Video Time-Lapse Microscopy of Phagocytosis and Intracellular Fate of Crystalline Nickel Sulfide Particles in Cultured Mammalian Cells

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ABSTRACT

The endocytosis and intracellular distribution of carcinogenic crystalline nickel sulfide (NiS) particles in Chinese hamster ovary cells were studied using time-lapse video recording with phase-contrast and bright-field optics. Crystalline NiS particles were phagocytosed by Chinese hamster ovary cells in regions of membrane ruffling. While these particles may remain bound to the cell surface for variable time intervals (min to hr), their internalization generally required only 7 to 10 min. Endocytosed crystalline NiS particles exhibited saltatory motion, and lysosomes were observed to interact repeatedly with the particles in a manner similar to that observed during the digestion of macropinosomes. Particles were never observed to be exocytosed from the cell, and with time, most of the internalized particles aggregated in the region around the nucleus. After 24 to 48 hr, particle saltation decreased to a point where the particle position became relatively fixed in the perinuclear region, and in some instances, this was associated with a conspicuous vacuole formation around the particles. It is concluded that the uptake and distribution of crystalline NiS particles occur by normal endocytic and saltatory processes as occur during the formation and breakdown of macropinosomes. The observed lysosomal interaction with phagocytosed cytoplasmic NiS may accelerate particulate nickel dissolution allowing entry of ionic nickel into the nucleus.

INTRODUCTION

The crystalline nickel sulfide compounds (NiS and Ni₃S₂) represent a class of extremely potent inducers of cancer in experimental animals (17, 26, 27, 30) and of neoplastic transformation in primary cell cultures (9–12, 23). In contrast, amorphous NiS exhibited no carcinogenic activity in experimental animals (27) and has been repeatedly shown to possess very weak cell-transforming activity (9–12). Recently, Costa and Mollenhauer (9, 10) related the potent carcinogenic activity of the crystalline nickel sulfide compounds to their selective phagocytosis by cell cultures, since amorphous NiS particles of similar respirable size (< 5 μm) were not endocytosed. Further studies by Abbracchio et al. (1–3) demonstrated that crystalline NiS particles had a negative surface potential (−28 mV), while amorphous NiS particles exhibited an overall positive surface charge (+9 mV). Since the interaction of the NiS particles with the cell membrane involved the particle surface, it was proposed that the negative surface charge was directly related to their endocytosis. The level of phagocytosis of crystalline NiS particles is not influenced by the components of tissue culture media or serum, since similar endocytosis of crystalline NiS occurs in a salts/glucose medium as in complete culture growth medium (1–3, 16). Recent studies by Heck and Costa (16) lend further support to this hypothesis, since chemical reduction of amorphous NiS particle surfaces with LiAlH₄ resulted in their avid endocytosis by cultured cells, and these chemically reduced amorphous NiS particles caused an incidence of transformation equaling that of the crystalline NiS in keeping with the direct relationship between phagocytosis and transforming activity.

It has been hypothesized that the carcinogenic potency of crystalline NiS particles is derived from the release of ionic Ni²⁺ inside the cell following their avid phagocytosis by facultative phagocytes (potential target cells for transformation capable of phagocytosis but not performing this function exclusively). Thus, particle dissolution is rate limiting in the delivery of oncogenically active ionic nickel across the nuclear membrane and into the nucleus, since this membrane represents a barrier to the interaction of particulate crystalline NiS with DNA (9). Studies by Abbracchio et al. (3) suggested that the dissolution of phagocytosed crystalline NiS particles was accelerated by several possible cytoplasmic events of which lysosomal interaction was implicated as the most probable, since the acidic pH of the lysosomes could enhance the dissolution of crystalline NiS particles. The purpose of the present study was 3-fold: (a) to further characterize the process by which crystalline NiS particles are phagocytosed; (b) to observe cytoplasmic lysosome-particle interactions; and (c) to determine particle movement and distribution inside the cell. For this study, we have used the technique of video time-lapse microscopy to obtain a continuous record of the interaction of crystalline NiS with the cell. Our results describe the phagocytosis process and lend support to the proposed lysosomal interaction with cytoplasmic crystalline NiS particles.

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: Dvorak-Stotler chamber from Nicholson Precision Instruments, Inc., Bethesda, Md.; crystalline NiS from Alfa Inorganics; acridine orange from Sigma Chemical Co., St. Louis, Mo.; McCoy's Medium 5A from Grand Island Biological Co., Grand Island, N. Y.

