Crystalloid Inclusion-Containing Macrophages in the Bone Marrow and Red Pulp of the Mouse, with Particular Relation to Age, Sex and Hydrocortisone Administration: Qualitative and Quantitative Electron Microscopy*

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Received December 6, 1982

Summary. Cytoplasmic inclusions, particularly crystalloid inclusions of macrophages in mouse bone marrow and splenic red pulp were qualitatively and quantitatively examined by electron microscopy.

In mice younger than 40 days of age, macrophages in the bone marrow contain a variety of inclusions, the majority of which are spherical phagolysosomes. In mice older than 60 days, needle-like crystalloid structures occur within the phagolysosomes. The crystalloids first appear at 60 days of age, and increase in amount with age. They exhibit a remarkable increase between 90 and 120 days in females and between 60 and 90 days in males. The crystalloids are significantly more abundant in adult males than in adult females.

Macrophages in the splenic red pulp have much more spherical inclusions than marrow macrophages. The crystalloid inclusions are also contained in the red pulp, but they are quite small in amount as compared with those in the marrow.

After administration of hydrocortisone, the amount of crystalloids increase significantly in marrow macrophages.

The crystalloids in macrophages are discussed with particular interest to their relation to erythropoietic activity in the hemopoietic tissue.

It is considered that macrophages may be involved in hemopoiesis. For example, they form so-called erythroblastic islands and are closely concerned with erythropoiesis (Bessis and Breton-Gorius, 1962; Seki and Shirasawa, 1965; Marton, 1975).

In this relation, electron microscope studies have demonstrated that macrophages in mouse bone marrow often possess cytoplasmic inclusions, which are characterized by the presence of crystalloids that are needle-like in appearance (Berman, 1967; Hudson, 1968). The crystalloid-containing inclusions have been reported to increase in number with age, although their functional significance is not fully understood (Hudson, 1969; Hudson and Shortland, 1974; Yang et al., 1979). The present study was undertaken to qualitatively and quantitatively examine, by electron microscopy,

*This work was supported by a grant from the Japanese Ministry of Education 1982 (No. 57480089).
macrophages in the bone marrow and the splenic red pulp, with particular reference to
the cytoplasmic inclusions in relation to age, sex and hydrocortisone administration.

MATERIAL AND METHODS

A total of 82 dd-mice of both sexes were used in this study. They were housed in
groups of five or six per cage and maintained under normal environmental conditions.
The animals were divided into two types of groups: normal untreated and hydrocor-
tisone-injected.

Normal group: This group consisted of females at 20, 40, 60, 90, 120, 150 days and
one year of age and males at 60, 90 and 150 days.

Hydrocortisone-injected group: Female mice were given two consecutive subcu-
taneous injections of 0.5 mg of hydrocortisone (Scheroson F, Schering) in 0.1 ml physio-
logical saline at 60 and 61 days of age and sacrificed 3, 10, 15 and 20 days after the
second injection.

In the two groups, five to nine mice were used on each date.

Electron microscopy
At the time of autopsy, the femurs and spleen were quickly removed after body weight
was registered. The femurs were cut open longitudinally by razor blades so that the
marrow was exposed to the fixative, and the spleen was cut into pieces after weighing.
The femurs and spleen were immediately fixed in an ice-cold mixture containing 5%
formalin and 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5, for 2 hrs. Then
the marrow was removed from the bone and cut into pieces. The pieces of the mar-
row and spleen were postfixed in 2% OsO₄ for 2 hrs at room temperature. The tissues
were immersed in 0.5% uranyl acetate overnight, then dehydrated in graded ethanols
and embedded in Quetol 812. Ultrathin sections were cut, stained with lead citrate,
and examined by electron microscopy.

Quantitative analysis
Morphometric studies were made on both the bone marrow and spleen. A random
sampling was obtained by photographing at low power (×1,000), and the quantitative
analysis of spherical phagolysosomes and crystalloid inclusions in macrophages was
made on photographs enlarged at a final magnification of 5,000 times. For the analysis,
on the average, 10 areas (2 fields from each of 5 blocks) were provided per animal.
For quantification of the phagolysosomes and crystalloid-containing inclusions, the
following parameters were used: For phagolysosomes, the cross-sectional areas of
phagolysosomes which lack crystalloids were measured by µm² by planimetry using
the Image Analysis System (Leiz-A. S. M.). The sectional area of phagolysosomes
per cell was calculated from both the total sectional area of phagolysosomes measured
and the number of macrophage nuclei in the same areas. For crystalloid inclusions,
the length of crystalloids contained was measured with a curvimeter. The length of
crystalloids per cell was estimated from both the total cumulative length of all the
crystalloids measured and the number of macrophage nuclei contained in the same
areas. More than 20 randomly selected nuclei were counted for each mouse, and 100
or more macrophages from each age and sex in the two groups were examined.

