ENDOCYTOSIS OF CATIONIC AND ANIONIC IRON COLLOID PARTICLES BY RAT MACROPHAGES

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By using anionic and cationic iron colloid particles, which are Prussian blue reaction positive and 5-250 nm in diameter, the surface charge-related endocytosis of rat macrophages was observed. The anionic particles were solely adhered to the surface of peritoneal and liver macrophages by exposing the cells for a short time period to the particles *in vitro* as well as *in vivo* by perfusion. The particles then were taken up selectively by macrophages, but not by neutrophils, eosinophils, lymphocytes, mast cells, fibroblasts and vascular endothelial cells, as observed by light and electron microscopy after intraperitoneal, subcutaneous and intravenous injections of the particles. The anionic iron colloid particles were taken up by macrophages, delivered to lysosomes and disintegrated quickly. Ferritin was first synthesized there and then dispersed into the cytoplasmic matrix. In contrast, cationic iron colloid particles were adhered to the surfaces of all kind of somatic cells and internalized by the process similar to adsorptive endocytosis. The ingested particles were found in thin canalicular structure or tiny vesicles being adhered to their luminal surfaces. The morphology of these compartment was much different from the phagosomes of macrophages formed by ingesting anionic iron colloid particles, and showed no delivery of the particles to lysosomes as long as 4 hr after the incubation. Some particles were found in lysosomes by another 5 hr of chase incubation. The results indicate that the "non-specific receptor mediated phagocytosis" by macrophages is induced by the binding of anionic macromolecules to the cationic receptor on the cell surface. And the adsorptive endocytosis will induce two kinds of different mechanism, one is currently accepted concept, the receptor mediated endocytosis and another one is the surface charge-mediated endocytosis.

In macrophages, the process of endocytosis can be divided into the following steps; contact between the cells and the substances, recognition, establishment of stable bonding, internalization and digestion. Many studies have been documented in which a variety of foreign substances are recognized by "non-specific receptor" on the surface of macrophages (27, 29), but the mechanism is not yet fully understood. We have previously demonstrated that net negative charge on the surface of foreign substances stimulates the recognition and attachment of the substances by macrophages (18, 22). Anionic substances were adhered to the cationic groups on the surface of macrophages then internalized and accumulated in lysosomes (18).

Abbreviations used. Fe-ChS: iron chondroitin sulfate, Fe-PVS: ferric hydroxide-potassium polyvinyl sulfate complex, RE: reticuloendothelial, Fe-Cac: ferric hydroxide-cacodylate complex, ACPase: acid phosphatase.

In this paper, we demonstrate how net charge of the substances and the surface of macromolecules are related to binding, uptake, and intracellular destination of the substances through morphologic observations on the endocytosis of anionic and cationic iron colloid particles by rat macrophages.

ENDOCYTOSIS OF ANIONIC IRON PARTICLES

Cationic groups on the cell surface of macrophages.

All kinds of somatic cells including macrophages have negatively charged surfaces, though, macrophages are less in net negative charge than other somatic cells as understood by their lower electrophoretic mobility (15). This means that negatively charged substances are easy to approach to macrophages than to other cells. In non-specific phagocytosis of a variety of foreign substances by macrophages, the electrostatic force of attraction between the substances and the cell surface will be essential for the adherence of the substances to the cell and the subsequent ingestion.

Previously we reported how macrophages or Kupffer cells developed cationic groups on their surface enough to form stable electrostatic bonding with anionic macromolecules through the perfusion experiment of rat liver with the anionic iron colloid particles, Fe-ChS (Iron chondroitin sulfate, Blutal; electron dense ferric granules of 5 nm in size, for medical use) (18).

