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In Vitro Interaction of Zeolite Fibers with Individual Cells (Macrophages NR8383): Measurement of Intracellular Oxidative Burst

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Inhalation of fibrous minerals such as asbestos and erionite can cause various lung diseases, including cancer. The mechanism by which these fibers induce disease is an area of active research. Interaction of fibers with lung macrophages leads to release of many substances. Among these, reative oxygen metabolites (which include hydrogen peroxide, superoxide, and possibly hydroxyl radicals) are proposed to cause cellular damage. In this paper, we report a method for observing intracellular hydrogen peroxide release as rat lung-derived macrophages (NR-8383) phagocytize erionite fibers. This is possible by observing the fluorescence of 2′,7′-dichlorofluorescein—the intracellular, oxidized form of 5 (and 6)-carboxy-2′,7′-dichlorodihydrofluorescin formed in the presence of newly released hydrogen peroxide. We are able to image the fluorescence within a single cell, thereby allowing us to get information on the spatial distribution of the metabolites.

The study of the pathogenesis of pulmonary toxicity related to inhalation of dusts is an active area of research.[1] In particular, asbestos, a naturally occurring hydrated silicate which was until recently used as a building material, has been associated with lung and pleural tumors as well as chronic lung disease.[2] Properties of dusts which are related to cell damage involve fibrous geometry,[3] size,[4] dissolution chemistry,[5] and surface properties.[6] The biological response to inhaled fibers begins with alveolar macrophages (AMs). Exposing AMs to asbestos produces a myriad of effects, including release of fibroblast growth factors, fibronectin, prostaglandins, plasminogen activators, platelet-derived growth factors, enzymes, and reactive oxygen metabolites (ROMs).[7] ROMs include superoxide, hydrogen peroxide, and hydroxyl radicals and are the end product of the oxidative burst. The mechanism of the formation of hydroxyl radicals is still being elucidated. These ROMs have been proposed as causative agents for mineral dust-induced diseases.[8]

Significant progress has been made in measurement in vitro of ROM release by macrophages upon phagocytosis of dust particles. Considering the complexity of the disease process, conclusions of a correlation between ROM production in these in vitro assays and the development of toxicity need to be carefully drawn. Nevertheless, ROM release upon phagocytosis is an important biological process, and its role needs to be clarified. The methods used to measure ROMs include reduction of cytochrome *c* by superoxide ion,[9] luminol chemiluminescence,[10] and reduction of scopoletin emission.[11] These measurements are typically performed on a large sample ($\sim 10^6$) of cells. Based on such assays, it has been noted that the fibrous geometry of particulates is an important factor in determining the magnitude of the oxygen metabolite release.[12] Recently, the role of the surface composition of the fiber as providing reactive "catalytic" sites that may stimulate the production of ROMs or enhance their effects has been discussed.[13] To definitively establish the basis of this hypothesis, it is necessary to produce fibers with well-defined geometric features and with a range of surface compositions. Also, in order to measure the ROM release by macrophages, the dosage of fibers needs to be controlled. It has been noted that fiber dosage should be expressed in terms of number of fibers per unit area (not simply as mass/area), since fiber number per unit *mass* will vary widely as fiber dimensions are altered.[14] In conventional assays, where a large number of cells are exposed to a large number of fibers, this control over fiber dosage is not possible. Moreover, with the possible exception of luminol assays, these methods measure the release of extracellular ROMs. An ideal technique would measure the ROMs released as a single cell interacts with a single, well-characterized fiber. Flow cytometric techniques have been developed to address the

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internal release of ROMs in single cells,[15] but temporal and spatial evolution within a given cell cannot be followed.

Recently, Reynolds and Hastings demonstrated the feasibility of measuring ROMs in cultured neurons at the single-cell level.[16] Their approach was based on the oxidation of 2′,7′-dichlorodihydrofluorescin by ROMs and detection of the fluorescence of the resulting product. Using the same dye system, we report here a method which makes it possible to monitor the formation of ROMs (in particular, H_2O_2) within a single macrophage after phagocytosis of mineral fibers.

