Ultrastructure and Cytometry of Monocytes and Lymphocytes of Mouse Peripheral Blood

Kazunobu SASAKI and Katsumi KIDA*

Department of Anatomy, Kawasaki Medical School, Kurashiki 701-01, Japan
**Department of Anatomy, Hokkaido University School of Medicine, Sapporo 060, Japan*

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ABSTRACT. Monocytes and lymphocytes in the buffy coat of the peripheral blood of adult mice were qualitatively and cytometrically examined by light and electron microscopy. Clear distinction between lymphocytes and monocytes could not be made in cell size by light microscopy. Two monocyte subgroups could be defined on the basis of the cell diameter: small and large monocytes. Blood lymphocytes consisted of granular lymphocytes and non-granular lymphocytes, and the latter could be divided into two subgroups: those with high N-C (nuclear-cytoplasmic) ratios and low N-C ratios. Non-granular lymphocytes with low N-C ratios and granular lymphocytes were similar in cell size distribution to monocytes, and large monocytes and granular lymphocytes were largely overlapped in the distribution of N-C ratios. The N-C ratio as well as the cell size is not the first criterion for identification of monocytes in mice. However, the occurrence of subsurface vacuoles and the abundance of cytoplasmic granules in monocytes were useful ultrastructural features for the distinction between mononuclear cells in the peripheral blood.

Key words: monocytes — lymphocytes — peripheral blood — ultrastructure — mouse

Blood monocytes belong to a heterogeneous family of cells constituting the mononuclear phagocyte system, MPS, which includes promonocytes in bone marrow and macrophages in tissues. Monocytes in the peripheral circulation are an immediate precursor of tissue macrophages in mature animals.[1] In comparison with lymphocytes and granulocytes in the circulation, monocytes are classically described as the largest leukocytes. It has been reported that human peripheral blood monocytes are not homogeneous in size and contain at least two subpopulations designated 'small' and 'large' monocytes.[2-5] Since the lymphocyte population of peripheral blood has been known to contain a small amount of larger lymphocytes possessing cytoplasmic granules,[6] some difficulties are encountered in making a distinction between monocytes and larger lymphocytes in smears. Ultrastructural observations on blood monocytes have been made in experimental animals as well as in humans.[7-10] However, little morphological information is available for distinction between peripheral blood mononuclear cells. The purpose of the present study is ultrastructurally

佐々木和信，木田勝己

and cytometrically to investigate monocytes and lymphocytes in the circulation of adult mice and to compare monocytes with macrophage precursors during an early embryonic life.

MATERIALS AND METHODS

Twelve mice of dd-strain from 90 to 120 days of age were used. Under chloroform anesthesia, half to one ml of blood was drain via cardiac puncture into plastic syringe containing heparinized physiological saline. Anticoagulated blood was diluted with an equal volume of 0.9% (w/v) NaCl in a test glass tube, 5 mm in caliber, and centrifuged at approximate 1000 $\times$g for 15 minutes. The supernatant plasma was removed by aspiration with a Pasteur pipette, and 4% paraformaldehyde-5% glutaraldehyde in 0.1 M cacodylate buffer (Karnovsky's fluid) was layered onto the buffy coat. In order to harden the buffy coat layer enough for preparation procedure, fixation took place at 4°C for 48 hrs, and, after cutting the tubes just above the level of the buffy coat, the solidified white layer was removed and processed for light and electron microscopy.

For *light microscopy*, whole buffy coat was embedded in methacrylate (HPMA, TAAB). Vertical sections were cut at 1 μm with glass knives on the microtome (Yamato, Japan) for semithin sections, and stained with May-Grünwald-Giemsa. For cytometrical analysis, 1 μm-sections were televised on a monitor, and mononuclear cells in the buffy coat were examined from the platelet layer down to the erythroid layer using an oil immersion objective ($\times$100). One hundred and fifty mononuclear leukocytes were randomly selected per one mouse and traced on transparent plastic sheets at a magnification of $\times$2,800. The cell and nucleus areas were measured with the aid of a computer-coordinating area-curve meter. The cell diameter was computed from the cell profile area, and the nuclear-cytoplasmic ratio (N-C ratio) was obtained from the nucleus area and cell profile area. Seven hundred fifty cells were observed from five mice for obtaining the distribution of cell diameter. In order to examine the size of neutrophils, the outlines of one hundred neutrophils were also traced.