Electron microscope observations of the liver revealed Fe-ChS particles adhered to the macrophages, but neither to the surface of endothelial cells nor of parenchymal cells. The electron opaque particles were found on the macrophage surface ranging regularly at certain distances. When cells from rat peritoneal exudate were incubated with Fe-ChS at 4°C *in vitro*, iron particles were found only on the surface of macrophages (Fig. 1), but not on the other peritoneal cells such as granulocytes, lymphocytes, mast cells and mesothelial cells. The results indicate clearly that distinct cationic groups are present on the cell surface of macrophages as well as Kupffer cells, as no other binding than electrostatic bonding was evidenced between tissue component and Fe-ChS by histochemical staining of fixed tissues with Fe-ChS at pH 7.0 (24). Thus the foreign substances are avidly taken up by the cells through electrostatic bonding between the cationic groups on cell surface and anionic substances.

Endocytic pathway.

The endocytosis of anionic iron particles, Fe-ChS and Fe-PVS (ferric hydroxide-potassium polyvinyl sulfate complex), by macrophages were studied on

FIG. 1. EM photograph of the distribution of anionic iron particles on the cell surface of a macrophage.

Peritoneal cells were treated with Fe-ChS at 4°C for 30 min. Note that anionic Fe-ChS particles are bound to the surface of macrophage (M), but not to the surface of eosinophilic leucocyte (E). Cells were stained with uranyl acetate and lead citrate. $\times 22,500$

FIG. 2. EM photograph of a rat peritoneal macrophage taken 1 hr after i.p. injection of anionic Fe-PVS particles. A number of Fe-PVS particles are found in phagosomes and some on the cell surface. Process of internalization also can be seen (arrows). Cells were stained with uranyl acetate and lead citrate. $\times 14,400$

FIG. 3. EM photograph of a rat peritoneal macrophage taken 6 d after i.p. injection of Fe-PVS. Numerous ferritin particles are scattered throughout the cytoplasmic matrix. Unstained section. $\times 60,000$

FIG. 4. EM photograph of a section of the rat liver excised 48 hr after i.v. injection of Fe-PVS. Fe-PVS particles are only found in the phagolysosomes or dense bodies of Kupffer cell (arrows). Tissue was stained with uranyl acetate and lead citrate. $\times 14,400$

FIG. 5. EM photograph of a section of the rat spleen excised 48 hr after i.v. injection of Fe-PVS. Macrophage has a number of decomposing Fe-PVS particles in the phagolysosomes. Tissue was stained with uranyl acetate and lead citrate. $\times 21,600$

rat peritoneal macrophages obtained at certain time intervals after an intraperitoneal injection of the particles (17). Fe-PVS prepared in this laboratory had a spherical and electron opaque iron hydroxide core with diameter of about 250 nm surrounded by radially arranged fibrous polyvinyl sulfate molecules. It also had strong negative

FIG. 6. LM photograph of spread preparation of the rat subcutaneous connective tissue taken 24 hr after the subcutaneous injection of Fe-PVS. Histiocytes have Fe-PVS, which appear as dark granules in the cytoplasm (arrows), but fibroblasts do not. Tissue was stained by Prussian blue reaction and post-stained with haematoxylin. $\times 520$

FIG. 7. LM photograph of spread preparation of the rat omentum taken 4 hr after i.p. injection of Fe-PVS. Note that Fe-PVS particles are solely taken up by macrophages in the milky spot of omentum (arrow), but never by fibroblasts and mesothelial cells. Tissue was stained by Prussian blue reaction and post-stained with haematoxylin. $\times 400$

electric charge. Its strong positive Prussian blue reaction is useful for light microscopy and the electron opaque core is for electron microscopy. Peritoneal macrophages found in the peritoneal fluid taken 1 hr after the Fe-PVS injection (0.055 mg Fe/ml, 3 ml) ingested Fe-PVS particles, but granulocytes, lymphocytes and mast cells did not. Light microscopy revealed many blue granules in the markedly swollen macrophages by Prussian blue reaction. Under electron microscopy, numerous Fe-PVS particles were found in the large phagosomes of macrophages and some in phagolysosomes (Fig. 2). At 2 to 3 d after the Fe-PVS injection macrophages had large phagolysosomes containing Fe-PVS particles in various stages of disintegration. Some phagolysosomes were scanty in contents. After 6 d macrophages had a number of phagolysosomes, but most of them were scanty or had only a few residual substances, with numerous ferritin particles scattered throughout the cytoplasmic matrix (Fig. 3). Fe-ChS was taken up by macrophages through the same route as Fe-PUS and ferritin was synthesized.

