.7% of the marrow nucleated cells at each age. Plasma cells are present in very small numbers in the marrow, but they appear to increase slightly with advancing age.

Proportions of dark and light lymphocytes

The proportions of dark and light small lymphocytes in both sexes at various ages are shown in Figures 8 and 9.

In males, as seen in Figure 8, dark small lymphocytes constitute more than 90%

Fig. 6. Frequencies of hemopoietic cells in the bone marrow in females at various ages. Bars represent standard deviations. *d* days, *y* year.

Fig. 7. Percentages of marrow lymphocytes in both sexes at various ages. Means with standard deviations at each age are indicated. *d* days, *y* year.

of marrow small lymphocytes during early life until 35 days. After 35 days, however, they decrease rapidly until 60 days when they make up 32.9% of the small lymphocyte population in the marrow. Thereafter dark small lymphocytes remain almost steady in proportion. On the other hand, light small lymphocytes are relatively few in early life, but they increase gradually with age. From 35 days to one year, they form a greater proportion (55.3 to 78.1%) of the marrow small lymphocytes.

In females, as presented in Figure 9, the majority of marrow small lymphocytes are dark small lymphocytes in early life until 35 days. Then dark small lymphocytes decrease progressively. After 60 days, however, the decrease of dark small

Fig. 8. Frequencies of marrow small lymphocytes in males. Bars indicate standard deviations of the total proportions of small lymphocytes. *d* days, *y* year.

Fig. 9. Frequencies of marrow small lymphocytes in females. Bars indicate standard deviations of the total proportions of small lymphocytes. *d* days, *y* year.

lymphocytes is not so marked in females as in males. Even at one year dark small lymphocytes account for 35.6% of the total small lymphocytes. On the other hand, light small lymphocytes undergo a gradual increase with age. In females older than 150 days, they are more than half of the total marrow small lymphocytes.

Larger lymphocytes increase in early life, but they decrease gradually after 20–35 days. In adults older than 60 days, the average proportion of larger lymphocytes is about 0.2%.

Discussion

In the bone marrow, it may be not always easy to identify lymphocytes with certainty under the light microscope. However, electron microscopy permits one to differentiate lymphocytes readily from the other hemopoietic cells. The proportion of marrow lymphocytes thus observed is low in both sexes in early life, but then increases rapidly until puberty is attained at about 35 days. After puberty, marrow lymphocytes are different in proportion between the two sexes. In males, the proportion of marrow lymphocytes shows a decline after 35 days, and then, from 60 days to one year, it remains less than half of the maximum value which has been attained at 35 days. In females, on the other hand, marrow lymphocytes remain almost unchanged after reaching maximum at 20–35 days. Thus, the proportion of marrow lymphocytes shows significant sex difference in adult mice older than 60 days. Since the sex difference in proportion of marrow lymphocytes becomes apparent after puberty, it is likely that the difference is due to the gonadal development.

Postnatal changes in immune functions have been suggested to occur depending on age and sex (ROWLEY et al., 1968; MICKLEM et al., 1973; WALFORD, 1974). However, little information is available on the morphologic changes of the immune system. As is generally known, the thymus undergoes involution after puberty. In the mouse, age involution of the thymus is more striking in males than in females (ITO and HOSHINO, 1961, 1963; SCHERZER et al., 1963). The sex difference in proportion of marrow lymphocytes appears similar in pattern to that in age involution of the thymus. It has previously been confirmed that the sex difference in the thymus involution is caused by the existence of the testis but not by that of the ovary (ITO and HOSHINO, 1963). At any rate, it is of interest that the marrow lymphocyte population which can be regarded as representing the primary immune elements, exhibits, like the thymus, primary or central lymphatic organ, significant sex differences. The cause of sex differences in marrow lymphocytes will be dealt with in a separate paper (SASAKI and ITO, in preparation).

Recently small lymphocytes in lymphatic tissues including the bone marrow have been classified by electron microscopy into two forms, dark and light (ABE et al., 1973; PEREIRA, 1974). As seen in the present results, marrow small lymphocytes which constitute about 97% of all the marrow lymphocytes are also classified into dark and light forms. The proportions of the two types of small lymphocytes show age-related changes. In early life until puberty, dark small lymphocytes form a vast majority of the marrow small lymphocytes, while light small lymphocytes are very few, in both sexes. After puberty, as the sex difference in proportion of lymphocytes becomes apparent, dark small lymphocytes decrease and light ones increase with advancing age. The decrease of dark small lymphocytes proceeds more markedly in males than in females. The selective decrease of dark small lymphocytes

seems to cause the decrease of marrow lymphocytes in males.

At present it is generally accepted that there are at least two populations of lymphocytes, thymus-derived (T) lymphocytes and bone marrow-derived (B) lymphocytes, which differ in immune function, life span, circulatory pathways and distribution patterns (review: GREAVES et al., 1973). Lymphocytes of the two lines can generally be distinguished by their specific surface characteristics such as surface antigens or receptors. The proportion and distribution of the two populations of lymphocytes have been reported in peripheral lymphatic tissues (RAFF, 1971; STUTMAN, 1972; GOLDSCHNEIDER and MCGREGOR, 1973; ROSSE, 1976). In the bone marrow, three types of lymphocytes have been identified by the surface antigenic markers: B-lymphocytes, T-lymphocytes and null lymphocytes bearing neither B nor T cell marker (review: ROSSE, 1976). In the marrow, the population size of T-lymphocytes is small (GOLDSCHNEIDER and MCGREGOR, 1973; BALCH and FELDMAN, 1974; RYSER and VASSALLI, 1974), and small lymphocytes with B-lymphocyte characteristics form a substantial population, although surface antigenic markers are not detectable when B-lymphocytes have been newly formed (OSMOND and NOSSAL, 1974a, b; RYSER and VASSALLI, 1974). Null lymphocytes devoid of definite surface markers also appear as small lymphocytes, and they are regarded as belonging to the most immature form (GOLDSCHNEIDER and MCGREGOR, 1973; OSMOND and NOSSAL, 1974a, b).