In the bone marrow of 60-day-old untreated and hydrocortisone-treated females,
the differential counts of nucleated hemopoietic cells were made on micrographs
enlarged to a final magnification of 3,500 times. For each mouse, more than 1,000 nucleated blood and hemopoietic cells were counted, and the frequency (%) of the hemopoietic cell series were obtained.

The values obtained were statistically evaluated by Student's t test.

RESULTS

A. General observations

1. Macrophages in the bone marrow

In the bone marrow, macrophages generally have an irregular profile with branching cytoplasmic processes extending between the surrounding hemopoietic cells. Their nuclei are round, oval or often reniform and are generally pale with solitary ring-shaped nucleoli. The cisternae of the rough endoplasmic reticulum and round or ovoid mitochondria are scattered throughout the cytoplasm. In younger mice, macrophages have well developed Golgi complex, whereas in aged animals, the Golgi complex is less developed and relatively inconspicuous.

At any age, macrophages have a variety of inclusions which appear to be derived from phagocytized erythroid cells (Fig. 1). The inclusions vary considerably in appearance and amount at different ages.

In mice younger than 40 days of age, most of the inclusions appear as spherical phagolysosomes with a diameter of 1-4 μm, of which the contents vary in electron density. A few inclusions contain myelin-like figures.

In mice older than 60 days, needle-like crystallloid structures are often seen within the phagolysosomes. Such crystalloids generally measure 1-5 μm in length and 50-300 nm in width. They are frequently seen together with a variety of heterogeneous debris and small electron-dense granules containing ferritin aggregates. The crystallloid-containing inclusions are often demilune, ellipsoid, spindle- or rod-like in profile (Fig. 2).

Fig. 1. Bone marrow of a young female mouse at 40 days of age. Macrophage (Mp) has a variety of inclusions, most of which appear as spherical phagolysosomes. ×5,000
In aged animals, 150 days to 1 year old, macrophages also contain a variety of inclusions, which are similar in appearance to those seen in younger mice. In addition, the crystalloid-containing inclusions measuring up to 10 μm in length are frequently found. The crystalloids are often arranged in parallel arrays. In some macrophages, they are so numerous that the crystalloid inclusions occupy a large portion of the cytoplasm (Fig. 3).

The cytoplasmic inclusions seen in marrow macrophages at 20, 60 and 150 days of age are schematically represented in Figure 4.

2. Macrophages in the splenic red pulp

In the splenic red pulp, macrophages also have many inclusions which are apparently derived from phagocytized blood cells (Fig. 5). In mice at 20 days to 1 year of age, the inclusions usually appear as spherical phagolysosomes measuring 0.5-4.0 μm in size. The crystalloid inclusions first appear at 150 days. Even in older animals at 1 year of age, splenic macrophages rarely contain crystalloid inclusions (Fig. 5b).

B. Quantitative observations

1. Area of spherical inclusions

In general, as mentioned above, the phagolysosomes lacking crystalloids appear spherical in shape. The sectional area of the spherical phagolysosomes per macrophage at various ages in female mice is shown in Figure 6. In the bone marrow, the area is almost constant, measuring 7.4-8.4 μm², until 90 days, but then it tends to decrease with age, measuring 4.3-5.5 μm² at 120 days to 1 year (Fig. 6a). Figure 7 shows the area in marrow macrophages of both sexes. The area is almost the same in both sexes at 60 days, but exhibits a decrease in males at 90 days when a significant sex difference is
apparent. At 150 days, however, no difference can be found between both sexes, because the area tends to decrease also in females as well as in males.

As shown in Figure 6b, the spherical phagosomes in splenic macrophages are small, 2.1 μm², in area at 20 days of age, but they have significantly increased at 150 days and 1 year. There is, however, no significant difference in area between both sexes at 150 days of age (Fig. 8).