Macrophages are distinguished by their special morphologic characteristics (36) and their carbon powder uptake activity, and by the specific lysosomal enzymes recognized histochemically. We, however, often met with difficulty in distinguishing them from other somatic cells. Because any of these characteristics are not specific to macrophages. For example, fibroblasts, neutrophils and endothelial cells take up some carbon powder (4, 6, 20), and also have histochemically demonstrable lysosomal enzymes like nonspecific esterase (21) and peroxidase (2, 25).

As supposed from the morphologic structure of Fe-PVS, the particles are covered fully with sulfate groups and have strong net negative electric charge. And as there

is no ionized cationic groups in tissues at pH 7.3 except those found on macrophage surface (24), Fe-PVS should be most useful tool for labeling of macrophages *in vitro* as well as *in vivo*. Actually, Fe-PVS injected into vein were taken up solely by reticuloendothelial (RE) cells like Kupffer cells in liver (Fig. 4) and splenic macrophages (Fig. 5), but not by capillary endothelial cells, parenchymal cells and others. We also could find histiocytes by injecting Fe-PVS into subcutaneous tissue (Fig. 6). Fe-PVS particles were never bound to the cell surface of fibroblasts and capillary endothelial cells nor taken up by them. When Fe-PVS particles were injected into peritoneal cavity, they were taken up by macrophages in peritoneal fluid as well as by those in the milky spot of omentum (Fig. 7), but not by other cells.

Ferritin synthesis in macrophages after uptake of Fe-PVS particles.

Injured or senescent red blood cells are mainly taken up by macrophages of the RE system, haemoglobin is degraded and iron is reutilized for the new haemoglobin synthesis in red cell precursors. Most of the surplus iron is stored as ferritin and haemosiderin in RE cells in various tissues and in liver parenchymal cells. Most report dealing with iron metabolism, however, focus on the liver parenchymal cells (10). Little is known about the mechanism of ferritin synthesis in macrophages or RE cells, and the travel of iron from these cells to red blood cells is still obscure.

So we observed the ferritin synthesis in macrophages, the primary iron storage cells in the body. Rat peritoneal macrophages taken at certain time intervals after an intraperitoneal Fe-PVS injection served as materials. The intracellular degradation process of Fe-PVS and the appearance of ferritin were examined by light and electron microscopy. The intracellular distribution of ferritin was also checked by the indirect immunofluorescence method (17). Within 5 hr after the injection, Fe-PVS particles were found in phagolysosomes of peritoneal macrophages by electron microscopy. Some of the Fe-PVS gross particles were found being disintegrated into minute iron hydroxide particles in the vacuoles. The disintegration proceeded with lapse of time and ferritin appeared in the phagolysosomes as early as 24 to 48 hr after the Fe-PVS uptake. Fluorescence microscope observations of smeared cells fixed in methanol and stained with rabbit anti-rat ferritin antibody and goat anti-rabbit fluorescein isothiocyanate-conjugated IgG revealed strong fluorescence in the phagolysosomes. At 5 to 6 days after the Fe-PVS injection, electron microscope observations revealed that ferritin was scattered diffusely throughout the cytoplasmic matrix (Fig. 3). Fluorescence microscopy showed strong fluorescence in cytoplasm with vacant vacuoles. It was consistent with the findings by electron microscopy that just mentioned above. The findings are clear evidence that ferritin is synthesized in the phagolysosomes first and then dispersed diffusely into the cytoplasmic matrix, where it lodged for more than 3 months.

