Interaction of Iron Oxide Fe_3O_4 Nanoparticles and Alveolar Macrophages *in Vivo*

B. A. Katsnelson, L. I. Privalova, M. P. Sutunkova, L. G. Tulakina*, S. V. Pichugova*, J. B. Beykin*,, and M. J. Khodos*****

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Aqueous suspension of magnetite nanoparticles with primary diameter of 10 nm were intratracheally administered into rat lungs. In 24 h, cells were isolated from bronchoalveolar lavage and examined under a transmission electron microscope. Alveolar macrophages demonstrated ability to actively uptake single nanoparticles and small aggregates composed of such particles, which then formed larger conglomerates inside fused phagosomes. Some of these mature phagosomes shed the membrane and free nanoparticles closely interacted with nuclear membrane and with cristae and mitochondrial membranes thereby inflicting pronounced damage to these intracellular structures. The loss of primary lysosomes can be viewed as indirect evidence attesting to the role played by diffusion of lysosomal hydrolytic enzymes in the final destruction of the alveolar macrophages provoked by nanoparticles.

Key Words: *nanoparticles; iron oxide*; *alveolar macrophage*

The development of nanotechnologies stimulated the appearance of a large family of materials containing nanoparticles conventionally defined as objects with at least one dimension <100 nm. Such materials are becoming widely used in various branches of biology and medicine. Specifically, nanoparticles (NP) produced from iron (II,III) oxide Fe_3O_4 (magnetite) are employed as *1*) selective drug carriers to target organs, *2*) markers controlled by external magnetic field to be accumulated in a cancer tumor where they became thermal killers of malignant cells being heated with alternating electromagnetic field; *3*) contrast medium for magnetic resonance tomography, etc.

A substance demonstrating low biological aggressivity even when applied as particles of micrometer range acquires pronounced cytotoxicity against alveolar macrophages (AM) *in vivo* and essential resorption toxicity at the systemic and organism levels when applied as NP with the size of 50 nm, and it becomes especially aggressive at the size of 10 nm [2,4]. However, the above studies did not confirm the views on defenselessness of biological organisms against pernicious effects of any NP [3,6,8] and, specifically, on poor efficiency of phagocytic mechanism of pulmonary self-purification from NP deposited in the deep airways. We have found that mobilization of AM (the major player in this mechanism) and, most important, neutrophil leukocytes (essential auxiliary effectors to this mechanism) on the free epithelial surface in the airways develops far more rapidly during the action of NP than during application of 1-μ iron oxide particles. In both types of phagocytic cells, the load with aggregated NP discernible under a light microscope is higher during exposure of the airways to 1-μ particles. These findings are corroborated by the data of semi-contact atomic force microscopy, which revealed far greater density of microscopic impressions on the cell surface viewed as manifestations of invagination of plasma membrane during phagocytosis.

Medical Research Center for Prophylaxis and Health Protection of Industrial Workers, Russian Trade and Sanitary Inspectorate; *Clinical and Diagnostic Center; **Institute of Immunology and Physiology, Ural Branch of Russian Academy of Sciences; ***Ural State Economic University, Ekaterinburg, Russia. ***Address for correspondence:*** bkaznelson@ymrc.ru. B. A. Katsnelson

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Our aim was to determine intracellular localization of magnetite NP in AM by transmission electron microscopy and to visualize cell damages at the ultrastructural level produced by NP. In accordance with previous studies, we chose NP with a diameter of 10 nm as the most toxic and most actively phagocytized particles for AM.

MATERIALS AND METHODS

The procedure of preparing 10-μ magnetite NP with a narrow size dispersion and suspending them in water solution, as well as the parameters of suspension, were described elsewhere [4]. Kinetics of NP aggregation was examined by the method of light transmission fluctuation (dynamic light scattering) employing a Brookhaven ZetaPlus universal suspension analyzer.