For *electron microscopy*, the buffy coat hardened after fixation in Karnovsky's fluid was cut into small pieces, post-fixed in 2% OsO_4 in the same buffer for 2 hrs and immersed in 0.5% uranyl acetate overnight. After dehydration in a graded series of ethanol, small pieces of the buffy coat were embedded in Quetol 812. Ultrathin sections were prepared on a Reichert OM-U3 ultratome, stained with lead citrate and examined by electron microscopy. For cytometrical analysis, 66 monocytes and 111 lymphocytes were examined on electron micrographs enlarged at a final magnification of $\times$15,000. In order to obtain the cell diameters and N-C ratios of lymphocytes and monocytes, the outline of the cells whose nuclei over 3.0 μm in diameter were traced on transparent plastic sheets from photomicrographs. The statistical differences of values obtained were evaluated by Student's t-test.

RESULTS

Light microscopic observations

The buffy coat, settling on the top of erythrocytes, consisted of platelets and leukocyte layers containing a mixture of granular leukocytes and mononuclear leukocytes including lymphocytes and monocytes (Fig. 1). Based

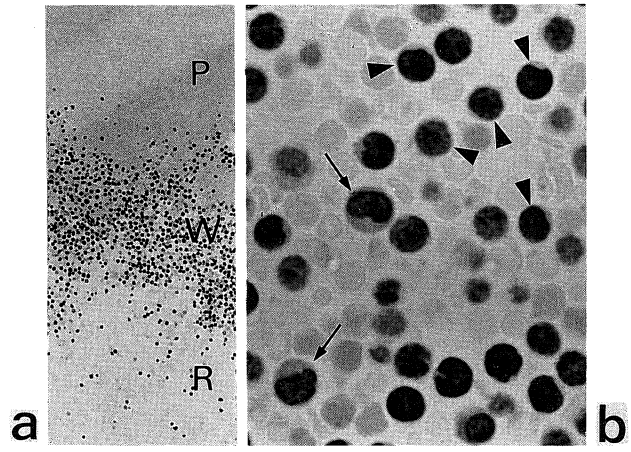

Fig. 1. Light micrographs of a buffy coat. May-Grünwald-Giemsa. a. A low-power micrograph. P : platelet layer, R : red blood cell layer, W : white blood cell layer. ×100. b. A high-power micrograph. Typical monocytes (arrows) with kidney-shaped nuclei and small lymphocytes (arrowheads) with scanty cytoplasm. ×1,000.

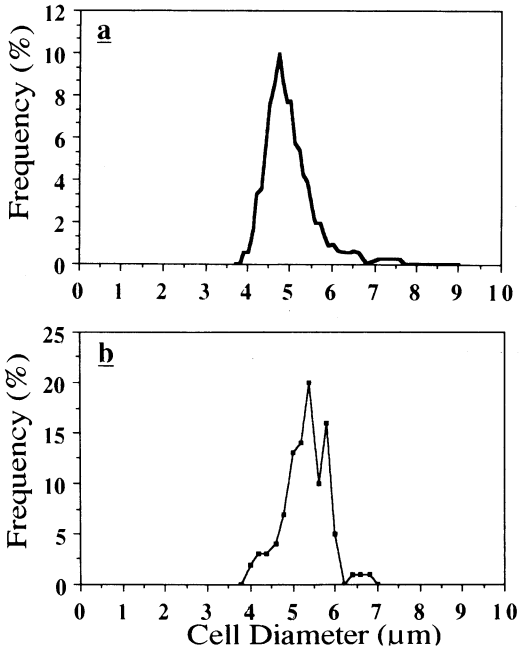

Fig. 2. Distribution of the cell diameter of mononuclear cells (a) and neutrophils (b) in a buffy coat.