The mineral we have examined is naturally occurring erionite. Erionite is a fibrous zeolite and was chosen for two reasons: (1) our research group has considerable experience with zeolite synthesis and characterization[17] and (2) erionite is one of the most potent carcinogenic fibers known. Exposure to erionite has been epidemiologically correlated with pleural and peritoneal mesothelioma in humans.[18] Thus, the use of erionite in cell cultures represents an excellent model system for developing ROM detection. The cell line of macrophages (NR8383) that we have chosen is derived from rat pulmonary alveolar macrophages and has been extensively characterized by Helmke and co-workers.[19] This line is characterized as having the potential of a profound oxidative burst following appropriate stimulation.

EXPERIMENTAL SECTION

Cell Culture. The cell culture (NR8383) was obtained through the cooperation of R. J. Helmke (University of Texas, Health Science Center, San Antonio, TX). The cells were cultured at 37 °C and 5% CO_2 using Hams F12 medium with 15% fetal bovine serum (Sigma Chemical, St. Louis, MO) and were maintained in 30 mL polystyrene culture flasks (Costar, Cambridge, MA). For use in the subsequent experimental procedures, aliquots of cells were grown in 15 mL polypropylene centrifuge tubes (Life Science Products, Denver, CO) to minimize cell adherence.

Erionite Fibers. The erionite sample was obtained from Minerals Research (Clarkson, NY). These samples were deposits from the northwestern United States. The erionite sample powder diffraction pattern was taken with a Rigaku Geigerflex D/Max 2B diffractometer using Ni-filtered Cu Kα radiation. Electron micrographs of the erionite sample were obtained with a JOEL JSM820 scanning electron microscope.

Reagents. The dye 5 (and 6)-carboxy-2′,7′-dichlorodihydrofluorescin diacetate (H_2DCF-DA) was obtained from Molecular Probes (Eugene, OR); 25 mg of H_2DCF-DA was dissolved in 5.0 mL of anhydrous DMSO (Aldrich Chemical, Milwaukee, WI) and divided into aliquots for freezer/desiccator storage. The buffer used during experiments performed with the ACAS 570 cytometer (ACAS buffer) was prepared from glucose and salts obtained from Jenneile Chemical (Cincinnati, OH) (except as noted) and contained 127 mM NaCl, 0.8 mM $MgCl_2$ (Mallinckrodt, Paris, KY), 3.8 mM KCl, 1.2 mM KH_2PO_4 (J. T. Baker Chemical, Phillipsburg, NJ), 1.2 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, Sigma Chemical). ACAS buffer was adjusted to pH 7.4 by addition of NaOH.

Cell−Fiber Contact. The following protocol was followed to establish cell−fiber contact. Cells (∼200 000/mL) were washed by centrifugation, removal of supernatant, and resuspension in ACAS buffer. The cells were allowed to equilibrate with buffer during 30 min incubation at 37 °C and 5% CO_2. Equilibrated cells were then exposed to H_2DCF-DA (final concentration, 60 μM) and incubated at 37 °C and 5% CO_2 for 40 min. The cells were then removed from incubation and immediately chilled (ice−water bath, 4 °C) to induce metabolic inactivity. Chilled cells were then subject to 1 mL of ACAS buffer (control) or 1 mL of fiber suspension in buffer (∼1.4×10^6 particles/mL), vortexed briefly (10 s), and then centrifuged (∼600 rpm for 5 min) at this same (4 °C) temperature in order to promote contact of the fibers with the cell surface. The supernatant containing debris and excess dye was removed and discarded. The pelleted cells (and cells with fibers) were immediately resuspended in chilled ACAS buffer. This cell−fiber mixture was then taken to the fluorescence spectrometer, transferred to a warmed Lab-Tek chambered coverglass (NUNC, Inc., Naperville, IL), and rapidly warmed to 37 °C in a temperature-controlled chamber on the microscope. Cells were then analyzed for fluorescence intensity. The first 5−8 min of this period involved allowing cells to settle and adhere to the coverslip bottom of the chamber. Since the chambered coverglass was clipped to a piezoelectrically driven stage (0.1 μm steps possible), and since the position of the stage is continually updated to the computer, cell position coordinates were recorded during this initial time as well. Once these position coordinates were obtained, the time involved in movement from one cell to another and back to a previous cell became trivial. Cells were selected for analysis primarily on the basis of representative morphology and, in the case of the erionite-exposed cells, contact with at least one fiber.

