**PROJECT TITLE:** INFLUENCE OF PARTICULATES ON OCCUPATIONAL LUNG DISEASE

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## 3. LIST OF ABBREVIATIONS

Benzo(a)pyrene - BaP
Pulmonary Alveolar Macrophage - AM
PAHs - Polycyclic aromatic hydrocarbons
dG - Deoxyguanosine
dA - Deoxyadenosine
TNF - Tumor necrosis factor
DNA - Deoxyribonucleic acid
BALF - Bronchoalveolar
TLC - Thin layer chromatography
HPLC - High performance liquid chromatography

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## 6. SIGNIFICANT FINDINGS

Summary of data. An in vitro AM culture system was used for assessing both phagocytosis and cytotoxicity of particles as well as metabolism, DNA-binding. The physical characteristics of the particles indicated that crystalline silica had a smaller surface area and a larger count median diameter than any of the amorphous silicas and that gelled had the largest surface area of all the particles tested including ferric oxide or aluminum oxide. Gelled silica based on equivalent doses given to AM was the most cytotoxic, consistent with its large surface area. All forms of amorphous silica had smaller particle sizes and were cytotoxic suggesting that more of the dose for the amorphous silicas entered the AM. The toxicity also appears related to the surface properties; those particles having more silanol groups were more cytotoxic. This may explain the fact that ferric oxide and aluminum oxide were not cytotoxic while silicas were. The dose of the silica is important in that more silanol groups are probably available on the crystalline silica surface and therefore a larger dose of amorphous silica must be incorporated into the AM in order to observe the same toxic effect as crystalline silica. Therefore dose, surface properties and particle size each affect cytotoxicity of silica to AM (see appendix for paper, Warshawsky in press).

These same properties appear to be important in the metabolic activation of BaP by AM. At a similar dose of BaP and particle, all of the BaP-coated particles produced more BaP dihydrodiols than BaP alone. The three amorphous forms of BaP-coated silica produced more 7,8-diol of BaP (in pmol/$10^6$ cells) than the BaP-coated $Fe_2O_3$ and BaP-coated crystalline silica. The 7,8-diol is considered to be the precursor to the ultimate metabolite of BaP. Although these metabolites were produced in low levels, it was sufficient to produce BaP-DNA adducts. In all three cases which were studied, BaP/ppt silica, BaP-crystalline silica and BaP-$Fe_2O_3$ in comparison to BaP alone, the BaP adduct patterns were altered such that adduct #2 was increased relative to adducts 1 and 3. Of these three the relative adduct levels for the precipitated form were greater than that of $Fe_2O_3$ or crystalline silica which is consistent with the greater surface area of precipitated silica. Adduct #1 is the +anti-diolepoxide of BaP bound to dG from P450 cytochrome metabolism while adduct 2 and 3 (have been found to be - anti-diolepoxide of BaP bound to dG and -anti-diolepoxide of BaP bound to dA, respectively) could involve non P450 cytochrome metabolism.

Morphometrically, we have shown that AM exposed to particle coated with BaP, responded differently than either BaP or particles alone at noncytotoxic doses. This approach would give a good estimate of AM activation as a result of coexposure which could be correlated with the biochemical parameters. Based on the data, BaP-coated particles at a single dose in comparison with BaP alone have an impact on the metabolism, DNA binding, the extent of adduction through P450 cytochrome metabolism, and AM activation and perturbation. Additionally for metabolism, DNA binding and cytotoxicity parameters, the surface area of the particle appears to play a major role.

## 7. USEFULNESS OF FINDINGS

There should be further investigation on the effects of different forms of silica such as foundry sands on the metabolism of BaP. It would be important to investigate both physiological changes which will have an impact on defense mechanisms but also on the physical characteristics of particles which seem to play a role in AM metabolism of xenobiotics. It would also be important to determine the role of AM in the lung with other cell types such as the peripheral cells in rat. Comparable intratracheal treatments of hamsters and rats with BaP-ferric oxide have been studied (43). Hamsters produced squamous metaplasia of the trachea and large bronchi (later tumors); in contrast the rat produced no such response but eventually peripheral lung tumors. There were no differences in the retention of BaP in lungs or tracheas. However, BaP was found in the epithelium of the hamster and not in the rat. It appeared that some mechanism prevented the diffusion of BaP in the rat. In terms of long-term objectives these studies will contribute to better understanding of how particulate-dependent factors alter BaP carcinogenic potential. Furthermore, information will be obtained that can be used to 1) to investigate sites and mechanism of action of BaP and/or its metabolic products in lung tissue in vivo, 2) provide insight as to the factors which influence macrophage metabolism and lung disease susceptibility and 3) develop AM to be used as biomarkers in molecular epidemiology and chemoprevention of cancer (96).

## 8. ABSTRACT

Epidemiologic and experimental studies indicate that particles and/or chemical carcinogens are important in the development of respiratory disease. Occupational exposure to silica often includes exposure to PAHs; silica has an enhancing effect on BaP induced lung carcinogenesis. Although the mechanism of cocarcinogenesis is unknown, several investigators have implicated BaP metabolism. An important biological response to inhaled particles is ingestion by AM and clearance from the lung. Since these cells have the capacity to metabolize BaP, it is possible that altered BaP metabolism leading to an enhanced carcinogenic potential occurs in the AM following phagocytosis of silica particles and adsorbed BaP. The long term objective of this research was to investigate the role that AM play in the particulate-dependent response of the lung to BaP via mechanisms involving BaP metabolism.

AM from hamsters and rats were incubated with particles and BaP-coated particles such as crystalline, and amorphous (precipitated, gelled, and fumed) silicas, ferric oxide, and aluminum oxide. Cytotoxicity endpoints were used to determine the noncytotoxic doses. Analyses were performed using extraction procedures, HPLC, $^{32}$postlabeling, morphometry, liquid scintillation spectrometry and reverse Salmonella mutagenesis assays. The data indicated that dose surface properties as well as particle size affect that cytotoxicity of silica to AM. Secondly, that BaP coated particles in comparison to BaP alone have an impact on metabolism DNA binding and the extent of adduction through P450 cytochrome metabolism. Lastly, the surface area of the particle appears to play a major role on the above parameters. In the evaluation of occupational hazards that may lead to increased susceptibility to lung cancer, the cocarcinogenic potential of an exposure is an important consideration. This research provided information on particulate modified BaP metabolism and contributed to our understanding of the involvement of AM in the mechanism of lung disease.