on the nuclear morphology and staining characteristics of granules, it is easy to distinguish cells of the granulocyte series from mononuclear leukocytes. The cell size distribution curves of mononuclear cells and neutrophils were shown in Fig. 2. The average cell diameter was 5.3±0.5 μm in neutrophils and 4.7± 0.5 μm in mononuclear leukocytes. Neutrophils were significantly larger in cell diameter than mononuclears ($p<0.01$). Mononuclear cells ranged from 4 to 9 μm in cell diameter, with a peak incidence at 4.7 μm. They contained a small population larger in cell diameter than 6.0 μm. A large proportion of mononuclears were small lymphocytes with round nuclei and narrow rim of

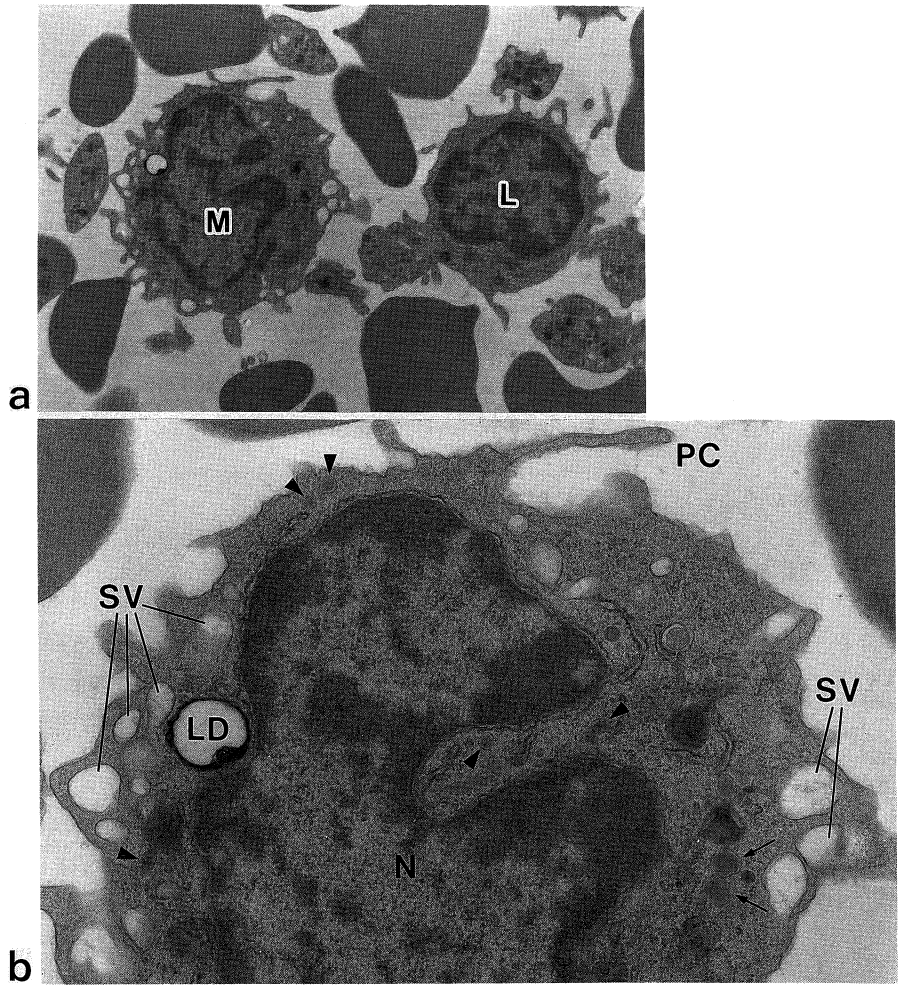

Fig. 3. Electron micrographs of a monocyte (M). a. Compared to a small lymphocyte (L), the monocyte is larger and its cell surface is more complex. The monocyte contains many subsurface vacuoles at the cell periphery. The vacuoles are variable in size and shape. ×4,800. b. Two kinds of granules, large (arrows) and small (arrowheads), are scattered throughout the cytoplasm of the monocyte. LD : lipid droplet, N : nucleus, PC : projection of cytoplasm, SV : subsurface vacuole. ×16,000.

clear cytoplasm without granules (Fig. 1b), and some of small lymphocytes had wide cytoplasm containing a few granules. Monocytes possessed a wide cytoplasm, and their eccentrically positioned nuclei were round or reniform in shape (Fig. 1b). Monocytes frequently contained small numbers of azurophilic granules and a few clear round vacuoles, 0.4–0.5 μm in diameter, in the cell periphery. Since monocytes displayed considerable variation in size, it was often difficult to distinguish monocytes from lymphocytes on the basis of their size and appearance on 1 μm-sections in light microscopy.