2. **Length of crystalloids**

The length of crystalloids included per macrophage in the bone marrow of female mice at various ages is shown in Figure 9. The crystalloid-containing inclusions are few in

![Figure 3](image-url)

**Fig. 3.** Bone marrow of a 150-day-old female. Crystalloid inclusions arranged in parallel arrays occupy a large portion of the cytoplasm of macrophage (Mp). ×7,000

![Figure 4](image-url)

**Fig. 4.** Schematic diagram of cytoplasmic inclusions of macrophages at various ages. *d* days of age.
number until 90 days, and the length of crystalloids per cell is nil to small until 90 days of age. At 120 days and later, the inclusions are frequently found and the length of crystalloids per cell is markedly increased.

Fig. 5. Macrophages in the splenic red pulp. a. Macrophage (Mp) in a 60-day-old female mouse. Phagolysosomes derived from phagocytized blood cells are seen in the cytoplasm. ×7,000. b. Macrophage (Mp) at the age of 1 year. Crystalloid inclusions (arrows) are very few in amount in the red pulp. ×10,000

Fig. 6. Area of spherical inclusions per cell in the bone marrow (a) and splenic red pulp (b) at various ages in female mice. Each point represents the mean ± SD. d Days, y year of age.
**Fig. 7.** Area of spherical inclusions per cell in the bone marrow of male and female mice. Each point represents the mean ± SD. *d* Days of age.

**Fig. 8.** Area of spherical inclusions per cell in the red pulp of male (*M*) and female (*F*) mice at 150 days of age. Each point represents the mean ± SD.

**Fig. 9.** Length of crystalloid per macrophage in the bone marrow (**a**) and red pulp (**b**) of female mice at various ages. Each point represents the mean ± SD. *d* Days, *y* year of age.
The length of crystalloids in the two sexes is shown in Figure 10. As seen in this figure, the length is almost the same at 60 days in both sexes. At 90 days, however, the crystalloid-containing inclusions are more numerous in males than in females, so that the length is significantly greater in males than in females (P<0.001). At 150 days of age, the crystalloids are also significantly greater in length in males than in females.

The length of crystalloids included in splenic macrophages of female mice is presented in Figure 9b. As seen in this figure, it is very small even at 1 year. Figure 11 shows the length of crystalloids in splenic macrophages in both sexes at 150 days of age. This figure indicates that the length per cell is greater in males than in females and that the crystalloid-containing inclusions in splenic macrophages are more numerous in males than in females. At 1 year of age, however, the crystalloids are almost one-tenth as small in length in splenic macrophages as in marrow macrophages.
3. Effects of hydrocortisone

In control females at 60 days, macrophages in the bone marrow contain few or no crystalloid-containing inclusions (Fig. 9). However, hydrocortisone administration at this age causes a significant increase of crystalloids in marrow macrophages. The length of crystalloids contained in marrow macrophages in females injected with hydrocortisone is shown in Figure 12. A remarkable increase is observed 10 days after injection and later.

In Figure 13, the proportion of each series of the nucleated blood and hemopoietic cells in the marrow normal and hormone-treated mice is presented. In the hydrocortisone-injected, erythroid cells show a decrease in frequency 3 days after injection. Twenty days after hydrocortisone injection, the percentage of nucleated erythroids falls to smaller than one-third of the normal. On the other hand, the granuloid cells increase in proportion following administration.

In splenic macrophages, on the other hand, hydrocortisone injection causes no increase of the crystalloid-containing inclusions.

DISCUSSION

As is well known, macrophages in the hemopoietic organ appear as highly phagocytic cells that contain a variety of heterogeneous inclusions derived largely from the degradation of phagocytized blood cells (reviews: Carr, 1973; Hudson and Shortland, 1980; Simon, 1980). They are considered to be concerned functionally and intimately with hemopoiesis (Bessis and Breton-Gorius, 1962; Seki and Shirasawa, 1965; Marton, 1975). In mice and rabbits, macrophages in the hemopoietic organ are known to possess not only spherical phagolysosomes but also peculiar long, straight and slender inclusions which, containing needle-like crystalloid structures, are referred to as crystalloid or paracrystalline inclusions (Moore et al., 1964; Berman, 1967; Hudson, 1968; Simon and Burke, 1970; Crichton et al., 1980).