## 9. BODY OF REPORT

### Background

Numerous epidemiologic and experimental studies indicate that various types of dusts, such as silica and ferric oxide ($Fe_2O_3$), and/or chemical carcinogens are important in the development of respiratory disease in the environment (1,2). An important aspect of these dust exposures is a cocarcinogenic effect when exposure to other carcinogenic agents occurs. For example, exposure to ferric oxide ($Fe_2O_3$) alone, is not associated with cancer induction (3). However, coexposure to $Fe_2O_3$ and carcinogens such as polyaromatic hydrocarbons (PAH) or ionizing radiation is associated with an increased incidence of lung cancer, when compared to control populations (3), and suggests a synergy resulting from exposure to the mixture. This is of importance since exposure to silica nearly always includes exposure to such substances as radon, asbestos and PAH (4).

Benzo(a)pyrene (BaP), a ubiquitous occupational and environmental pollutant is the product of incomplete combustion associated with fossil fuels and cigarette smoke (5). It is known that the biological activity of BaP is influenced by its metabolic fate (6) such that the target tissue must be exposed to an appropriate level of reactive metabolites which alter critical cell regulatory mechanisms. Although it is likely that several factors contribute to the particulate-related susceptibility to BaP lung carcinogenesis, the studies outlined in the proposal specifically address the role of BaP metabolism by AM. An important biological response to inhaled particulates is ingestion by AM and release of oxygen radicals such as superoxide anions and a variety of cell mediators into the surrounding tissue. Many chemical agents can be metabolized by AM to products that may be more active than the parent compound (7,8). It is possible that altered BaP metabolism leading to an enhanced carcinogenic potential occurs in the AM following phagocytosis of silica particulates and adsorbed BaP. It is proposed that silica affects AM metabolism of BaP such that the efficacy of BaP to induce lung tumors is potentiated; the potentiation can be due to either altered P450-mediated metabolism or by oxidative metabolism induced by ingestion of particles.

Silica and Lung Disease. Silica is a common industrial particle. It has been estimated that approximately 250,000 workers in 114 occupations are exposed to silica flour alone (9) and over 500,000 workers are exposed to synthetic amorphous silica (10). There have been many epidemiologic and experimental studies relating silica exposure and the development of fibrotic lung disease (11). These studies have indicated that all forms of crystalline silica can cause fibrotic lung disease; for any quartz polymorph the severity of the disease appears to be related to dose. Coating silica particles with $Al_2O_3$ has an inhibitory effect on the development of fibrotic reactions (12,13). There is limited evidence to indicate that crystalline silica is carcinogenic in humans who have not developed silicosis, while amorphous silica is not carcinogenic in humans (4). Silica, administered either by inhalation or intratracheal (IT) installation, has been shown to elicit a carcinogenic response in rats of two strains and both sexes (14,15) and it has been hypothesized that silica either directly induces lung cancer or causes fibrosis leading to cancer (16). In the hamster, however, silica did not produce a carcinogenic response and is usually

not associated with fibrosis (17,18).

Exposure to silica often includes exposure to PAH (4). Epidemiologic studies have concluded that there is a significant excess of lung cancer among foundry workers (19). It has been implied that other organics and metals act as carcinogens and particles act as irritants, cocarcinogens and promoters (20).

Workers are also exposed to many other types of dust, such as ferric oxide, which do not appear to cause completely debilitating lung disease. It has been estimated that approximately 450,000 individuals are exposed to ferric oxide in 150 occupations (9). Numerous epidemiologic studies have examined the effect of exposure to ferric oxide in the workplace. In many of the cohorts, persons exposed to a mixture of ferric oxide and potential carcinogens such as PAH or ionizing radiation had an increased incidence in various forms of cancer, particularly lung cancer when compared to a control population. Workers exposed to only ferric oxide had no increased incidence in any form of cancer when compared to control populations (2,3).

PAH-Particulate In Vivo Studies. Animal models demonstrate that a particle such as crystalline and amorphous silica and foundry sands enhance the carcinogenicity of BaP in the lung. The hamster and the rat have been used as in vivo models for studying the interaction of PAHs and particulates (21). The hamster has been commonly used because lung carcinomas in hamsters more closely resemble the human in terms of tumor pathology, histogenesis and ultrastructural morphology (22,23). The hamster has a low incidence of spontaneous pulmonary tumors and is highly resistant to pulmonary infections.

A variety of particulate carriers have been used in the hamster model. BaP in combination with ferric oxide increased the incidence of tumors of bronchiogenic origin following intratracheal administration (22,25). Similar increased respiratory co-carcinogenic responses have been reported for other particulates, such as amorphous silica (25), titanium dioxide (26), India ink (27), carbon black (26), iodine (28) and magnesium oxide (29). Results with aluminum oxide (29) and manganese dioxide (28) did not show cocarcinogenic responses with BaP. Crystalline silica and foundry sands, in conjunction with BaP, caused an increased carcinogenic response in the hamster in the range similar to that of ferric oxide and BaP (17). Animals receiving only the particles did not develop any tumors.

Metabolic Activation of BaP. As with all PAHs, it is not the BaP itself but rather its metabolites which are responsible for the majority of biological effects formed through cytochrome P450 metabolism. BaP phenols, dihydrodiols, epoxides and diol-epoxides have demonstrated different degrees of cytotoxicity, mutagenicity and carcinogenicity (6). The rate of BaP metabolism is decreased in the presence of particulates in the lung, such as asbestos (30), ferric oxide (31), cigarette smoke (33), and flyash (36). However the metabolic pathways appear altered and binding of BaP metabolites to macromolecules (33) increased in comparison to BaP exposures alone. BaP may also be metabolized to DNA binding species through the action of tissue peroxidases which involve peroxyl radicals associated with prostaglandin H synthesis and lipoxygenase catalyzed oxygenation reactions (34-36). By quantitating the extent of conversion of (+)-BaP-7,8-diol to diol-epoxide products, the relative contribution of cytochrome P450 (+syn-

diol-epoxide) and noncytochrome P450 (-anti-diol-epoxide) processes related to epoxidation can be estimated. This approach has been used to demonstrate noncytochrome P450 dependent epoxidation of (+)-BaP-7,8-diol in cultured cells, cultured hamster trachea, freshly isolated mouse epidermal cells and epidermal homogenates and mouse skin in vivo (34-36). $^{32}$P-postlabeling systems which can resolve adducts produced by each pathway have been developed (34-35,37).