NP suspension was administered intratracheally to female outbred albino rats in a single dose of 2 mg in 1 ml sterile deionized water, because aggregation developed too rapidly in physiological solution. The procedure of suspension intake into a syringe and its visually controlled intratracheal administration were performed within a 28-sec interval after a short-term pause in ultrasonication characterized by minimum NP aggregation. Control rats received 1 ml NP-free intratracheal water.

In 24 h, a cannula was introduced into the trachea. The cannula was connected to Luer syringe filled with 10 ml physiological saline. The suspension was slowly instilled into the lungs driven by the weight of piston. During instillation, both rat and syringe were held vertically. Then the rat and the syringe were turned upside-down to collect the pulmonary lavage into the syringe. The collected lavage was transferred into cold siliconized tubes and centrifuged for 30 min at 3000 rpm. The cell pellet was fixed in 2.5% glutaraldehyde and postfixed with OsO_4 for 2 h. Then it was washed with 0.2 M phosphate buffer and dehydrated in ascending alcohols and in acetone. The specimens were placed into acetone-araldite (1:1) mixture for 24 h and embedded into araldite for polymerization at 37°C for 24 h and then at 50-60°C for 2-3 days. Ultrathin sections were prepared in Leica EM UC6 ultratome and examined under a Morgagni 268 electron microscope.

More than 200 sections from 15 rats were examined and 254 images of macrophages were obtained at magnification ranging from ×3500 to ×140,000.

RESULTS

Figure 1 shows a typical electron microscope image of a peripheral part of AM from a rat exposed to magnetite NP. Individual extracellular NP and their preformed tiny aggregates composed of 2-3 primary NP

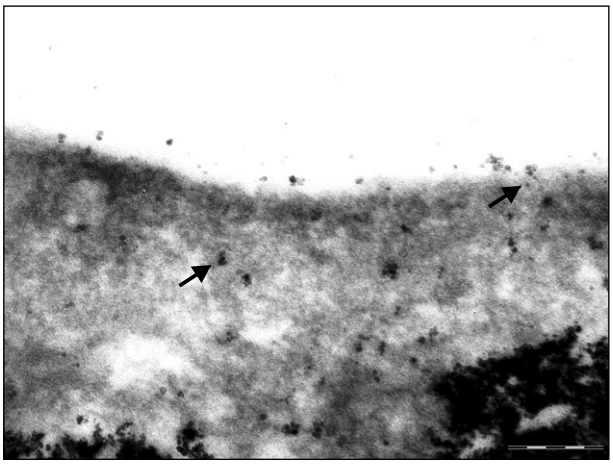

Fig. 1. Phagocytosis of magnetite NP by AM. Arrows show small phagosomes, ×14,000.

were arranged in close proximity with plasmalemma and some of them directly contacted with it. Intracellularly, these particles were located within the vacuoles separated from the environmental cytoplasmic matrix by vacuolar membrane (tonoplast). These tiny phagosomes were produced by separation of plasmalemma invaginations. Figure 1 shows a phagosome in a close contact with the internal contour of plasmalemma, evidently, just after separation from it. Next to it, some NP are located to be engulfed by the developing process of invagination.

We found no images that would attest to similar phagocytosis of preformed large micrometer aggregates observed in AM under a light microscope at ×1000 [2,4]. However, electron microscopy revealed a large number of such aggregated inside AM, and in most cases, they were surrounded by two-contour membrane, *i.e.* they were located inside a large endosome (phagosome) formed after fusion of smaller phagosomes (Fig. 2). Such fusion is known to result from an active process with involvement of actin [5].