The findings are inconsistent with the current concept of the ferritin synthesis in hepatocytes, in which ferritin is synthesized first in the cytoplasm by free polyribosomes (11, 12), and then accumulated in lysosomes by a process of autophagy (5). Thus, it is generally accepted that hepatocytes take up iron from transferrin which is internalized through receptor mediated endocytosis (1). Macrophages phagocytize senescent red blood cells to decompose haemoglobin for reutilization of iron. Therefore, the iron in macrophages from either haemoglobin or other phagocitized iron compound is the first material of iron reutilization. The ferritin

FIG. 8. Immunohistochemical demonstration of ferritin in liver tissue taken 3 d after i.v. injection of Fe-PVS. Kupffer cells show strong intracytoplasmic staining. PAP method. $\times 250$

synthesis in hepatocytes should be made mainly by using iron absorbed from intestinal tract or circulating transferrin iron. Hepatocytes do not phagocytize red blood cells. Therefore, there would be some difference in the pathway of ferritin synthesis with the ingested iron compounds between macrophages and hepatocytes.

Intravenously injected Fe-PVS particles also were utilized effectively for ferritin synthesis in Kupffer cells of liver. Electron microscopic and immunohistochemical observations on rat liver tissues removed at varying time intervals after the intravenous injection of Fe-PVS showed clearly the process of ferritin synthesis in liver. Rat liver tissues were removed and fixed in Zamboni fixative (37) for immunohistochemistry and 2% glutaraldehyde for electron microscopy. Ferritin appeared first in some Kupffer cells as early as a few hours after Fe-PVS injection. Thereafter, the ferritin positive Kupffer cells increased in number for up to 3 days. Most of Kupffer cells in the peripheral area of each lobule were greatly swollen with the heavily laden ferritin in the cytoplasm (Fig. 8). Hepatocytes had hardly any ferritin at this stage. At 4 days, ferritin distribution pattern was markedly changed. The number of ferritin positive Kupffer cells greatly decreased. In contrast, hepatocytes around the central vein became ferritin positive, some were strongly stained in granules in the cytoplasm by immunohistochemical method. These evidences suggest the possibility that ferritin synthesized in Kupffer cells by taking up Fe-PVS particles was secreted into tissue spaces and taken up by parenchymal cells through receptors for ferritin (13) and stored there.

ENDOCYTOSIS OF CATIONIC IRON PARTICLES

Surface anionic sites.

Using several electron dense macromolecules like cationized ferritin (3, 8, 28), cationized hemeundecapeptide (7) and cationic iron colloid (Fe-Cac, ferric hydroxide-cacodylate complex) (9, 23, 26), the study of cell surface anionic sites

FIG. 9. EM photograph of the rat aortic wall, fixed with glutaraldehyde and incubated with cationic Fe-Cac at pH 7.3 for 5 hr. The Fe-Cac particles are uniformly adhered to the luminal surface of endothelial cells. L, Lamina elastica. Tissue was lightly stained with uranyl acetate and lead citrate. $\times 14,400$

FIG. 10. EM photograph of endothelial cells of rat aorta taken 10 min after the perfusion with Fe-Cac at pH 7.3. Note that Fe-Cac particles are randomly distributed on the luminal surface of endothelial cells and are found within tiny vesicles in the cytoplasm. Unstained section. $\times 36,000$

has substantial progress. Fe-Cac has been utilized to demonstrate the anionic sites on the surface of capillary endothelial cells and in glomerular basement membrane of mammals (24, 25) as well as of fish (33, 34).

Electron microscope observations of the glutaraldehyde fixed tissue sections of rat aorta incubated with Fe-Cac at pH 7.3 revealed that the iron particles were uniformly found on the luminal surface of endothelial cells (Fig. 9). When Fe-Cac

Figs. 11 and 12. EM photographs of sections of the rat liver excised 10 min after the perfusion of Fe-Cac. Fe-Cac particles are found randomly on the surface of Kupffer cell (Fig. 11) as well as endothelial cell (Fig. 12), and are taken up by them (arrow heads). Tissue was stained with uranyl acetate and lead citrate. Fig. 11 and 12 $\times 27,000$

particles were introduced into rat aorta and portal vein by perfusion *in situ*, the particles were bound to the cell surface of endothelial cells and some in small pits or vesicles, showing the way of transendothelial transport (Fig. 10). In liver sinusoidal wall they also were found in Disse's spaces being adhered to the surface of microvilli of parenchymal cells (24). The particles were also adhered to Kupffer cells and

taken up by the cells in the similar way as that of endothelial cells (Figs. 11 and 12).