Ultrastructure of mononuclear leukocytes

The mononuclear leukocytes essentially consisted of lymphocytes and monocytes. Although the peripheral blood contained plasma cells, the number was negligible.

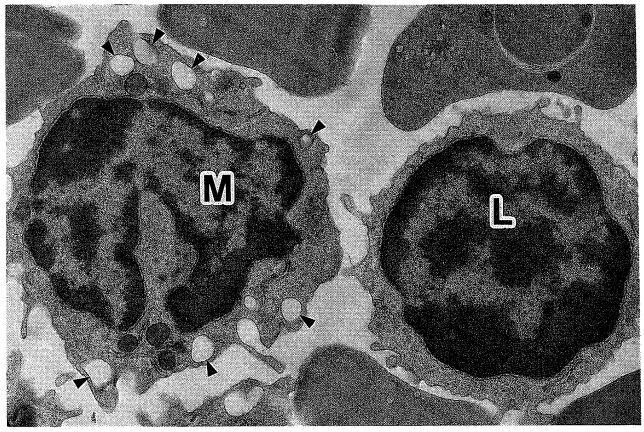

Fig. 4. A small monocyte (M) and a lymphocyte (L) with narrow cytoplasm. The monocyte has many subsurface vacuoles (arrowheads). The size difference between both cells is not obvious. ×7,000.

1. Monocytes

Monocytes were generally round, and their nuclei were kidney-shaped or deeply indented (Fig. 3a). Some nuclei were deeply indented, showing two or three profiles of nuclear masses in ultrathin sections. The nuclei were situated eccentrically with a thick margin of heterochromatin and contained ring-shaped nucleoli with a thin rim of heterochromatin. The cell surface showed a small number of short microvillous projections, approximately 0.5 μm long. Monocytes possessed subsurface vacuoles which were one of the most prominent features that distinguished monocytes from lymphocytes (Figs. 3 and 4). The vacuoles were round, 0.2–0.5 μm in diameter, or elliptical, with a short axis of 0.3–0.4 μm and a long axis of 0.5–1.0 μm. The vacuoles generally occurred in groups at the cell periphery. The counts of subsurface vacuoles gave a mean of 7.2 ± 3.6 per cell section. Some vacuoles showed rod-like profiles in section. Monocytes contained granules of two types, which were discerned by size, small and large (Fig. 3b). Both granules were limited by a single membrane and possessed an electron-dense homogeneous matrix. A clear halo separated the granule contents from the limiting membrane. Small type granules, composing approximately 76% of the granules, were round, 100–150

nm in diameter or rod-shaped, 100 nm in width and 300 nm in length. The remaining 24% were large type granules, 300-400 nm in diameter. The granules and Golgi complexes were often situated in the nuclear indentation. The counts of granules gave a mean number of 9.8±5.2 per cell section. The cytoplasm contained a few round mitochondria, short strand of rough endoplasmic reticulum, free ribosomes and multivesicular bodies. The average cell diameter of monocytes was 6.2±0.5 with a range from 5.5 to 7.5 μm, and the mean N-C ratio was 0.77±0.27. The scattergram of cell diameter against N-C ratio in monocytes is shown in Fig. 5a. The majority of them were 5.5 -7.0 μm in cell diameter and 0.5-1.0 in N-C ratio.