Fluorescence Imaging. Images of the fluorescing cells were obtained with a commercial spectrometer manufactured by Meridian instruments. This instrument, the ACAS 570 laser cytometer, has been employed by previous researchers for quantitative fluorescence measurements including, among others, the measurement of exogenous peroxide within cells,[20] the measurement of intracellular glutathione levels,[21] and (as mentioned above) the measurement of ROMs within neurons.[16] For the experiments described herein, the ACAS 570 used an acoustooptically modulated Ar ion laser tuned to 488 nm to excite the fluorescence in the cell, and emission at wavelengths > 515 nm (filter) was detected with a photomultliper tube. The microscope optics used for focusing the laser also allow visualization of the sample volume. The capability of the microscope to perform confocal imaging allows collection of fluorescence from thin (0.8 μm) sections of the cell as rapidly as 1 min/slice, depending on the size of the image.

RESULTS AND DISCUSSION

Nature of the Erionite Sample. A protocol was set up to focus only on small fibers (length < 10 μm) of the erionite sample. This size would be conducive to complete phagocytosis. This was done by dispersing the as-obtained material in buffer. The suspension was vortexed and allowed to stand for 5 min. During

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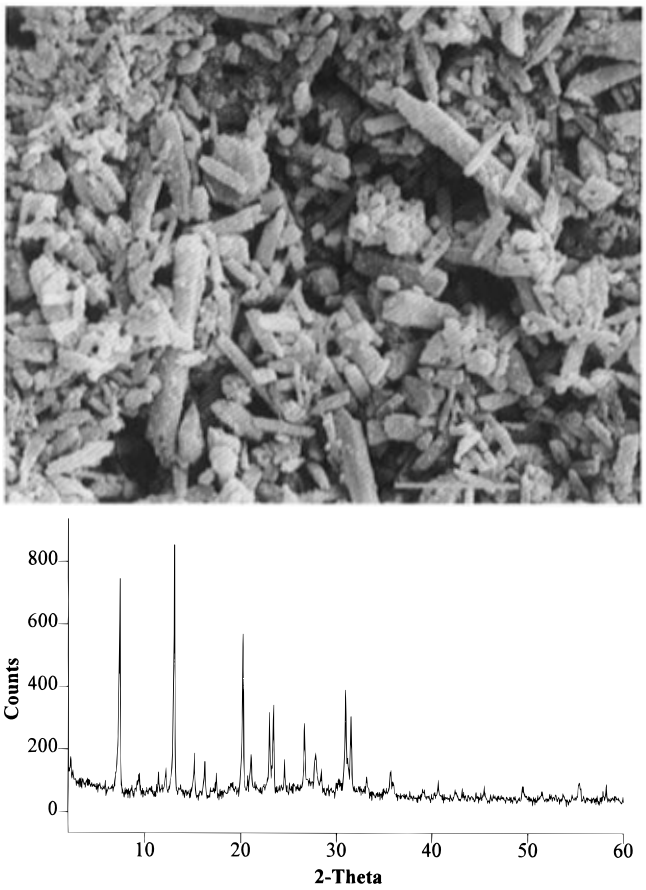

Figure 1. (a, top) Scanning electron micrograph of a dispersion of erionite fibers (3000× magnification; scale bar = 10 μm). (b, bottom) Powder X-ray diffraction data from the erionite sample.

this time, most of the sample settled out, leaving only a cloudy supernatant. Samples used in this study were collected from the upper part of this cloudy zone. For example, Figure 1a shows the SEM micrograph of such a sample. It is to be noted that, besides fibers, smaller colloidal dust is also present. The confirmation that these particles are erionite comes from the powder diffraction pattern shown in Figure 1b. These peaks are characteristic of the erionite structure.[22]

Phagocytosis. It was essential to establish that the alveolar macrophage cell line was readily capable of phagocytosis. Figure 2 shows an optical micrograph indicating a fiber (arrow) to be present within the cell, thus illustrating that completion of the phagocytic process had already occurred.

Loading of Reduced Dye into the Macrophage. The strategy that we followed to introduce the reduced form (H_2DCF-DA, **I**) into the cells is an adaptation from previous studies.[15b] Figure 3 is a schematic representation of this process. The reduced, esterified dye form (**I**) enters the cell and is deesterified by (nonspecific) cellular esterases to form **II** (H_2DCF). This is expected, since Helmke and co-workers have reported that the NR8383 cell line exhibits high, nonspecific esterase activity.[19a] The oxidation of **II** to the fluorescent form **III** (DCF) by ROM is detected. Oxidation of H_2DCF in aqueous media has been shown