Retention of PAH-Coated Particulates in Lung. The enhancing role of particles can be due to the transport of the chemical compound by the particles into the interstitial tissues of the lungs. The particulates can penetrate through the epithelium and can be phagocytized by macrophages in the interstitial spaces or in the alveolar lumina (24). Organics may then be eluted from the particles and diffuse into the surrounding lung tissue (38). It has been suggested that the particle effect is nonspecific and perhaps is merely a means of providing longer residence times in target tissue (38,40). On the other hand more recent studies indicate that the residence time of carcinogens adsorbed on particles in the deep lung is very short, on the order of seconds or minutes (41). Physical characteristics and surface properties are all important in the retention abilities of the particles. In lung perfusion studies, particulates (ferric oxide, flyash, crude air particulate) decreased the rate of appearance of BaP in the blood and altered the metabolic pattern (33), possibly as a result of altered bioavailability of BaP adsorbed to the particulate (33). Phospholipid vesicles and rat liver microsomes take up more BaP in the presence of particulates when compared to uptake without particulates (42). In addition, retention in the lung of BaP-coated silica could lead directly to lung cancer or fibrotic changes characteristic of silicosis followed by a carcinogenic response as a result of cells dividing in the presence of BaP (16).

Evaluation of these data requires consideration of species. For example, comparable intratracheal treatments of hamsters and rats with BaP-ferric oxide have been studied (43). Hamsters developed squamous metaplasia of the trachea and large bronchi (later tumors); in contrast the rat produced no such response but rather an initial granulomatous response and epidermoid metaplasia in the peripheral lung followed by tumors. No differences in the retention of BaP in the lungs were found except that BaP was present in the epithelium of the hamster but not in the rat. Both species showed BaP-containing AM in the tracheobronchial lumen. These findings suggested that in the hamster BaP diffused from the luminal macrophages through the mucociliary layer into the tracheobronchial epithelium but in the rat some mechanism prevented the diffusion of BaP (43). Diffusion of the AM through the interstitial tissue from the lungs to the bronchi and trachea is also possible (21).

Role of AM. Mechanisms mediated by AM and surrounding cells, therefore, must consider the effect of particles on the metabolic activation of organics such as BaP. Human and dog AM (44-47) can metabolize BaP and phagocytize BaP-coated particulates, such as ferric oxide and diesel particulate (45,48) with subsequent release of BaP and metabolites (44,48,45) and binding to DNA and protein (48). Co-incubation of human AM with V79 cells showed that AM could metabolize BaP to induce ouabain-resistant mutations and sister chromatid exchange (49). Other studies with $Fe_2O_3$ coated with BaP have resulted in increased mutagenic responses (50-52). It is apparent that the metabolism of BaP-coated particles by AM may

be very important in the overall development of lung disease.

Additionally there is considerable evidence that AM from different sources produce eicosanoids (arachidonic acid metabolites) (53-56) and that the production can be modulated by stimuli such as crystalline silica (54). It has also been shown that AM respond to stimuli through phagocytosis by releasing reactive oxygen intermediates as well as lipid oxidation products through noncytochrome P450 metabolism (8,53,57). Therefore, it seems reasonable that similar noncytochrome P450 metabolism reactions of BaP take place in the AM and which can be measured by $^{32}$P-postlabeling. This mechanistic approach would be in line with the altered BaP adduct patterns which we have observed for BaP-coated particulates versus BaP alone (see Results and Discussion).

Efforts have been made to identify potential biomarkers of pulmonary toxicity to better predict and interpret responses in the lung. Investigations have focussed on the underlying pulmonary responses to dust exposure such as silica and titanium dioxide. Bronchoalveolar lavage fluid (BALF) was analyzed for lactate dehydrogenase, total protein and $\beta$-glucuronidase and differential BALF levels were more pronounced due to exposure to silica (58,59). Potential biomarkers of pulmonary toxicity have also focused on AM release of a variety of lipid and protein mediators which can recruit and activate cells involved in tissue injury and repair e.g. epithelial cells and this activity have been thought to play an important role in the pathogenesis of lung disease. Macrophage-derived cytokines such as tumor necrosis factor (TNF) and interleukins are thought to be important in local inflammation and tissue repair (60). Silica, ultrafine particles of titanium dioxide, and coal dust have been shown to stimulate AM release of TNF and interleukin 1 and 6 (60-64). These cytokines have a number of proinflammatory activities which include neutrophil activation, stimulation of eicosanoid biosynthesis and activation of cells to release cytokines (60). It therefore seems reasonable that cytokine release as a result of particle exposure would also stimulate the induction of noncytochrome P450 metabolism of BaP in the epithelial cells of the target tissue relative to cytochrome P450 metabolism. This is supported by the observation that TNF$\alpha$ appears to inhibit P450 gene expression in cells as in the case of human adrenal cells (86) which would lead to more noncytochrome P450 metabolism.

The relative role of different types and surface properties of silica particles on the metabolic activation and binding of PAHs in AM has not been well studied except for those we described here. It has been reported that 0.5 to 2 $\mu$m was the optimum particle size for crystalline silica for AM cytotoxicity (65) but it is not clear as to whether surface area played a role in AM cytotoxicity. Crystalline silica toxicity appeared to be related to surface properties, those particles having silanol groups are more cytotoxic (silica versus aluminum oxide, Section 3), due to interaction with phospholipids on the surface of AM. However, other studies have shown that amorphous forms of silica with larger surface areas and available silanol groups are more cytotoxic than crystalline silica at equivalent doses in both peritoneal and pulmonary macrophages (66-68). Additionally, it has been shown that particles in cigarette smoke, carbon black or diesel exhaust result in AM recruitment with subsequent accumulation of particles (69-72) as well as the fact that AM metabolism of BaP was enhanced in AM from smokers relative to nonsmokers (95). Therefore, the overall objective

was to gain a better understanding of how particles with defined particle size and surface area will influence the metabolism of BaP by AM. Our basic hypothesis was that particulates can influence AM metabolism of BaP such that the effectiveness of BaP lung genotoxicity is potentiated. In other words, particulates could optimize the action of BaP by favoring metabolic pathways of BaP metabolism in the AM towards production of active metabolites and subsequent release to the surrounding tissue. Alternatively, particles may also affect the intracellular metabolism and binding of BaP in the AM versus metabolites which are being released into the surrounding media.

## Specific Aims

The specific aims were to determine 1) the ability of AM to phagocytize BaP - Coated forms of silica including crystalline and amorphous silica relative to $Fe_2O_3$ and $Al_2O_3$ that do and do not enhance BaP carcinogenicity, respectively, 2) the pattern of formation of metabolites from BaP-coated particles incubated with AM, 3) the adduct pattern of BaP derived from BaP-coated particles incubated with AM and 4) the mutagenic potential of mixtures of BaP metabolites present in the media of AM cultures exposed to BaP-coated particles.