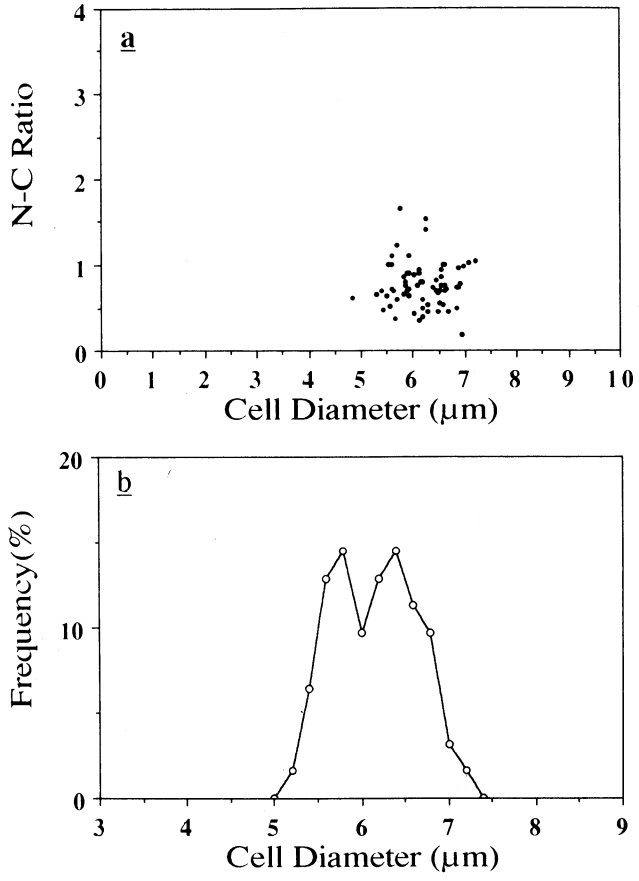

Fig. 5. Scattergram of cell diameter against N-C ratio in monocytes (a) and frequency distribution of cell diameter of monocytes (b).

Two monocyte subgroups could be defined on the basis of cell diameter (Fig. 5b) : small monocytes (cell diameters <6.0 μm) and large monocytes (cell diameters >6.0 μm). The N-C ratio was 0.80±0.28 in small monocytes and