Methods

Time-Lapse Video Recording. CHO³ cells in the Dvorak-Stotler...
chamber were observed with a Leitz Diatr ret inverted microscope using a x 63 oil lens (phase) N.A. 1.4. The platform of the Leitz microscope was enclosed in a plexiglass box where the temperature was maintained at 37° by a hair dryer (on warm setting) connected to a Yellow Springs Instrument Co., Inc., Termistemp temperature controller (Model 71A). The microscope was equipped with a 50-watt tungsten halogen lamp illuminator, a Ploemopak fluorescence vertical illuminator, and a 100-watt mercury and 75-watt xenon epilluminator (28).

An RCA (Model TC 1030) silicon-intensifier target television camera was mounted directly by a Leitz adapter to the microscope (28). The signal from the camera was patched into a Panasonic time-lapse videotape recorder (Model NV-8030) and a Panasonic video monitor (Model NV-5300). All observations reported here were with oil immersion x 63 objective (made with the Dvorak-Stotler chamber) and recorded at regular speed or at an 18/1 time lapse on 0.5-inch Hitachi video tape (R-176) (28).

Tape records of video images were photographed either from the monitor as single frames or during continuous playback. Polaroid black and white photographs were taken using a Polaroid Model CU-5 land camera (5-inch lens), equipped with a Polaroid Cathode Ray Tube hood (Model 69-49) (12-inch diagonal, at f/2, 1/15 sec). Prints were also made from 16-mm film converted from videotape.

Cell Culture. CHO cells grown in monolayer were maintained in McCoy's Medium 5A supplemented with 10% fetal bovine serum in an atmosphere of 5% CO2/95% air. Cells were removed from the monolayer by trypsinization, and approximately 1000 cells were introduced at regions of active cellular ruffling. Fig. 1, d and e, demonstrates the progressive formation of a membrane envelope around the particle (t = 5.7 to 6.0 min). Fig. 1, f and g, (t = 6.9 to 8.7 min) records the final passage of the particle to the inside of the cell (total time = 8.7 min). The membrane ruffling is greatly diminished in Fig. 1g as indicated by the return of the clearly defined cellular edge with the remaining crystalline NiS particle (short arrow) still bound to cell surface. Following endocytosis, the particle moves from the site of uptake by a process of saltatory motion, and the path of motion can be followed in Fig. 1h. The internalized particle in Fig. 1h (arrow) was followed for an additional 10 min, at the end of which it has traveled approximately 10 μm. The uptake of the crystalline particle represents the first step of in vitro transformation diagrammed in the model in Chart 1.

Movement of NiS Particles to Perinuclear Region. Fig. 2 shows the movement of internalized crystalline NiS particles from a region of membrane ruffling to the perinuclear area. Fig. 2a shows a particle at 0 sec. Twelve min later, the particle has traveled approximately 20 μm in distance and has encountered a phase-dense lysosome (Fig. 2b). Fig. 2c documents the path of particle from 0 time (*) to its position at 13.2 min. A bright-field micrograph at 24 min (Fig. 2d) shows the light-opaque nickel particle in close proximity to the nucleus. The particle also appears inside a large vacuole which may have formed as a result of lysosomal fusion.

The movement of the particle, once inside the cells, is characterized as saltatory movement. Saltatory motion is distinguished from simple Brownian movement by sudden particle movement, usually of many μm and at velocities in excess of those that they may have had prior to saltation or discontinuous jump (20).