## Procedures and Methodology

Chemicals. $^{14}C$-labeled BaP (Amersham, 20 $\mu$Ci/mmole) and unlabeled BaP (Aldrich) were checked for purity by TLC and HPLC. If necessary BaP was further purified by use of a neutral alumina column with benzene or toluene as the eluant followed by recrystallization in benzene-isopropanol. BaP metabolite standards supplied by the NCI Chemical Repository are as follows: 9,10-diol, 7,8-diol, 4,5-diol, 4,5-epoxide, 3,6-quinone, 6,12-quinone, 3-OH, 7-OH, 9-OH, and conjugates of BaP. All compounds will be handled under yellow light to prevent photooxidation (31). (+)Anti-diol-epoxide-dG, (-)anti diol epoxide dA and (-)anti diol epoxide dG are available in our laboratory. The latter two were made available from Larry Marnett for the determination of noncytochrome P450 metabolism.

Crystalline and amorphous silica, and ferric oxide particles that were used were characterized by this laboratory as described in Appendix 1.

BaP and Particle Preparation for Plate Assay. Stock solutions of pure labeled ($^{14}C$ BaP) and pure unlabeled BaP were prepared in toluene and handled under lights equipped with yellow filters to prevent photodecomposition. Those stock solutions were used in all experiments. All handling of BaP solutions were done under lights with yellow filters. The stability of the stock solution were determined periodically by HPLC.

The noncytotoxic dose of the appropriate particulates were placed in a vial, and an aliquot of the $^{14}C$-BaP solution were evaporated to dryness under a gentle stream of nitrogen. The particulates were resuspended in 1 ml RPMI-1640 just before the use in any experiment as described later. The suspension was vortexed or stirred with a magnetic stirrer to ensure that the BaP-coated particulates will in essence be unagglomerated before use (87). The methods used have been described by other investigators, (45), for BaP-coated diesel particles, (88), for BaP-coated asbestos, $Fe_2O_3$, silica and carbon black, and through similar

methods (40), and (22), for BaP-$Fe_2O_3$. The potential for BaP recrystallization is a function of a number of nucleation sites, temperature, concentration, crystal habit growth time and system turbulence. Pure BaP crystals were not noted in prior batches of coated particles prepared in this laboratory and documentation of conditions were maintained for this investigation. The number of nucleation sites were kept to a minimum by the use of the most pure grade of solvent; additionally, the particulate to be coated will be prewashed with the solvent and filtered to remove ultrafines. The evaporation time was kept as short as possible, to limit the size of recrystallization of BaP, should formation be initiated. Temperature and turbulence rate were carefully noted.

Animal Handling and Treatment. The environmental conditions of the animal rooms were automatically controlled and continuously monitored. Room temperatures are set at 74°F + 2°F and relative humidity at 45% ± 10%. Light cycle was 12 hours light and 12 hours dark. Syrian golden hamsters were obtained at 6-8 weeks old and quarantined two weeks. The two-week confinement was to insure that basal enzyme levels had been maintained; prior exposure to P-450 inducing agents would increase the rate of metabolism and give misleading results.

Macrophage Culture. AMs were harvested by tracheal lavage from male Syrian hamsters. All animals were kept on a standard light cycle (12/12). Animals were anesthetized with pentobarbital sodium ip (100 mg/kg) and exsanguinated by cutting the abdominal aorta. The diaphragm was incised, and the trachea was cannulated with a blunt 18 gauge 1 1/2" needle. AMs were obtained by lavaging the lung approximately ten times with a total of 50 mls of cold, sterile saline. The lungs were manually massaged during the lavage procedure. The lung washings were not used if the lungs appeared abnormal; i.e., contain areas of pus, or if the lavage fluid contains visible blood. The cells were counted by a Coulter B counter. Cells (2-3 x $10^6$ cells/dish) were placed in tissue culture dishes (35 x 10 mm) in 2.5 ml of the culture RPMI-1640 medium containing 0.1% gentamicin, 25 mM L-glutamine, 0.2% sodium bicarbonate and 2 mg/ml BSA (pH 7.2). The cells were incubated at 37°C in a humidified atmosphere and 5% CO2. After two hours the cultures were washed two to three times with fresh culture medium to remove any nonadherent cells. The cells were then fed 2-2.5 ml culture medium and incubated for an appropriate amount of time. Dishes were used for cell cultures to ensure culture viability for at least a 24-hour exposure to BaP. This long exposure time was necessary to maximize DNA binding. Cell viability was used as general indices of long-term culture viability and of AM cytotoxicity to particles. Cell viability was assessed prior to placing the cells in culture by exclusion staining utilizing erythrosin B.

Particulate-Induced Cytotoxicity. Particles were resuspended in media and administered to tissue culture dishes which contain 2-3 x $10^6$ cells previously incubated for 1 1/2-2 hours. The dishes were then be placed in an incubator with a humidified atmosphere at 5% $CO_2$ at 37°C. At the end of the incubation period (24 and 48 hours), dishes (each type of particle and control) were be removed from the incubator. Additional time points and dishes per treatment were added as necessary depending on the results. The media was removed form each dish and centrifuged. The AM was removed from the dish and washed until no radioactivity could be removed. The washes were discarded. The AM were assayed for viability as described.

Morphometric Analyses. Cultured macrophages ((confluent) $3x10^6$ per square mm of aclar) were washed in cold PBS, then fixed for 20' in an isoosmolar glutaraldehyde/paraformaldehyde phosphate buffered fixative containing calcium salts and sucrose. They were washed once with phosphate buffer, post fixed in 1% osmium tetroxide, dehydrated stepwise in ethanol solutions, then embedded in a thin layer of Spurr resin. After polymerization, the aclar was peeled from the Spurr leaving the cells behind. This was trimmed and reembedded in beem capsules. Thick sections (1 $\mu$m) were placed on glass slides and stained with toluidine blue, thin sections were placed on naked copper grids and stained with uranyl acetate and lead citrate.

Morphometry was conducted in two stages, 1) light microscopy, and 2) two levels of electron microscopy. The former served as a check for sampling, since it included an analysis of many more cells per group. For light microscopy, the cells were captured using JAVA software, video camera and Zeiss photomik. Then a perimeter of the entire cell and the nucleus were traced, and area of each determined. At least 200 cells from each group (divided among five replicate culture wells) were used. With electron microscopy, using grids derived from the same samples used for light microscopy, the entire macrophage was photographed (2500X (montaged from two or more negatives)). Perimeter/area of the cell (including processes which are not visualized at the light microscopic level) was traced, number of mitochondria, phagolysosomes counted, and perimeter/area of the nucleus were obtained. At a magnification of 6000X, phagolysosmes were photographed, and enlarged 3X at printing. The perimeter/area of each phagocytosed particle, and of the surrounding phagolysosomal membrane was obtained. The number of macrophages, and particles/phagolysosomes counted was dependent upon the standard error of the mean obtained for each group. Previous studies have indicated that 10 macrophages, and 50 phagolysosomes should produce a standard error of less than 10% of the mean at the electron microscopic level. These samples were divided among the five replicate cultures for each group.