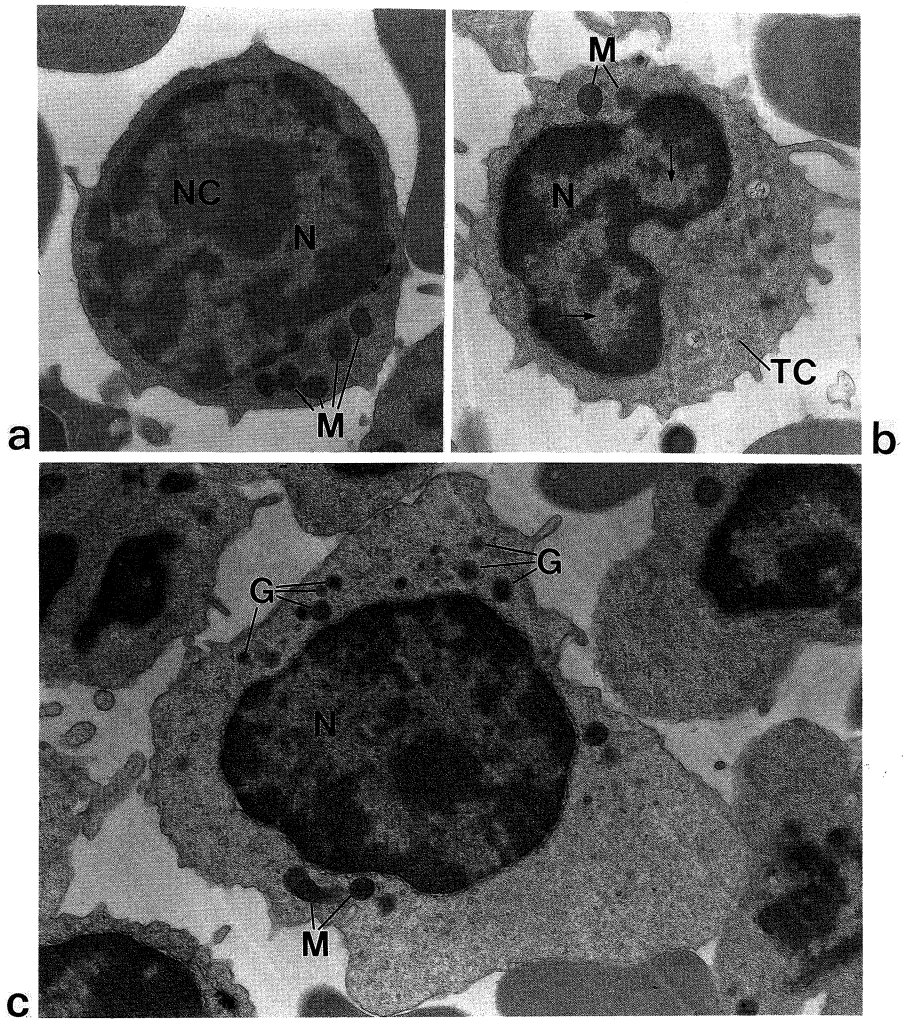

Fig. 6. Three types of lymphocytes in peripheral circulation. a. A non-granular lymphocyte with a high N-C ratio is the most frequent lymphocyte group in peripheral circulation. × 8,000. b. A non-granular lymphocyte with a low N-C ratio. The nucleus contains one or two nuclear bodies (arrowheads), and tubular complexes (TC) are often seen in cytoplasm. ×8,000. c. A granular lymphocyte. The cytoplasm is generally voluminous, and the granules (G) are scattered throughout the cytoplasm. M: mitochondria, N: nucleus, NC: nucleolus ×8,000.

0.74 ± 0.27 in large monocytes, but the difference was statistically not significant. The monocyte differing in respect to cell size appeared similar in morphology, and showed no ultrastructural difference between two subsets. Little difference was noted in cell diameter between small monocytes and small lymphocytes (Fig. 4).

2. Lymphocytes

The majority were smaller than 5.0 μm in nuclear diameter, and smaller than 6.0 μm in cell diameter, being classified as small lymphocytes. According

to the presence of cytoplasmic granules, peripheral blood lymphocytes could be divided into two groups: non-granular and granular lymphocytes.

1) Non-granular lymphocytes

Non-granular lymphocytes which formed a majority of blood lymphocytes could be classified into two subgroups: those with high N-C ratios and with low N-C ratios.