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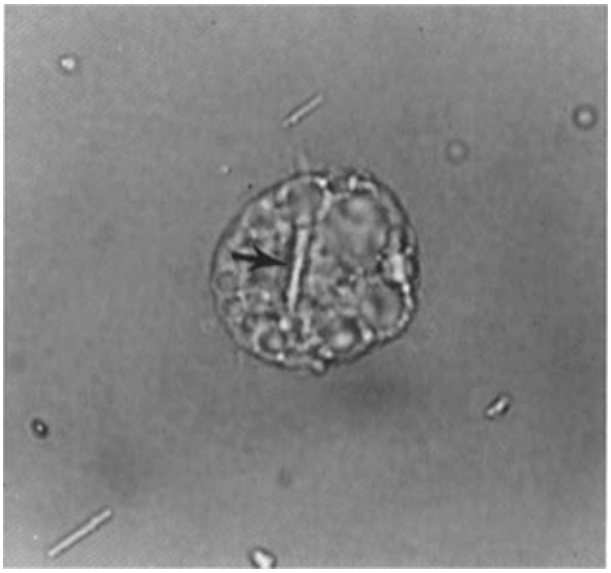

Figure 2. Optical micrograph of phagocytosis of fiber (arrow) by a macrophage.

to occur by H_2O_2 in the presence of either peroxidase or ferrous iron.[15b,23] Oxidation by superoxide ion remains dubious. The fluorescent form (**III**) has an absorption maximum at 504 nm and an emission maximum at 523 nm (communication from Molecular Probes, Inc.).

Imaging of Intracellular Fluorescence. For all color images (Figures 4 and 5), color intensity reflects fluorescence intensity according to the color scale provided. White and red reflect stronger signals (and, therefore, stronger fluorescence intensity), while dark violet and black represent nearly no signal.

Figure 4 shows individual sections of fluorescence imaged from one control cell and one erionite-exposed cell. Both cells were imaged 30 min after the previously described warming of the cold cell suspensions was begun. Clearly, the cell exposed to erionite exhibits stronger, more intense fluorescence and is indicative of higher levels of intracellular peroxide levels. Although the pictured control displays a lower level of intensity as compared to the erionite-exposed cell, the control cell fluorescence is clearly nonzero. Previous researchers have noted nonzero controls for oxidative metabolism and have attributed this background oxidation to factors such as mitochondrial respiration.[24]

Figure 5 depicts several images from an erionite-exposed cell. These represent thin (0.8 μm) cross sections of this cell along the vertical axis taken over a period of about 4 min. The first of these slices was begun 10 min after the warming process described above was initiated. The possibility of observing fluorescence from sections results from the confocal application of the ACAS 570 instrument, which provides submicrometer resolution in the vertical direction. The distribution of the oxidized dye (DCF) is not uniform throughout the cell. This is in sharp contrast to control cells (such as the one depicted in Figure 4), where the distribution of the fluorescence is quite uniform. Phagocytosis is thought to trigger ROM release primarily within the phagocytic vacuole surrounding the fiber. Because of the way we expose our cells to fibers, there is the strong possibility that a single cell can phagocytize multiple fibers as well as colloidal particles that are typically present in the fiber sample. The

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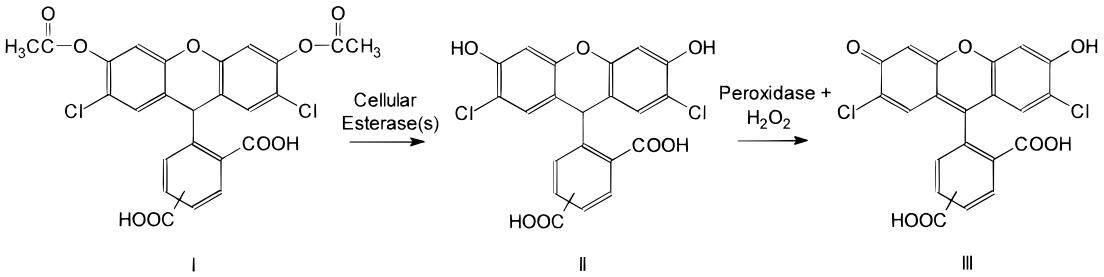

Figure 3. Forms of the fluorescin/fluorescein dye relevant to measuring the oxidative burst.

