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Project Summary

Ultrastructure and X-Ray Microanalysis of Macrophages Exposed to Non-Criteria Pollutants, with Emphasis on Certain Metals

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It is well known that the alveolar macrophage represents one of the important first lines of defense of man and other species against inhaled environmental pollutants. The study was undertaken to increase understanding of the effects of a wide variety of environmental pollutants on alveolar macrophages.

The objectives of this study were as follows: 1) to investigate the ultrastructural effects of certain soluble metals on alveolar macrophages, 2) to investigate the ultrastructural effects of realistic and quasirealistic airborne respirable particles on alveolar macrophages, 3) to develop new techniques not currently available in the literature that would permit study of the subcellular movement (metabolism) of *ions* which either enter cells from solution or which enter cells from surfaces, of particles be they outside cells or within phagolysosomes, and 4) to use these new techniques to study as many soluble ions and respirable particles as possible.

The study resulted in the development of a variety of new techniques which permit investigators to freeze-fix alveolar macrophages and thus to trap normal ions and xenobiotics in their *in vivo* location. Using these techniques, we have studied the subcellular metabolism of numerous soluble ions such as cadmium, nickel, vanadium, copper, cobalt, and maganese as well as a number of particles including 2-5 micron coal fly ash particles with or without coatings of PbO or NiO, and particles of PbO, Pb_3O_4, and NiO. These studies have shown that this work is possible and have pointed clearly to future studies involving new, more sensitive imaging techniques such as secondary ion microscopy. These new techniques will be explored in the future.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Interest in this area began with a series of experiments using NiO and MnO_2 particles Rabbit alveolar macrophages (RAMs) phagocytized these particles and then fixed them with conventional techniques. These cells were then embedded in Epon and studied by SEM/EDX. Several new mapping techniques were developed which worked quite well in the STEM mode

and which have permitted clear illustration of the location of these particles within cells

Next, further study sought to demonstrate location of a soluble metal within the cytoplasm of the cell exposed *in vitro*—not to particles, but to a metal in solution. For the initial experiments, manganese was selected. Manganese was demonstrated within the cytoplasm of cells but it was demonstrable only in electron dense cytoplasmic precipitates In addition, a wide variety of ultrastructural effects of manganese was described. During these studies, manganese could not be detected elsewhere in the cytoplasm of these cells even though the cells had been incubated with rather high levels Also, at this time preliminary experiments with soluble cadmium *in vitro* were initiated. Again, a large number of ultrastructural effects of cadmium using conventional chemical fixation with glutaraldehyde and conventional embedding in Epon were described and published However, even though a galaxy of ultrastructural changes were present, including fascinating nuclear inclusions not previously described in the literature, using this conventionally prepared material it was not possible to detect the location of cadmium even though its presence was very likely High levels had been used for the exposure and subcellular changes were very pronounced.

Physiologists have shown that physiologic ions such as sodium and potassium are redistributed tremendously during chemical fixation and that the only way to preserve their true *in vivo* location is to use freezing techniques. Experiments with manganese and cadmium seem to support that observation for physiologic ions as well as xenobiotics No published papers were available, however, describing how to snap-freeze mammalian cells in suspension so that they could be serially sectioned using cryoultramicrotomy. It was important to cut sections of these cells since this is the only way to get inside of them to map their ultrastructure. Therefore, the first requirement was to develop new techniques for the freezing of macrophages either in suspension or as monolayers. These techniques were used throughout these experiments.

This study shows that cadmium produces a lamellar nuclear inclusion which we have proven to contain cadmium by microprobe analysis Interestingly, similar lamellar nuclear inclusions were observed in RAMs exposed to nickel, either in soluble or particulate form, as discussed below and in RAMs exposed *in vitro* to soluble copper. These inclusions were not observed in RAMs exposed to manganese, lead, cobalt, or iron. In addition to the experiments noted in this manuscript, the following *new* experiments with cadmium were performed.