In mice, as seen in the results, the crystalloid inclusions are found in macrophages of the bone marrow and the splenic red pulp which is hemopoietic—largely erythropoietic—in function (Bozzini et al., 1970; Sasaki et al., 1982).

In younger mice, the most frequent inclusions in macrophages are spherical in shape, lacking crystalloids. Needle-like crystalloid structures first appear in the spherical inclusions in marrow macrophages at 60 days, and then they appear to become elongated in shape (Fig. 14). Thus, such spherical inclusions contain two components; crystalloids and small

Fig. 13. Frequencies of various hemopoietic cell series in the bone marrow after hydrocortisone injection (H. C. Inj.). Bars represent standard deviations.

Fig. 14. Schematic diagram of formation of crystalloid-containing inclusions.
dense granules composed of ferritin aggregates. The crystalloid inclusions gradually accumulate in macrophages. In older mice, macrophages in the bone marrow are often filled with the crystalloid inclusions.

In the mouse, the crystalloid inclusions are known to increase in number with age (Hudson, 1969; Hudson and Shortland, 1974; Yang et al., 1979). Our results also indicate that in the bone marrow, crystalloid inclusions increase in amount with age. As expressed by the length of crystalloids, they exhibit a prominent increase between 90 and 120 days of age in females and between 60 and 90 days in males. On the other hand, the spherical inclusions which contain no crystalloids remain constant in amount at 20 to 90 days, and then they slightly decrease. In the splenic red pulp, the spherical inclusions are small in amount at 20 days in females, and then they progressively increase with age. In adults, the area of spherical inclusions per macrophage is almost two to three times larger in splenic macrophages than in marrow ones. In the splenic red pulp, the crystalloid inclusions also occur in macrophages, but they first appear at 150 days of age. Even at 1 year of age, there are very few crystalloid inclusions in the spleen, when compared with those in the bone marrow. Morphological changes of the phagolysosomes shown in Figure 14 seem to take place on a much more extensive scale in the marrow than in the spleen.

Formation of the crystalloid inclusions appears to be independent of the amount of the spherical inclusions.

In the bone marrow and spleen, as shown in the results, the length of crystalloids per cell is different between the two sexes. In the bone marrow at 90 and 150 days of age and the spleen at 150 days, the crystalloids per macrophage are larger in size in males than in females. Thus, the formation of crystalloid inclusions is more marked in males than in females. On the other hand, no sex difference is seen in the area of spherical inclusions per cell in the bone marrow and splenic red pulp.

After administration of hydrocortisone, the crystalloid inclusions increase significantly in amount. The increase in the length of crystalloids becomes apparent 10 days after injection. Administration of phenylhydrazine that is known to accelerate red cell destruction causes an increase in the number of crystalloid inclusions in the mouse bone marrow (Hertzberg and Orlic, 1980). As shown in Figure 13, hydrocortisone administration causes a significant decrease in the frequency of erythroblasts. The decrease is thought to be caused by erythroblast breakdown. Thus the increase of the crystalloid inclusions after hydrocortisone injection is considered as a result of marked erythrophagocytosis by macrophages. Thus, it is likely that the crystalloid inclusions are derived from degradation of phagocytized erythroids in normal mice as well.

In the bone marrow and spleen, as is generally known, macrophages are functionally associated with developing erythroid cells. They frequently form erythroblastic islands (Bessis et al., 1978), and the surrounding erythroblasts are nursed by centrally located macrophages. The erythroblasts are considered to receive ferritin and other substances, such as subsequently utilized in the synthesis of hemoglobin. Thus, the islands are generally regarded as representing a morphological and functional unit of erythropoiesis (Bessis and Breton-Gorius, 1962; Seki and Shirasawa, 1965; Marton, 1975). From these considerations, it is probable that macrophages undergo morphological changes in association with erythropoietic activity. Thus, formation and increase of the crystalloid inclusions in macrophages are likely to be related to functional changes in erythropoietic activity. As reported in our previous paper, postnatal erythropoiesis in the bone marrow is different in activity from that in
the spleen (Sasaki et al., 1982). The difference in erythropoietic function between the bone marrow and spleen may be reflected by the amount of the crystalloid inclusions in macrophages in these hemopoietic tissues.

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