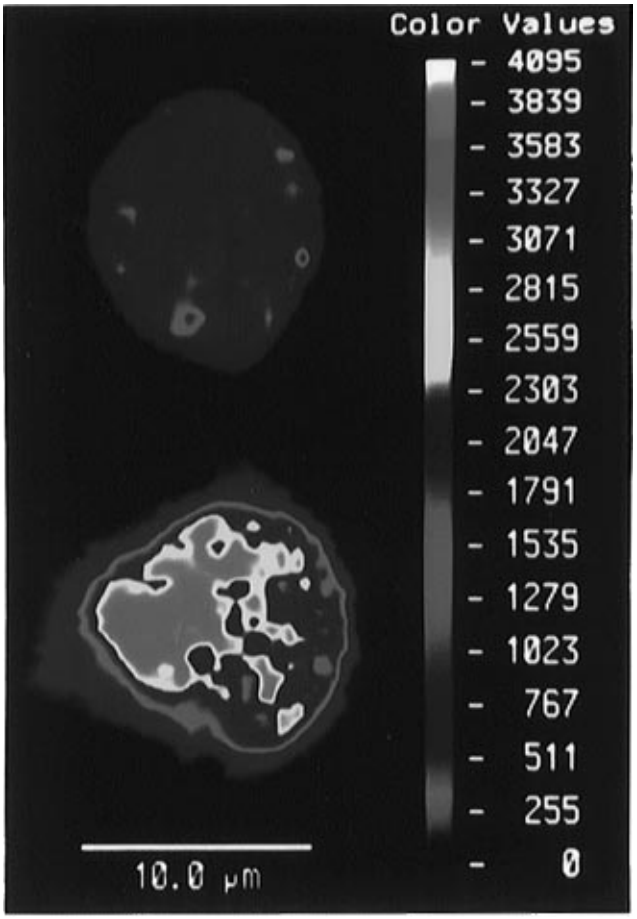

Figure 4. Fluorescence image of macrophage: top, control cell; bottom, cell exposed to erionite fibers. Data taken after 30 min of exposure (colors are artifical and indicate fluorescence intensities).

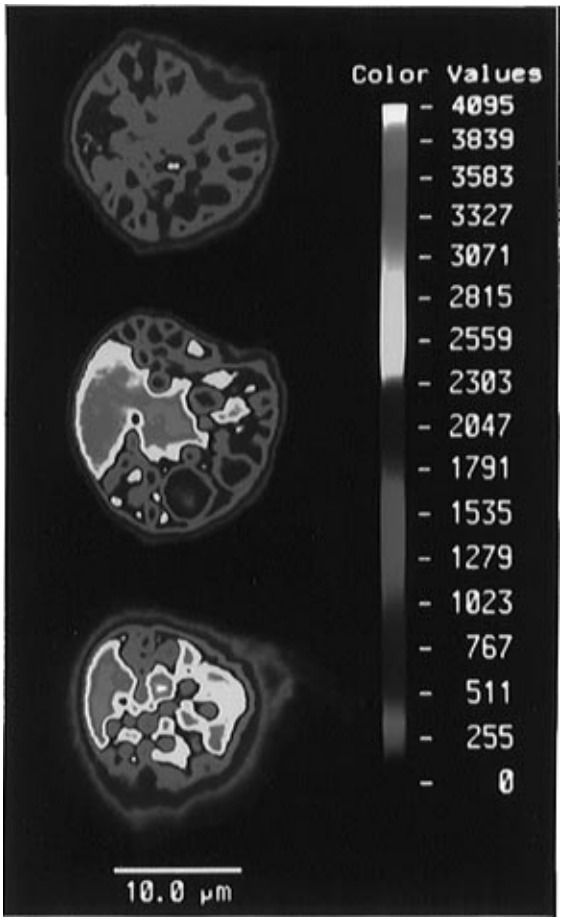

Figure 5. Fluorescence images of a fiber-exposed cell taken along thin (0.8 μm) cross sections along a vertical axis after 10 min of exposure.

inhomogeneous distribution of fluorescence shown in Figure 5 may be reflecting the localization of multiple particles within the cell.

We have demonstrated in this study that the oxidative burst produced within a single macrophage cell upon interaction with erionite fibers can be monitored. Currently, the limitation of the technique is that it does not allow for the introduction of a single, specific fiber into the cell. Resolution of this problem would make it possible to study single cell−single fiber interaction and is under investigation. Nevertheless, the ability to spatially locate the fluorescence is an advantage of the present technique over flow cytometry.

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