RESULTS

Uptake of Crystalline NiS Particles. Because of their opaque nature, crystalline NiS particles are readily observable during phagocytosis. Following 2-hr treatment of CHO cells with NiS, cells were observed under bright-field optics to first locate those cells bearing NiS particles attached to their surfaces. Fig. 1 shows a sequence taken from videotape record of the internalization of a crystalline NiS particle by a CHO cell. Fig. 1a (t = 0) shows 2 surface-bound nickel particles which were first located as 2 black spots by bright-field optics. With phase contrast, both appear white in the figure. Fig. 1, a through h, filmed at 18/1, illustrates the sequence of events which occur during phagocytosis of one of these particles (long arrow). Within 4 min (Fig. 1c), a phase-dense region begins to form beneath the particle on the right. The particle on the left has disappeared from focus as a result of its position on a developing membrane ruffle (note the loss of the sharp demarcation of the edge of the cell which could be observed previously in Fig. 1, a and b). Internalization was observed frequently at regions of active cellular ruffling. Fig. 1, d and e, demonstrates the formation of a membrane envelope around the particle (t = 5.7 to 6.0 min). Fig. 1, f and g, (t = 6.9 to 8.7 min) records the final passage of the particle to the inside of the cell (total time = 8.7 min). The membrane ruffling is greatly diminished in Fig. 1g as indicated by the return of the clearly defined cellular edge with the remaining crystalline NiS particle (short arrow) still bound to cell surface. Following endocytosis, the particle moves from the site of uptake by a process of saltatory motion, and the path of motion can be followed in Fig. 1h. The internalized particle in Fig. 1h (arrow) was followed for an additional 10 min, at the end of which it has traveled approximately 10 μm. The uptake of the crystalline particle represents the first step of in vitro transformation diagrammed in the model in Chart 1.

Movement of NiS Particles to Perinuclear Region. Fig. 2 shows the movement of internalized crystalline NiS particles from a region of membrane ruffling to the perinuclear area. Fig. 2a shows a particle at 0 sec. Twelve min later, the particle has traveled approximately 20 μm in distance and has encountered a phase-dense lysosome (Fig. 2b). Fig. 2c documents the path of particle from 0 time (*) to its position at 13.2 min. A bright-field micrograph at 24 min (Fig. 2d) shows the light-opaque nickel particle in close proximity to the nucleus. The particle also appears inside a large vacuole which may have formed as a result of lysosomal fusion.

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Movement of NiS Particles to Perinuclear Region. Fig. 2 shows the movement of internalized crystalline NiS particles from a region of membrane ruffling to the perinuclear area. Fig. 2a shows a particle at 0 sec. Twelve min later, the particle has traveled approximately 20 μm in distance and has encountered a phase-dense lysosome (Fig. 2b). Fig. 2c documents the path of particle from 0 time (*) to its position at 13.2 min. A bright-field micrograph at 24 min (Fig. 2d) shows the light-opaque nickel particle in close proximity to the nucleus. The particle also appears inside a large vacuole which may have formed as a result of lysosomal fusion.
Lysosome-crystalline NiS Interaction. Fig. 3 shows a sequence from a video recording of lysosome-particle interaction. Fig. 3, a to c, follows the movement of a lysosome from the cell periphery toward a phagocytosed NiS particle and records its binding to the particle-phagosome. In later sequences (not shown), the lysosomes were observed to detach from the particle and then bind to the same particle. This process of binding and detachment followed by reattachment was repeated over and over. Fig. 3d definitely demonstrates the presence of the large number of particles that this cell has accumulated.

It is of interest to note the perinuclear aggregation of NiS particles. The 2 largest particles in Fig. 3, near the nucleus, moved only slightly during the entire sequence, which covered 17 min of real time.

Lysosome-Particle Interaction Studied by Acridine Orange. Lysosomes were identified by their phase-dense appearance and their ability to accumulate acridine orange. Fig. 4 shows the interaction of 2 lysosomes with a NiS particle. Fig. 4, a and b, is of a video recording of a phase-contrast sequence which shows the approach and interaction of the lysosomes with the particle. The acridine orange-acquiring lysosomes are easily distinguished by their fluorescence and can be seen to be in close contact with the particle. It is difficult to distinguish whether the fluorescence at the particle edge is the result of fusion between lysosome and the membrane envelope containing the particle or merely a reflection off the particle of the lysosomal fluorescence. However, subsequent vacuolization of some particles aggregated around the nucleus suggests that fusion of lysosome with particles must occur.

Aggregation of Crystalline NiS Particles around the Nucleus. As was observed in Fig. 2, crystalline NiS particles, following phagocytosis, move to the perinuclear region. With time, aggregations of particles were observed around the nucleus. After 24 hr, their saltatory motion is greatly diminished, and the particles remain relatively fixed in their positions. Phase microscopy (Fig. 5a) suggested the presence of particles around the nucleus, and bright-field microscopy (Fig. 5b) confirmed that the nuclear aggregated particles were in fact crystalline NiS. The aggregation pattern shown in Fig. 5 is a typical finding at times as long as 48 hr following endocytosis of crystalline NiS particles. Particles were not observed to be exocytosed from the cell at times to 48 hr.