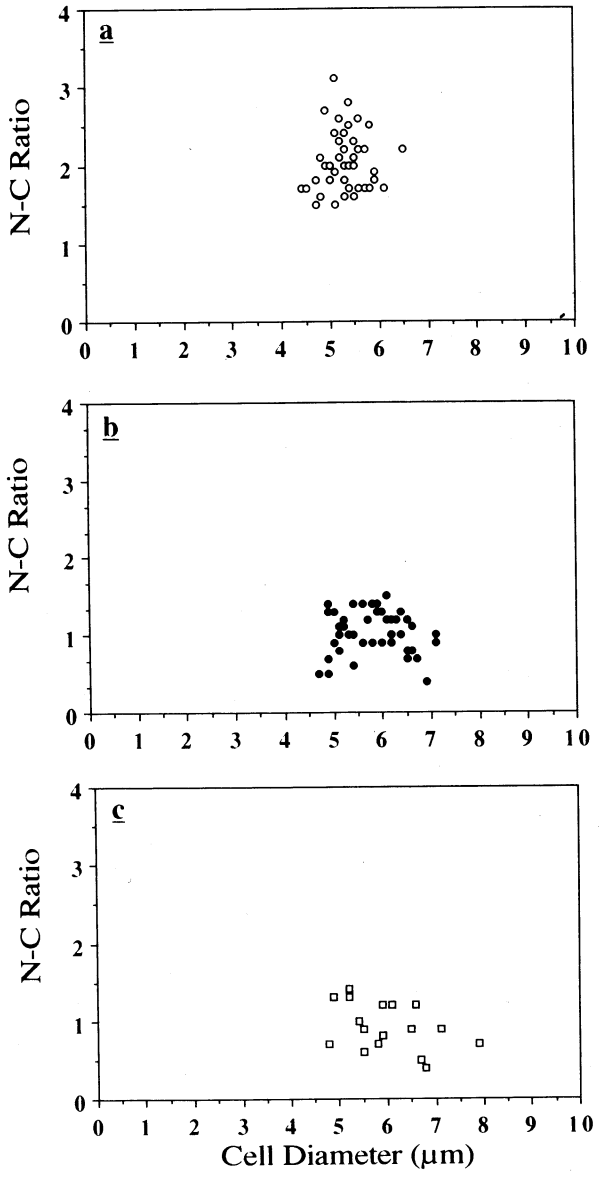

Fig. 7. Scattergram of cell diameter against N-C ratio in non-granular lymphocytes with high N-C ratios (a), non-granular lymphocytes with low N-C ratios (b) and granular lymphocytes (c).

a. Non-granular lymphocytes with high N-C ratios : The lymphocytes were rounded, having a round nucleus with a narrow rim of cytoplasm (Fig. 6a). The cell surface showed a few short villous projections. The nucleus contained one or two nuclear bodies and a nucleolus, 0.7–1.0 μm in diameter, surrounded by heterochromatin. The nuclear bodies were round, 0.3–0.6 μm in diameter, consisting of homogeneous fibrillar material surrounded by a clear halo. The cytoplasm was scanty, and cell organelles were not well developed. As shown in Fig. 7a, the lymphocytes were 4.2–6.5 μm in cell diameter, 5.3$\pm$0.4 μm on the average, and 1.5–3.1 in N-C ratio, a mean of 2.0$\pm$0.4. The majority were smaller than 6.0 μm in cell diameter and around 2.0 in N-C ratio.

b. Non-granular lymphocytes with low N-C ratios : Compared with the small lymphocytes with high N-C ratios, the profiles of these cells were irregular. The nucleus was sometimes indented, containing one or two nuclear bodies (Fig. 6b). Micropinocytotic vesicles were seen at the cell surface. The cytoplasm contained sparse cisternae of rough endoplasmic reticulum and many free ribosomes. The mitochondria, which were generally round in profile, the Golgi apparatus, multivesicular bodies and tubular complexes, which consisted of an accumulation of tubules measuring 50 nm in diameter and vesicles, were situated in the nuclear indentation. As shown in Fig. 7b, the cell diameter ranged from 5.0 to 7.0 μm, 5.8$\pm$0.6 μm on the average, and the N-C ratio, 0.5 –1.5, a mean of 1.1$\pm$0.3. The scattergram showed that one third of non-granular lymphocytes with low N-C ratios were larger than 6.0 μm in cell diameter.

2) Granular lymphocytes

The cell surface often showed a few short microvillous projections, but no subsurface vacuoles were visible in the cytoplasm. Some of the granular lymphocytes had an indented nucleus. As shown in Fig. 6c, the lymphocytes were characterized by the occurrence of spherical granules which were situated together with organelles such as mitochondria, Golgi apparatus and short profiles of rough endoplasmic reticulum. The granules ranged from 100 to 300 nm in diameter, and the majority of them were small, 100–200 nm in diameter. The small granules, possessing homogeneous matrix, tended to be clustered. Larger granules tended to have a more electron-dense content. Counts of granules ranged 3 to 8 per cell profile, giving a mean number of 4.3$\pm$2.0. The granules in lymphocytes were significantly lower in number than those in monocytes ($p<0.01$). The granular lymphocytes had a diameter of 5.0–8.0 μm with the average of 6.0$\pm$0.8 μm, and larger lymphocytes over 6.0 μm in cell diameter were present in considerable numbers (Fig. 7c). The N-C ratio ranged from 0.3 to 1.5 with the average of 0.92$\pm$0.30.