The other images, where no such phagosomal membrane was detected attesting to free location of NP aggregates in cytoplasm, probably demonstrate the secondary phenomenon related to degradation of phagosomal membrane under the damaging effect of NP. Most clearly, such destructive effect can be seen in the places where a free NP aggregate makes a close contact with the membranes of other organelles (especially frequently with mitochondria) or with nuclear membrane (Fig. 3). In mitochondria, NP docked to membrane or cristae filling in some cases the entire matrix. In such cases, the abnormalities in two-contour membrane structure could be seen accompanied with degradation of cristae and clarification of mitochondrial matrix. In contrast to crude fraction of atmospheric dust, which after *in vitro* interaction with macrophage-

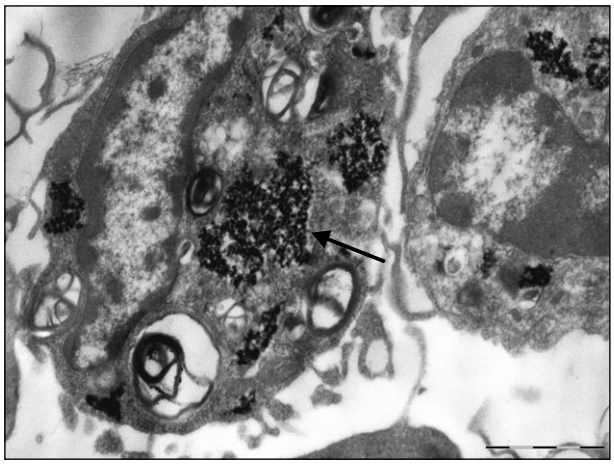

Fig. 2. Magnetite NP aggregated inside large phagosomes (arrow), ×8900.

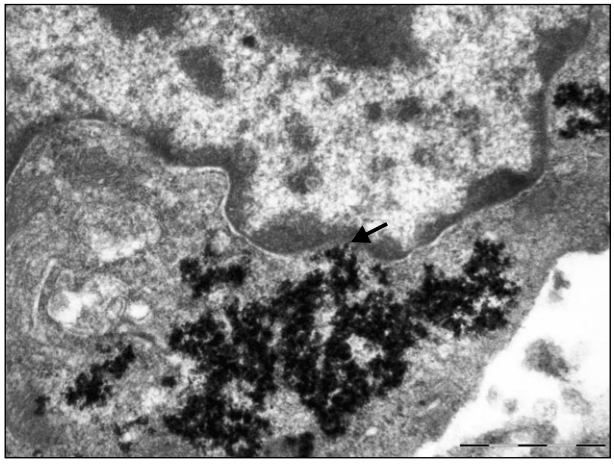

Fig. 3. Contact of loosely located aggregate of magnetite NP with nuclear membrane (arrow), where a destruction of two-contour membrane structure is clearly seen, ×71,000.

like cells could be found only inside phagosomes, ultrafine fraction particles (<150 nm) contact and damage mitochondria triggering oxidative stress [7].

In our experiments with magnetite NP, there were virtually no primary lysosomes in striking contrast to the control animals, which demonstrated large amount of such organelles in AM. Probably, this difference resulted from the damaging effect of NP on Golgi apparatus, which is responsible for lysosome formation. However, it cannot be excluded that the absence of primary lysosomes in the experimental group resulted from controlled fusion of these organelles with numerous phagosomes culminating in production of phagolysosomes (secondary lysosomes). Probably, the release of lysosomal hydrolytic enzymes into cytoplasm after damage to phagolysosomal membrane is supplementary mechanism of cell destruction. The role of this self-digestion of macrophages during the exposure of the airways to microparticles is firmly established and considered as a cornerstone in the classical theory of the cytotoxic mechanisms of such particles (specifically, the quartz ones) aimed at the macrophages [1]. Electron microscopy demonstrates a large number of completely destroyed AM with release of NP and their aggregates into the intercellular space.

Thus, this study showed that despite some published opinions [3], AM can recognize and phagocytize even extremely small NP. Here in addition to passive penetration of such NP across the plasmalemma, active physiological process is going on according to fundamental phagocytosis pathways of solid particles. There are reasonable data to believe that formation of intracellular aggregates of NP is primarily related not only to their especial tendency to physical aggregation characteristic of all NP (and magnetic NP in particular) in a liquid medium, but also to the abovementioned physiological process at the stage of fusing of small phagosomes into large conglomerates that demonstrate this tendency. Finally, the data obtained showed that similar to mineral microparticles [1], the prime course of NP cytotoxicity against AM is membranolytic activity.

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