DISCUSSION

The endocytosis of crystalline NiS particles and its intracellular fate are summarized in Chart 1. This scheme is based in part on our video-recorded observation of uptake and lysosomal interaction from 20 different CHO cells and from other studies (1–3, 8–11) reported by this laboratory. The first step in NiS particle carcinogenesis is an endocytic process which brings the particle into the cell enclosed in a phagocytic vesicle. The surface property of the NiS particle is an important determinant for phagocytosis. Abbracchio et al. (1–3) and Heck and Costa (16) have demonstrated that crystalline NiS particles used in this present study have a negative surface potential, while amorphous NiS particles are characterized by a positive surface potential. Video time-lapse studies of amorphous NiS particle interaction with CHO cells show that these particles reversibly bind to the cell surface and are not phagocytosed. The binding and uptake of crystalline NiS particles appear to occur at ruffling areas of the cell membrane. Since the cell membrane has an overall net negative surface charge, it is possible that, in these ruffling regions, the membrane charge is altered, resulting in a favorable electrostatic interaction with negatively charged crystalline NiS particles. Parallel uptake studies performed in a simple salts/glucose medium showed similar results and exclude the possibility that serum proteins, amino acids, or other components modify the phagocytosis of NiS particles (16). We have also observed that, once crystalline NiS particles bind, they tend to remain attached to the cell membrane unlike amorphous particles. These and many other observations shown in Chart 1 and described in other parts of this paper were easily made using time-lapse microscopy and add considerable new information to our previous studies using static light and electron microscopy (8, 9).

The phagocytosis of single NiS particles can be sequentially followed in Fig. 1. As described by Silverstein et al. (24), the cell surface membrane applies closely to the particle, so that the initial phagosome usually corresponds in size and shape to those of the particle (Fig. 1h). Fig. 1, c to e, records the formation of phase-dense membrane folds around the particle. Fig. 1f may represent the opening of the membrane invagination just before final fusion of the membrane folds. The endocytic vesicle or phagosome of each particle taken up has its own characteristic size and shape. Changes in vesicle size may result from later fusion with lysosomes.

Following phagocytosis of crystalline NiS, there is a short lag period during which there is limited lysosomal involvement while the particle is actually moved from the ruffling region (Fig. 2). This may loosely be defined as the second step in nickel particle carcinogenesis in which the particle is moved from the cell periphery to the perinuclear region, during which time substantial lysosomal interaction occurs. In extended cellular processes (Fig. 2), particles are observed to saltate in a longitudinal direction toward the nucleus. Similar observations have been reported by Freed and Lebowitz (15) using time-lapse cinemicrography of cultured Hela cells treated with carbon particles. The latter studies presented evidence linking long saltatory movements with microtubules (15). Linear arrays of microtubules were proposed to be responsible for moving particles to the cytocenter (i.e., in close proximity to the Golgi apparatus and other structures of the smooth endoplasmic reticulum). In our study, many particles were observed to occasionally reverse direction of saltation, but the net movement of all particles was to the perinuclear region where, with time, they aggregated around the nucleus.

The initial interaction of lysosomes with NiS particles was similar to that observed by Willingham and Yamada (29) between macrophages and lysosomes. This phenomenon, termed piranhalysis, is characterized in mouse 3T3 fibroblasts by the fragmentation of pinosomes mediated by repeated interaction with lysosomes. This piranhalytic interaction between lysosomes and NiS-containing phagosome was observed in this study. This period is schematically illustrated in Chart 1 which provides the average time interval of active lysosomal attack.

This contact may result in the exposure of the particle to the acidic content of lysosome. The chemical inference from these findings and the studies of Abbracchio et al. (1–3) suggest that this cytoplasmic interaction accelerates the intracellular dis-
solution of particulate crystalline NiS to ionic nickel.

Particle saltation became greatly reduced with time once the particle reached the vicinity of the nucleus. After 24 hr, particle movement became restricted, limited to a slight rocking action of the particles in a relatively fixed position. No particles were observed to be exocytosed from the cells, even after 48 hr. At 24 hr, distinct vacuoles could be detected around some particles. Vacuolization may in part be due to the fusion of lysosomes with NiS-containing phagosome. The slight fluorescent crescent at the edge of the particle in contact with the lysosome (Fig. 5c) may represent lysosome content entering the phagosome in the region between the phagosome membrane and the particle surface.