Data from the morphologic studies was analyzed using SAS (for the PC, and generating means, standard deviations, standard errors of the means, and General Linear Model for group interactions. Significance was obtained from the analyses when $p \leq 0.05$.

Metabolism of BaP by AM. A noncytotoxic dose of the selected BaP-coated particle or BaP in media was administered to culture dishes which contain 2-3 x $10^6$ cells. Upon completion of each incubation period (6,12, and 24 hours) the media was removed from dishes (control-BaP alone and three treatments of BaP-coated particulates containing silica and ferric oxide) and centrifuged at 500 xg for ten minutes. The dishes were washed until no radioactivity could be removed and the washes were combined with the original media so that the medium from each treatment could be analyzed separately. The AM was also removed from each dish and washed to remove all radioactivity as described. These washes were also combined with the original medium from each dish. The medium containing the washes from each treatment was centrifuged at 500 xg for ten minutes, decanted, and frozen until analysis. The particles were discarded. An aliquot of the AM suspension was then tested for viability as described previously.

The amounts of BaP and its metabolites in both media and AM were determined by

extracting a portion of the media and AM several times with a mixture of ethyl acetate and acetone (2:1 by volume). The organic extracts were evaporated to dryness under $N_2$ at 40°C and stored at -20°C until HPLC analyses. Aliquots of media and extracted media and aliquots of the extracted AM suspensions following centrifugation were solubilized and decolorized and the radioactivity determined. The dried samples were reconstituted in 100 $\mu$l chloroform just prior to HPLC.

HPLC Analyses. All $^{14}C$ scintillation counting were performed on Tri-Carb Packard Liquid Scintillation Spectrometer. Ten $\mu$l samples prepared in 100 $\mu$l of chloroform were chromatographed on a Waters HPLC with a variable wavelength UV detector under the following conditions: Whatman ODS 10 m C-18 column 4.6 mm x 25 cm at ambient temperature with a flow of 1 ml/min with a water methanol gradient from 80-100% methanol over a 30 minute period. A HPLC chromatogram was recorded using a mixture of the metabolite standards prior to running samples. Samples were then chromatographed and the fractions collected individually. The fractions were then quantified by counting using a cocktail of Scintiverse Scintillation Media; the counting efficiency is determined by either using an internal toluene standard or a standard quench curve. All data collection, data reduction and statistics were handled by computer programs. All samples were processed under nitrogen and subdued yellow lighting to minimize photooxidation (31). The compounds were collected, the solvent evaporated off, and the residue dried and stored for further analysis.

$^{32}$Postlabeling for DNA Binding. A noncytotoxic dose of selected BaP-adsorbed particulate resuspended in media and control BaP was administered to AM on culture glass dishes. At the end of each incubation period the medium was removed from each dish and the residual BaP determined. The cells will be pelleted. The particles were extracted and analyzed for residual BaP and discarded and the DNA was isolated by phenol extraction. The $^{32}P$-postlabeling of these DNA samples were also as described (34,37,91,92). In brief, the DNA samples were hydrolyzed to 3'-phosphodeoxynucleotides with spleen phosphodiesterase and micrococcal endonuclease. We found that hydrolysis pH and time must be adjusted for each type of sample to increase adduct yields (93). The 3'-phosphodeoxynucleotides were then 5'-labelled with $\gamma^{32}P$-ATP and polynucleotide kinase. Hemminki and Segerbäck (personal communication) found that the conditions of labelling (in particular, time) can affect the yield. As with the digestion to 3'-phosphodeoxynucleotides, this was determined with early samples and then held constant for the remainder of the study. The normal 3',5'-bisphosphodeoxynucleotides were removed from the adducts by overnight thin layer chromatography (TLC). The adducted nucleotides were resolved from each other using further multi-dimensional TLC. Adducts were visualized by autoradiography and quantified by scintillation counting of the resulting adduct spots. To increase the sensitivity of the method for these samples, the 3'-phosphodeoxynucleotides were further digested with Nuclease $P_1$ prior to labelling. The length of this incubation was also adjusted to maximize adduct yields. Each sample was labelled with 100-200 $\mu$Ci [$^{32}P$]ATP and polynucleotide kinase. Analysis of normal nucleotides was performed to determine if there was excess [$^{32}P$]ATP in each sample. These measures were necessary to ensure that the available adduct nucleotides was sufficient [$^{32}P$]ATP to be labelled quantitatively.

The relative adduct labeling was determined as described in Reddy et al. (94).

Results from at least duplicate analyses were expressed as relative adduct labeling times $10^9$ to avoid small numbers.

Mutagenicity

Preparation of Samples. Initial studies assessed the BaP-particulates for mutagenicity at 24 and 48 hours. Depending on our initial results the concentration time points and number of plates/treatment were varied in order to obtain sufficient material for the mutagenicity studies. As we have described, aliquots of the organic extracts of the media were set aside for these studies. One half of the ethyl acetate extracts was evaporated to a 1 ml volume at which time 1 ml of DMSO was added. The evaporation proceeded to remove the remainder of the ethyl acetate. The second half of ethyl acetate extract was evaporated to dryness and was subjected to HPLC with a methanol:water mixture to separate unmetabolized BaP from the metabolites (73). The water-methanol mixture of metabolites was then prepared for the assay on the same occasion as the non-chromatographed extract in the manner described above.

Salmonella Mutagenicity Assays and Data Analyses. Initial studies involved both reverse and forward Salmonella mutagenesis assays based on data reported by this laboratory that have shown complementary data for BaP and its metabolites (97). Both assays have been used for assessing biological mixtures (73,97). As the research progressed the mutation assays were assessed as to which would be more appropriate assay for these mixtures. Initially, the dose range of the parent compound and their products and/or mixtures of products were in the range of 1-25$\mu$/ml. Liver homogenates (S9) for mammalian metabolism were prepared from male Sprague-Dawley rats. Animals were pretreated by i.p. injection of 3MC 40 mg/kg and asceptically excised livers were homogenized in three volumes cold buffer (0.5 M KCl, 0.05 M Tris HCl). The supernatant fraction from 20 minutes centrifugation at 9000 g was dispensed into aliquots, and stored at -80°C until use. Reverse mutation plate incorporation assays using Salmonella typhimurium strains TA98 and TA100 were done according to published procedures (73,97). For activation assays, 10, 15, 50, or 100 $\mu$l S9/plate plus cofactors were used. Positive responses were judged by two criteria: 1) numbers of revertant colonies greater than twice the number obtained spontaneously; 2) evidence of a sample-dose related increase in revertant colonies. Numbers of revertants less then the spontaneous controls or the presence of pinpoint $His^+$ colonies were taken as evidence of toxicity. Duplicate plates were done for each experimental point and all assays were repeated. Salmonella strain TM677 was used in forward mutation assays with resistance to 8-azaguanine as the end point (97). Micro-volume suspension assays (total volume 0.1 ml) were done using 1 $\mu$l test material. For activation assays, S9 was added so as to constitute 1 or 10% of the assay volume. Duplicate assay tubes were prepared for each experimental point. After a two-hour incubation at 37°C, the bioassay suspension was diluted and plated in triplicate for selection of mutant organisms diluted again, and then plated in triplicate for bacterial toxicity. Numbers of revertant/$10^5$ surviving cells were calculated from the 6 mutagenicity and the 6 toxicity plate counts. Positive responses were judged as those with numbers of revertants/$10^5$ survivors which were greater than the upper 99% confidence limit of the historical spontaneous controls and the controls from the day of the assay.