Fe-Cac particles injected into subcutaneous tissue of rat were taken up by all kinds of cells there, i.e. lymphocytes, granulocytes, histiocytes and fibroblasts. Twenty four hours after the injection all of cells had Prussian blue positive granules adhered to the cell surface or taken into the cells. When the cells from rat peritoneal fluid were incubated with Fe-Cac particles at 37°C *in vitro*, the particles were adhered to the surfaces of all kinds of cells and taken into the cytoplasm (Figs. 13–15). These findings clearly indicate that all kinds of somatic cells are able to adsorb the cationic Fe-Cac particles to the surfaces by the electrostatic attraction force, and then internalize them through adsorptive endocytic pathway.

Endocytic pathway in macrophages.

A variety of morphological studies on the endocytic pathway of cationic macromolecules have been carried out in fibroblasts, smooth muscle cells and macrophages. Cationized ferritin has been found to enter the cells through adsorptive endocytic mechanism (16, 31, 32). The cationized ferritin internalized was concentrated in lysosomes and moved back to the cell surface directly or via the Golgi apparatus, suggesting the recycling of the plasma membrane (14, 30).

Cationic Fe-Cac particles electrostatically bound to the cell surface were internalized in the cell by the way of cationized ferritin (19). Though, we have noticed that there are some difference on the intracellular fate of the tracers between cationized ferritin and Fe-Cac. Rat peritoneal macrophages were exposed to Fe-Cac *in vitro* for varied time length and examined for electron microscopy. Fe-Cac was first found in tiny vesicles or tubular profiles. These vesicles showed Fe-Cac labeling on their luminal surfaces (Fig. 15). The vesicles were independent of each other being retained in the deeper area of the cell, showing no delivery of the particles to lysosomes. In order to find out how or when these iron containing vesicles fuse with lysosomes, histochemical staining for acid phosphatase (ACPase) was carried out. Rat peritoneal macrophages first were exposed to Fe-Cac at 37°C for 2 hr. After washing, the cells were reincubated in iron-free medium for 2, 5, 10 and 15 hr, respectively. Glutaraldehyde fixed cells were stained for ACPase and observed under electron microscopy. Reaction product for ACPase appeared within large vacuoles containing iron particles at 5 hr after the chase incubation, showing fusion of the vacuoles with lysosomes. After 10 to 15 hr of chase incubation in iron-free medium, macrophages had large rounded or irregular phagolysosomes containing both iron particles and reaction product for ACPase. Again ferritin was scattered diffusely throughout the cytoplasm at this stage, as demonstrated by electron microscopy and by immunofluorescence microscopy with anti-rat liver ferritin antibody. Thus, some Fe-Cac entered into phagolysosomes of macrophages fairly a long time period after the ingestion, and was utilized for ferritin synthesis. It is the problem, however, to be solved whether or not some particles are sent to the cell surface by membrane recycling pathway like cationized ferritin.

FIGS. 13–15. EM photographs of rat peritoneal cells exposed to Fe-Cac at 37°C for 1 hr (Fig. 14, Lymphocyte) and 2 hr (Fig. 13, Eosinophilic leucocyte; Fig. 15, Macrophage). Fe-Cac particles are found on the surfaces of all kinds of cells. Fe-Cac is also seen within the intracellular vesicles, and in cell surface invagination (Fig. 14, arrow). These vesicles show Fe-Cac labeling of their inner surfaces. Cells were stained with uranyl acetate and lead citrate. Fig. 13. $\times 27,000$, Fig. 14. $\times 36,000$, Fig. 15. $\times 27,000$

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