It remains to be determined to which population each of the two types of small lymphocytes, dark and light, confirmed by electron microscopy would correspond. Dark small lymphocytes in the marrow are ultrastructurally similar to thymic cortical small lymphocytes (ABE and ITO, 1970; ABE et al., 1973). Thus it seems likely that dark small lymphocytes in the marrow may, like thymic cortical small lymphocytes, be short-lived in nature and that they may be included into newly formed immature lymphocytes. It has been reported that immature small lymphocytes lacking surface characteristics in the marrow constitute about half of the entire population of marrow lymphocytes (GOLDSCHNEIDER and MCGREGOR, 1973; OSMOND and NOSSAL, 1974a, b). In adult mice, as shown in the results, dark small lymphocytes occupy almost half of all the marrow lymphocytes. Thus dark small lymphocytes in the adult marrow appear to be almost similar in proportion to immature small lymphocytes. Light small lymphocytes in the marrow morphologically resemble thymic medullary small lymphocytes, which are thought to differentiate into T-lymphocytes (ABE and ITO 1970). Thus, they may be included in T-lymphocytes. However, it has been known that T-lymphocytes contained in the marrow are very few during the entire life (GOLDSCHNEIDER and MCGREGOR, 1973; BALCH and FELDMAN, 1974; RYSER and VASSALLI, 1974). Therefore all the light lymphocytes in the marrow would not be T-lymphocytes. Recently it has been demonstrated that the marrow contains not only long-lived T cells but also long-lived B cells and that the latter forms increase with advancing age (MILLER and OSMOND, 1975). It is possible, therefore, that light small lymphocytes may represent long-lived B-lymphocytes as well as T-lymphocytes and that they may be more differentiated than dark lymphocytes as known in the case of the thymus.

In the bone marrow, the existence of a pluripotent hemopoietic stem cell has recently been confirmed (reviews: YOFFEY, 1973; ROSSE, 1976). The stem cells have not yet been morphologically identified, but they are generally considered to be very similar in appearance to marrow lymphocytes. Stem cells in the marrow have been

estimated to constitute only about 0.1% of the nucleated cells (Dicke et al., 1973). Therefore, even if stem cells in the marrow are included in lymphocytes, they would be only a very small fraction of the total lymphocyte population, and age-related changes in proportion of marrow lymphocytes would not essentially be affected by the content of stem cells.

In addition to small lymphocytes, the marrow also contains larger lymphocytes, referred to as transitional cells, which have been thought to be progenitors of small lymphocytes (OSMOND and EVERETT, 1964; OSMOND, 1967; YOFFEY and COURTICE, 1970; ROSSE, 1973; YOFFEY, 1974). In the mouse marrow, larger lymphocytes, which are similar in morphology to transitional cells, are also observed. The larger lymphocytes, though relatively few, appear to be similar in the course of frequency in life to dark small lymphocytes. It remains uncertain, however, whether or not larger lymphocytes may be really precursors of small lymphocytes.

Other hemopoietic cells will be briefly referred to here. Many qualitative and quantitative studies have been made on hemopoietic nucleated cells in the bone marrow for various mammals (SABIN et al., 1936; BURKE and HARRIS, 1959; FAND and GORDON, 1957; HARRIS and BURKE, 1957; HUDSON et al., 1963; MILLER and OSMOND, 1974; BJÖRKHOLM and ERNSTRÖM, 1975; WICKRAMASINGHE, 1975). The proportions of various lines of the nucleated cells appear to vary more or less from species to species. In the mouse marrow, erythroid cells form the greatest proportion of the nucleated cells in early life and then tend to decrease gradually with age. Granuloid cells and plasma cells tend to increase gradually with advancing age. Megakaryocytes are very few throughout life. These results obtained in the mouse marrow are generally in accordance with those ones of earlier studies.

マウス骨髓リンパ球の出現頻度の性差—電子顕微鏡による研究

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生後各期の雌雄両性 dd-マウスを用いて、骨髓リンパ球を電子顕微鏡レベルで観察した。

雄性マウスで骨髓血液有核細胞に対するリンパ球の比率は、生後4日で4%，20日で14%，35日で21%であり、以後減少し、60日から1年の齢期で約8%の値をとる。一方、雌性マウスの骨髓リンパ球は、生後4日で6%であり、20日から1年の齢期で15~18%の値をとり、その出現頻度は60日齢以後 雄性と比べて有意に高い。

骨髓リンパ球の約97%は小リンパ球であり、骨髓小リンパ球は超微形態のうえで2型、すなわち暗調小リンパ球と明調小リンパ球とに区別される。暗調小リンパ球は生後早期から35日まで雌雄両性ともに小リンパ球の90%を占めるが、以後 雌雄ともに減少し、とくに雄性では著明な減少を示す。一方、明調小リンパ球は 雌雄両性ともに加齢とともに増加する。

これら暗、明2型の骨髓小リンパ球の機能的意義を、最近唱えられているリンパ球の免疫機能や幹細胞との関連のもとに考察した。

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