## Results and Discussion

Data in Detail. Isolation of AM. We developed an assay the phagocytic ability of particles by AM. Male Syrian hamsters and Sprague Dawley rats (8-10 wks old) were used. Primary AM were isolated by tracheal lavage as reported (8). The lavaged fluid was centrifuged, the supernatant was discarded and the pellet was resuspended with RPMI-1640 medium. An aliquot of the cell suspension was used to identify the purity of the AM cellular differential staining (Diff-Quik stain set Sci. Product Inc.). An aliquot of the cell suspension was removed to determine AM numbers using a hemocytometer and selected for AM based on size and morphology and AM viability by dye exclusion using erythrosin B (75). The average viability for each isolation was on the order of 95-98%, 2.5-3 million AM were obtained per animal. Two and a half ml of fresh RPMI-1640 (containing different concentrations of particles, BaP or BaP-coated particles) were administered to the AM, and viability (24 and 48 hours) assayed twice. One of the major objectives of this work was to culture AM without fetal calf serum since it was thought that the serum would interfere with the planned metabolic studies by removing free metabolites from the media. By adding back BSA at a concentration of 2 mg/ ml to the Petri dish the viability stabilized at 86.5%$\pm$0.9 at 24 hours (n=6) to 77.0%$\pm$1.0 at 48 hours (n=4).

Determination of the Physical Characteristics of Particles. The physical characteristics of the particles are reported in Table 1 and represent values which are typical of human exposure. All of the particles were of respirable size with at least 98% less than 5 microns. The count median diameters for aluminum oxide and amorphous silicas were equal to or less than 0.38 microns while for crystalline silica the count median diameter was 0.83 microns, ferric oxide was 0.32 microns. The larger surface areas for the aluminum oxide and the amorphous silicas were generally consistent with the small median diameters and the size distributions in that the values ranged from 124.8 to 253.1 $m^2/g$. For crystalline silica with larger count median diameter, the surface area was 4.3 $m^2/g$. Neither precipitated nor gelled silica contained any detectable crystalline silica; fumed silica contained 1.6% crystalline silica. Crystalline silica had a smaller surface area and a larger count median diameter than any of the amorphous silicas. Photomicrographs of fumed silica showed marked clumping of the individual submicron particles; this possibly contributes to the lower-than-expected surface area determination (Appendix 1, paper in press).

Determination of AM to Phagocytize Particles and the Cytotoxicity of Particles to AM. Comparative viability studies of the AM in the presence of ferric oxide, aluminum oxide, or the four forms of silica (crystalline, gelled, fumed, precipitated silica) were undertaken to determine noncytotoxic doses during phagocytosis. Doses of particles ranged from 0.0 to 0.5 mg/plate. The viability of the hamster AM in the presence of aluminum oxide up to the highest dose was similar to controls. After 24 and 48 hours, the viability of the AM for aluminum oxide was approximately 80 and 70%, respectively. Similar results were obtained with ferric oxide with the exception of an apparent small cytotoxic response ($P \leq 0.01$) of the AM at the highest dose, 0.5 mg. In the presence of silica, the viability of the hamster AM was similar to controls up to a dose of 0.01 mg except for precipitated silica, where the viability was 57% at 48 hours. The percent viability decreased with increasing dose and time. Zero percent viability occurred at 0.1 mg of gelled silica. For precipitated, fumed and crystalline, zero viability was observed at 0.5 mg. Viability at 48 hours was

consistently lower than at 24 hours. Similar results for all the particles were obtained for the Sprague Dawley rat AM. The crystalline silica had a smaller surface area based on an equivalent dose and a larger count medium diameter than any of the amorphous silicas; the gelled silica had the largest surface area of all the particles tested. The cytotoxicity data on the silica interactions with AM were generally consistent with this physical characteristic in that the particle with the largest surface area was the most cytotoxic i.e. gelled silica was the most cytotoxic which was consistent with its large surface area (Appendix 1, paper in press).

TABLE 1. Physical Characteristics of Particles

| Particle | Size Distribution (microns) | Median Distribution (microns) | Count Surface Area ($m^2/g$) | X-Ray Diffraction |
|---|---|---|---|---|
| $Al_2O_3$ | 99% <5[a]<br>80% <1 | 0.36[a] | 198.4[b] | |
| $Fe_2O_3$ | 98.9% ≤5[b]<br>91.5% ≤1 | 0.32[b] | 10.8[b] | |
| Silica Crystalline | 99% <5[c]<br>50% <1.0 | 0.83[c] | 4.3[b] | |
| Amorphous Gelled | 99.8% <5[d]<br>93% | 0.27[d] | 253.1[b] | crystalline silica not detected[d] |
| Fumed | 99.9% <5<br>99.5% <1[a] | (submicron 15-50 nm)[e] | 196.2[b] | 1.6% crystalline silica[e] |
| Precipitated | 98% <5[d]<br>85% <1 | 0.38[d] | 124.8[b] | crystalline silica not detected[d] |

[a]NIOSH 1992
[b]Analyzed by Micromeritics, Norcross, GA 1992
[c]Stettler et al. 1991
[d]Groth et al. 1981
[e]Analyzed by DataChem, Salt Lake City 1992

A possible explanation of the observed effect is the presence of silanol groups on the particle surface. The silanol groups associated with crystalline silica have been hypothesized to play an essential role in its cytotoxicity (76). Silanol groups are also found on amorphous silica (77). It should be noted that gelled and precipitated in aqueous solution are covered with silanol groups while pyrogenic silica (fumed) are only partly covered with silanol groups; the remainder are covered with siloxane groups (78). The test materials with the largest surface area present more silanol groups for interactions with phospholipids on the surface of the AM (65). This would explain why ferric oxide and aluminum oxide were not cytotoxic or at best weakly cytotoxic at these doses while silicas are. The dose of the silica is related to surface area, and the total silanol groups available. In a comparison of gelled and precipitated

silica, the metal impurities in the precipitated silica were greater than the gelled but the cytotoxicity was greater in the gelled silica (10). The crystalline silica had little in the way of impurities but was not more cytotoxic than precipitated silica (79).