The close proximity of the particles to the nucleus as shown in Fig. 5 represents a third stage of the nickel particle carcinogenesis where the release of nickel ions would result in their delivery across the nuclear membrane. Since examinations of numerous electron micrographs of cultured cells treated with crystalline NiS revealed that no particulate nickel entered the nucleus (8, 9), yet other studies demonstrated the presence of ionic nickel in the nucleus (12), dissolution of crystalline NiS particles may be analogous to a carcinogenesis activation step, and the involvement of lysosomes is key to this process. Ionic nickel binding proteins such as metallothionein may accelerate the dissolution of the particles by altering particle surface equilibrium between dissolved and particulate nickel. The cytoplasmic dissolution of crystalline NiS particles would be greatly accelerated by the acidic pH of the lysosomes and by the rapid removal of ionic nickel on the particle surface through its chelation to metal binding proteins.

Since ionic nickel appears to be the ultimate carcinogen in mediating the genotoxic effects of crystalline NiS, it is noteworthy that ionic nickel can lead to DNA-protein cross-linking (6), DNA strand breaks (6, 22), chromosomal aberrations (19), decreased fidelity of transcription (25), and other genetic effects (4) which would contribute to its induction of neoplastic transformation (5, 7, 12, 13, 21). The reason for the potent effects (4) which would contribute to its induction of neoplastic transformation of Syrian hamster cells exposed in vitro to nickel subsulfide (12), dissolution of crystalline NiS particles may be analogous to a carcinogenesis activation step, and the involvement of lysosomes is key to this process. Ionic nickel binding proteins such as metallothionein may accelerate the dissolution of the particles by altering particle surface equilibrium between dissolved and particulate nickel. The cytoplasmic dissolution of crystalline NiS particles would be greatly accelerated by the acidic pH of the lysosomes and by the rapid removal of ionic nickel on the particle surface through its chelation to metal binding proteins.

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REFERENCES

Fig. 1. Phagocytosis of crystalline nickel sulfide particles. Particle phagocytosis was recorded on videotape at 18/1 time lapse from which Polaroid photographs were taken. In a to f, there is a total time lapse of 12 min. In a (0 min), 2 crystalline NiS particles (white appearance under phase contrast) become bound to the CHO cell surface. One particle (long arrow) will be phagocytosed; the other particle (short arrow) remains on the cell surface. \( \times 1700 \).
**Fig. 2.** Movement of internalized NiS particle to perinuclear region. Movement of a single particle (black arrow) was followed for 24 min. a to c, phase-contrast photographs in which the particle appears white; d, a bright-field view where the particle appears dark. It can be seen in d that other particles have also entered the cell.

**Fig. 3.** Lysosomal interaction with internalized crystalline NiS particles. CHO cells were treated with crystalline NiS (20 µg/ml) for 2 hr, washed, and incubated for 16 hr in complete medium. Video time lapse was 18/1. Total real time elapsed between a and d is 17 min. In a to d, the interaction of NiS particles (white arrow) with a lysosome (black arrow) is followed. d, a bright-field view at 17 min. NiS particles are dark in appearance (light opaque). The particle indicated by the arrow is the same particle in a to c. P, particle; L, lysosome; N, nucleus, × 1700.
Fig. 4. Acridine orange staining of lysosome interacting with crystalline NiS particles. CHO cells were first treated with 20 µg of crystalline NiS per ml for 2 hr, washed, and incubated for 3 additional hr before treating with acridine orange (1 mg/ml) as described in "Materials and Methods." a (0 min) and b (40 sec), the approach and binding of 2 lysosomes to a crystalline NiS particle video recorded with phase-contrast optics; c (45 sec), the fluorescent lysosomes bound to the particle. lys, lysosome; P, particle; N, nucleus, × 1700.

Fig. 5. Aggregation of crystalline NiS particles around nucleus. CHO cells treated with crystalline NiS (20 µg/ml) for 2 hr, washed, and incubated in fresh medium for 48 hr. a, a phase-contrast photograph of NiS particles around the nucleus; b, a bright-field photograph of the same cellular region. Bright-field microscopy allows for easy identification of the particles. × 1700.
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