Since Chinese hamster ovary (CHO) cells possess fewer lysosomes and more mitochondria than do RAMs, these cells were exposed to soluble cadmium as had been done previously for RAMs. The results to date of this work are that cytoplasmic densities similar to those noted in RAMs are still present and these are positive by microprobe analysis for calcium, magnesium and cadmium. The presence of magnesium is strong evidence that these inclusions are in mitochondria. Freeze substituted images are consistent with but do not absolutely unambiguously prove that hypothesis since the mitochondria appeared damaged by the cadmium and thus are difficult to recognize as mitochondria. The striking finding was that cadmium did not produce nuclear inclusions in CHO cells even though it did at the same doses and times produce nuclear inclusions in RAMs. Thus, CHO cells are not a suitable model for studying the pathophysiology of cadmium with respect to its effects on nuclei. Cadmium did produce clumping of heterochromatin in treated cells but this did not appear to be a specific finding in the same sense that cadmium nuclear inclusions are.

Since the cytoplasmic densities observed in previous experiments with RAMs exposed to cadmium also gave peaks for phosphorus when studied by EDX, and because of the presence of extracellular cadmium phosphate precipitates, these experiments were repeated using special phosphate and sulfate free medium. When compared to cells exposed to regular media, control cells exposed to this special media without cadmium exhibited more numerous homogeneous nuclear bodies at 4 hours, swelling of ER cisternae, and peculiar flocculant mitochondrial matrix densities. Cells in this special media treated with cadmium did exhibit characteristic lamellar nuclear inclusions but fewer than observed in previous experiments with regular medium. No cytoplasmic densities at all were observed. Homogeneous nuclear bodies were extremely common. No extracellular precipitation of any sort was seen in frozen sections Thus, this special media did prevent the problem of extracellular precipitation noted previously but the lack of phosphate (probably) seems to have blocked the formation of mitochondrial densities containing cadmium and blocking partially the formation of cadmium specific nuclear bodies

Microprobe analysis of frozen sections revealed - in *marked* contrast to previous experiments with regular media - a surprising lack of detectable cadmium. Nuclear inclusions were magnesium, calcium and phosphorus positive, but only occasionally exhibited a trace of cadmium No other cadmium localization was detected. Perhaps the absence of phosphate prevented cadmium transport into cells (phosphate is, in some instances, necessary for calcium transport). Or, perhaps the phagocytosis of cadmium phosphate precipitates is necessary to get large amounts of cadmium into cells in a hurry (4 hours), bypassing normal membrane barriers. Experiments to test these hypotheses are underway

A series of experiments were conducted on a mutant strain of CHO cells developed by Dr. Ken McCarty, Sr , Department of Biochemistry, Duke University. These CHO cells are resistant to cadmium and can successfully bind large amounts of cadmium intracellularly without visible toxic effects An induced metallothionein is responsible for the remarkable behavior of these cells. Four different variables were studied "Wild" CHO cells with and without cadmium and mutant CHO cells with and without cadmium were incubated on glass and Thermonox coverslips for 4 hours (wild) and 48 hours (mutant) in 0.1 mM cadmium chloride. At the end of the incubation, these coverslips were snap frozen in liquid nitrogen and freeze-dried on an Edwards freeze-drying apparatus or in a rotary vacuum pump chamber. After a light carbon coating, these cells were studied by SEM-EDX Some coverslips were rinsed in medium without cadmium before snap freezing. The principal findings to date are· 1) The glass coverslip is a poor substrate because it contributes to high background radiation and to numerous spurious peaks including silicon, zinc, titanium, and chromium On the other hand, Thermonox coverslips are an excellent substrate as there is no increased background radiation. 2) The mutant cells exposed to cadmium do exhibit cadmium peaks and there are differences between cells. No cadmium

peaks were seen in "wild" cells with or without cadmium exposure. These studies were done using the spot probe mode since cell size varies significantly. 3) "Wild" cells treated with cadmium were rounded. "Wild" cells not treated with cadmium and most mutant cells th *or* without cadmium were relatively flat 4) Sulfur peaks were consistently higher in treated mutant cells than in any of these other groups, possibly due to the presence of the induced metallothionein. 5) The results with rinsed vs unrinsed cells were the same.

In addition, perhaps the most interesting results of this work with CHO cells concerned viral particles. Experiments with CHO cells in two different laboratories have shown that treatment with cadmium causes the production of a type RNA virus, probably a tumor virus In view of the fact that cadmium is a carcinogen, we feel this hitherto unreported finding is a significant discovery

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The complete report, entitled "Ultrastructure and X-Ray Microanalysis of Macrophages Exposed to Non-Criteria Pollutants, with Emphasis on Certain Metals," (Order No PB 81-157 935, Cost $14 00, subject to change) will be available only from.

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