The results support the hypothesis that the silica is taken up into the phagosomes in the cytoplasm (80). The silica then by surface action through the silanol groups damages the membrane of the phagosomes resulting in the release of hydrolytic enzymes from lysosomes into the AM cytoplasm. The process then repeats itself as the particles which are released are phagocytized in a continuing cycle. The more silanol groups available the more severe the cytotoxic response. In this set of experimental results, gelled silica demonstrates a highly cytotoxic response which may indicate the presence of silanol groups on the very large surface area; the particles are available inside the phagosome following uptake. In support of this hypothesis, it has been indicated that amorphous silica can denature protein and disrupt cell membranes (78).

BaP was assayed with this procedure in hamster AM in a dose response manner from 5 to 15 $\mu$g and was found to be noncytotoxic. When BaP-coated $Fe_2O_3$ was added to hamster AM at two doses of $Fe_2O_3$ at 5 $\mu$g of BaP, no cytotoxicity was observed at 24 hrs. Similar results were seen with BaP-coated-$Al_2O_3$. BaP-coated forms of silica at the highest noncytotoxic doses i.e. crystalline, fumed and precipitated silica at 0.05 mg and gelled silica at 0.01 mg. were found to be noncytotoxic for the hamster AM (data not shown).

Morphometric Analyses. To develop a better understanding of the interaction of BaP-coated particles with AM, we developed a morphometric approach for assaying cytotoxicity and activation of AM following exposure to BaP-coated particles similar to that described by others (81,82,83). The techniques are described in detail in the methods section.

The relative amounts of phagocytosed material (inclusions) can be quantified, and the size of the phagolysosomes in which inclusions are found can be measured. The closer the phagolysosome and the phagocytosed particles are in size, the milder the cytologic reaction to the internalized structure. If there are very large phagolysosomes, then the reaction to the ingested material would be considerable. When the particle is very toxic, then only small amounts of material are internalized, and the percent inclusion in the entire cytoplasm is also small. The reverse is true of very non-toxic particles, where 80% or so of the entire cytoplasm can be occupied by the phagolysosome, and the ratio of particle to the entire AM cytoplasm, are both measures of toxicity of that particular type of particle. Secondly, AM which have been stimulated, have very large nuclei, and are generally larger in size than nonactivated AM. Mean nuclear and cell area can be determined in relation to the type of particle ingested and the volume density of particle within the AM determined. These data can then be related to biochemical parameters. Thirdly, surface phenomenon which alter cell activity can be measured as cytoplasmic blobbing and flattened pseudopodia, while hypertrophy of the cytoplasm can be measured by the increase in nuclear area and cytoplasmic area (84,85).

In studies with light microscopic morphometry of AM (Table 2) cell and nuclear areas were analyzed. BaP was given at 3 $\mu$g and all particles were added at 0.05 mg and 3.5-3x$1^6$ cells/plate. There was no significant difference between macrophages treated with BaP and DMSO+BaP in terms of nuclear area or cell area. There was a significant increase in nuclear area and/or cell area if $Fe_2O_3$ or silica, either crystalline or precipitated, was co-ingested with BaP relative to BaP alone. There was also a significant difference (in nuclear area) between the different silicas when incubated with BaP. $Fe_2O_3$ alone was less effective at increasing nuclear and cell area than $Fe_2O_3$+BaP. The data indicate that AM exposed to BaP-coated particles respond in manner which is different from that seen with either BaP or particle alone.

TABLE 2. Morphometric Analyses of Hamster AM

| Alveolar Macrophages | Nuclear Area ± S.E. | Cell Area±S.E. |
|---|---|---|
| BaP (3 $\mu$g) | 6.0 ± 1.0 | 28.2 ± 3.8 |
| BaP DMSO (3 $\mu$g) | 7.0 ± 0.6 | 26.2 ± 2.5 |
| Aluminum Oxide (0.05 mg) | 5.7 ± 1.0 | 22.9 ± 3.9 |
| $Fe_2O_3$ (0.05 mg) | 7.1 ± 0.7 | 28.1 ± 2.5 |
| BaP-$Fe_2O_3$ (0.05 mg) | 9.0 ± 1.1* | 42.4 ± 4.3* |
| Fumed Silica (0.05 mg) | 7.2 ± 0.6 | 26.4 ± 2.5 |
| BaP Crystalline (0.05 mg) | 6.9 ± 0.6+ | 38.8 ± 2.5* |
| BaP PPT Silica (.05 mg) | 9.1 ± 0.8* | 34.0 ± 3.2* |

*Significantly different than BaP alone and/or BaP/DMSO $p \leq 0.05$
+Significantly different from BaP ppt silica (precipitated-ppt)

Determination of Metabolic profiles from BaP-coated Particles. Previous cytotoxicity studies of particles were used to determine the dose of particle for metabolism studies (0.5 mg $Fe_2O_3$, 0.05 mg crystalline, fumed and precipitated silica and 0.01 mg gelled silica). To 3 $\mu$g of BaP per dose and particles, 20 ml of acetone was added. A volume:weight ratio was calculated and the appropriate aliquot of the particle mixture was taken while the mixture was vortexed, placed into a 60°C oven until the acetone had evaporated. Then $^{14}$C-BaP was dissolved into 50 $\mu$l acetone and added to the particle, vortexed and heated in a 60°C oven. Sample was then stored at 4°C. Observation of particles in media (under an inverted fluorescence microscope) indicated that all of the BaP was associated with the particle under study. EM work indicated that the particles were of the same size as precoated forms.

After lavaging hamster AM with PBS and aliquoting cells for count and viability, 3x $10^6$ cells were plated using 2.5 ml RPMI-1640 media for 1.5 hours (Figure 1). The AM were then washed with media and BaP-coated particles were then added for 24 hours. The media was removed and extracted with ethyl acetate. The

nonextractable media was counted. The AM were removed from the plate by adding 0.25% trypsin at room temperature for 30 min. The AM and a media wash of the plate were combined and spun. The wash was counted and the pellet was resuspended in media and gently agitated. The AM suspension was extracted with ethyl acetate (The same procedure was used to extract the BaP-coated particle controls without AM in order to determine the amount of extractable BaP). The ethyl acetate extracts were evaporated to dryness under nitrogen and stored for HPLC analysis. The nonextractable cell suspension was counted. The particle pellet from the AM suspension was counted and combined with the ethyl acetate extract count of the plate itself.