DISCUSSION

The present cytometrical observations in semithin light and electron microscopy reveal that blood monocytes in mice are generally larger in cell size than lymphocytes, but that no clear distinction can be made between lymphocytes and monocytes on the cell size distribution in light microscopy. Circulating lymphocytes are conventionally classified by cell size into small,

medium and large, and they have been known ultrastructurally to display heterogeneity.[11] Ferrarini et al.[12] identified, in the human blood, the four different subgroups of blood lymphocytes by electron microscopy, L1, L2, L3 and L4 cells, and they reported that L2 cells, characterized by the presence of cytoplasmic granules, have the ultrastructure of T-cell subpopulations bearing receptors for IgG. Several studies have indicated an association between granular lymphocytes and natural killer activity.[13,14] The distribution pattern seen in the present scattergram of blood lymphocytes by electron microscopy shows that non-granular lymphocytes with high N-C ratios vary in diameter between 4.2 and 6.5 μm and that the considerable population of non-granular lymphocytes with low N-C ratios and granular lymphocytes are over 6 μm in cell diameter. Monocytes and lymphocytes at the ultrastructural level are largely overlapped in cell size distribution. In particular, small monocytes exhibit the same cell size distribution as non-granular lymphocytes with high N-C ratios. Large monocytes, 7.2 μm in maximum cell diameter, do not exceed nongranular lymphocytes with low N-C ratios and granular lymphocytes in size. In the case of mice, therefore, it is controversial that blood monocytes are the largest in circulating leukocytes. The cell size and N-C ratio are not the first criterion for identification of monocytes in peripheral blood in the mouse.

As stated in the results, monocytes, either small or large, can ultrastructurally be distinguished from circulating lymphocytes by abundance of cytoplasmic granules and features of the cell surface. Concerning the number of the granules, monocytes have 24 granules on the average per cell profile in rabbits and 59 in man.[7] The number of granules in monocytes is smaller in mice than rabbits and humans but more than twice in granular lymphocytes. Phase contrast observation reveals that the most striking feature of monocytes is the presence of the ruffled plasma membrane at the cell surface.[15] The cell membrane invaginations resulting from the membrane ruffling are one the ultrastructural features of all the cells of the MPS.[8,16-18] The vacuoles or vesicles due to the cell membrane invaginations in cells of the MPS have been called 'subsurface vacuoles' in human spleen macrophages,[18] 'peripheral vacuolar profiles of irregular shape' in mouse macrophages,[17] or 'peripheral lacunae' in human circulating monocytes and in hamster peritoneal macrophages.[8,16] As shown in the results, circulating lymphocytes have microvillous projections on the cell surface, but the subsurface vacuoles or vesicles are rarely seen. Compared to macrophages, the surface projections are not developed in monocytes, but can permit ready identification of monocytes.

Blood monocytes as a member of the MPS are known to be an immediate precursor of tissue macrophages in adult life. During the intrauterine life, mature macrophages even prior to the start of marrow hemopoiesis appear in the mouse. The peritoneal cavity of mouse embryo contains two types of mononuclear cells; mature macrophages and small mononuclear cells.[19] Macrophages first appear at 11 days of gestation,[19] and small mononuclear cells appear at 9 days in the extra-embryonic coelom and vitelline vessels.[20] Studies with antigen F4/80 as a macrophage marker reveal that monocyte-like macrophages appear in the mouse yolk sac at day 9-10.[21] Ultrastructural study on the embryonic liver shows that the sinusoids at the beginning of hepatic hemopoiesis contain two forms of free mononuclear cells; mature macrophages and small mononuclear cells which are similar in ultrastructure to mature

macrophages except for large heterophagosomes.[22] Two forms of mononuclear cells have many clear vesicles at the cell periphery and long cytoplasmic extensions. On the basis of the ultrastructural morphology, small mononuclear cells are a possible candidate for progenitor of macrophages from the yolk sac. Small mononuclear cells range in cell diameter 7-12 μm in the liver sinusoids.[22] Mononuclears less than 7 μm in cell diameter are not present in embryonic liver sinusoids at the beginning of hepatic hemopoiesis. Since monocytes in peripheral circulation of adult mouse are 5-7 μm in cell diameter, macrophage progenitors from yolk sac are considerably larger in cell size than monocytes. Distinct difference exists in cell size between macrophage precursors before hepatic hemopoiesis and in adulthood, and embryonic macrophage precursors from the yolk sac are larger than monocytes which are produced in bone marrow to become tissue macrophages after birth.

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