The data indicate (Table 3) that although the metabolism of BaP at this dose is not high, there are differences in the metabolic pattern seen in the media which can be considered important. The major pathway is through the dihydrodiol formation. The 7,8-diol metabolite which is the precursor of the ultimate metabolite 7,8-diol-9,10-epoxide is higher in all of the particle associated BaP relative to BaP alone. This would suggest that BaP-coated particles are metabolized more readily through the active pathway than by BaP itself which would suggest a change in the metabolic pattern due to particles. (This will become more apparent with DNA binding data.) This has been seen before in our lung perfusion (33) and macrophage cell suspension studies (8). Of the particles studied, the values for the 7,8-diol were higher for the amorphous silicas than that of $Fe_2O_3$ or crystalline silica. No other significant differences were seen in the distribution of BaP in media and AM (data not shown).

TABLE 3. (pmol/$10^6$ cells)±S.E. Metabolism in Hamster AM

| | 9,10-diol | 4,5-diol | 7,8-diol |
|---|---|---|---|
| BaP n=4 | 3.5±0.1 | 0.6±0.1 | 0.3±0.1 |
| BaP-ppt n=5 | 6.4±0.3[a] | 1.2±0.1[a] | 1.4±0.0[a] |
| BaP-Fumed n=5 | 7.6±0.8[a] | 1.5±0.1[a] | 1.4±0.1[a] |
| BaP-Gelled n=3 | 2.8±0.4 | 0.7±0.1 | 1.0±0.1[a] |
| BaP-$Fe_2O_3$ n=6 | 4.5±0.1[a] | 0.8±0.1 | 0.7±0.1[a] |
| BaP-Crystalline n=6 | 6.4±0.4[a] | 0.8±0.1 | 0.6±0.1[a] |

[a]Significantly different from BaP alone at p=0.05

The level of DNA-binding from BaP-coated particles. As previously described in the section above the BaP-coated particles were incubated with AM and after 24 hours the AM were isolated. The DNA was isolated using a phenol extraction method and analyzed by $^{32}P$-postlabeling techniques.

The data indicate that both qualitatively and quantitatively that the BaP-adduct patterns have been altered by the presence of particles (Table 4 and Figure 2). In all cases, ppt, crystalline or $Fe_2O_3$ adducts #2 is the predominant adduct as opposed to the presence of all three adducts for BaP alone. The adducts in

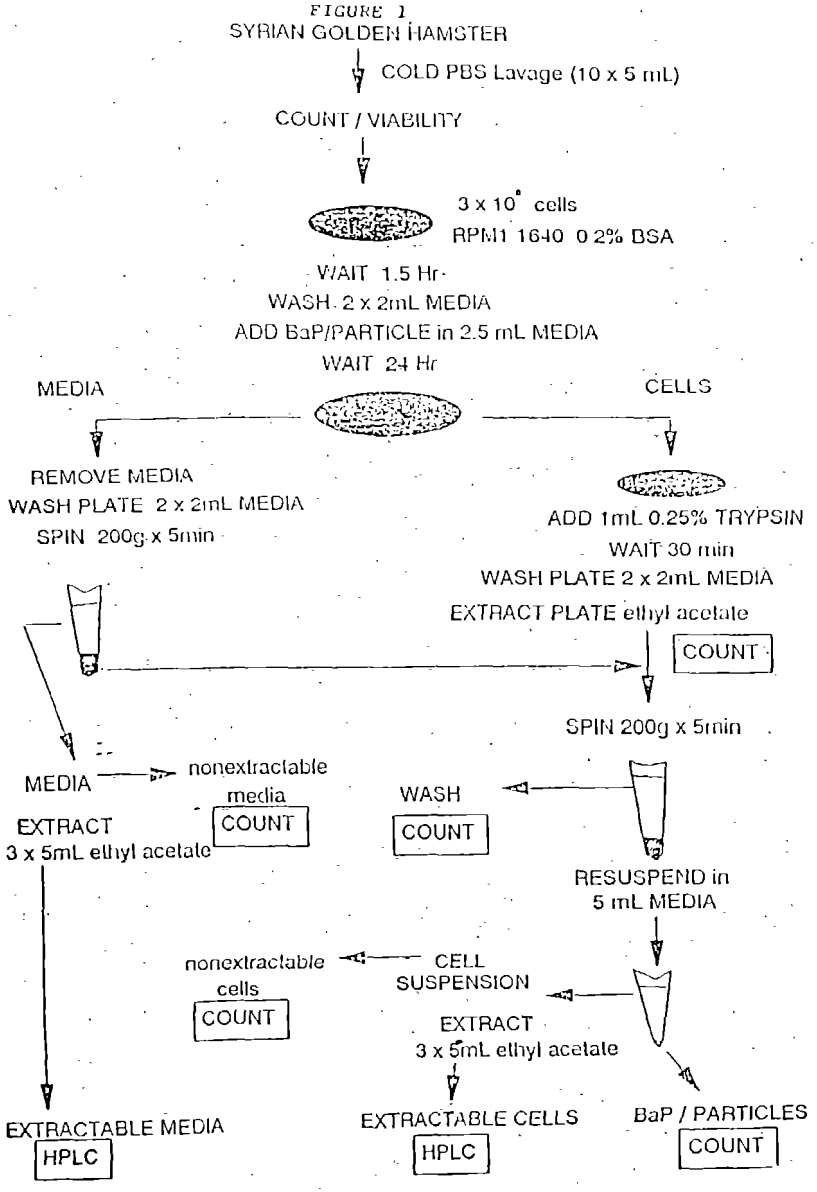
FIGURE 1
SYRIAN GOLDEN HAMSTER
COLD PBS Lavage (10 x 5 mL)
COUNT / VIABILITY
3 x 10⁶ cells
RPMI 1640 0 2% BSA
WAIT 1.5 Hr
WASH 2 x 2mL MEDIA
ADD BaP/PARTICLE in 2.5 mL MEDIA
WAIT 24 Hr
MEDIA
CELLS
REMOVE MEDIA
WASH PLATE 2 x 2mL MEDIA
SPIN 200g x 5min
ADD 1mL 0.25% TRYPSIN
WAIT 30 min
WASH PLATE 2 x 2mL MEDIA
EXTRACT PLATE ethyl acetate
COUNT
SPIN 200g x 5min
MEDIA
nonextractable media
COUNT
EXTRACT
3 x 5mL ethyl acetate
WASH
COUNT
RESUSPEND in
5 mL MEDIA
nonextractable cells
COUNT
CELL SUSPENSION
EXTRACT
3 x 5mL ethyl acetate
EXTRACTABLE MEDIA
HPLC
EXTRACTABLE CELLS
HPLC
BaP / PARTICLES
COUNT

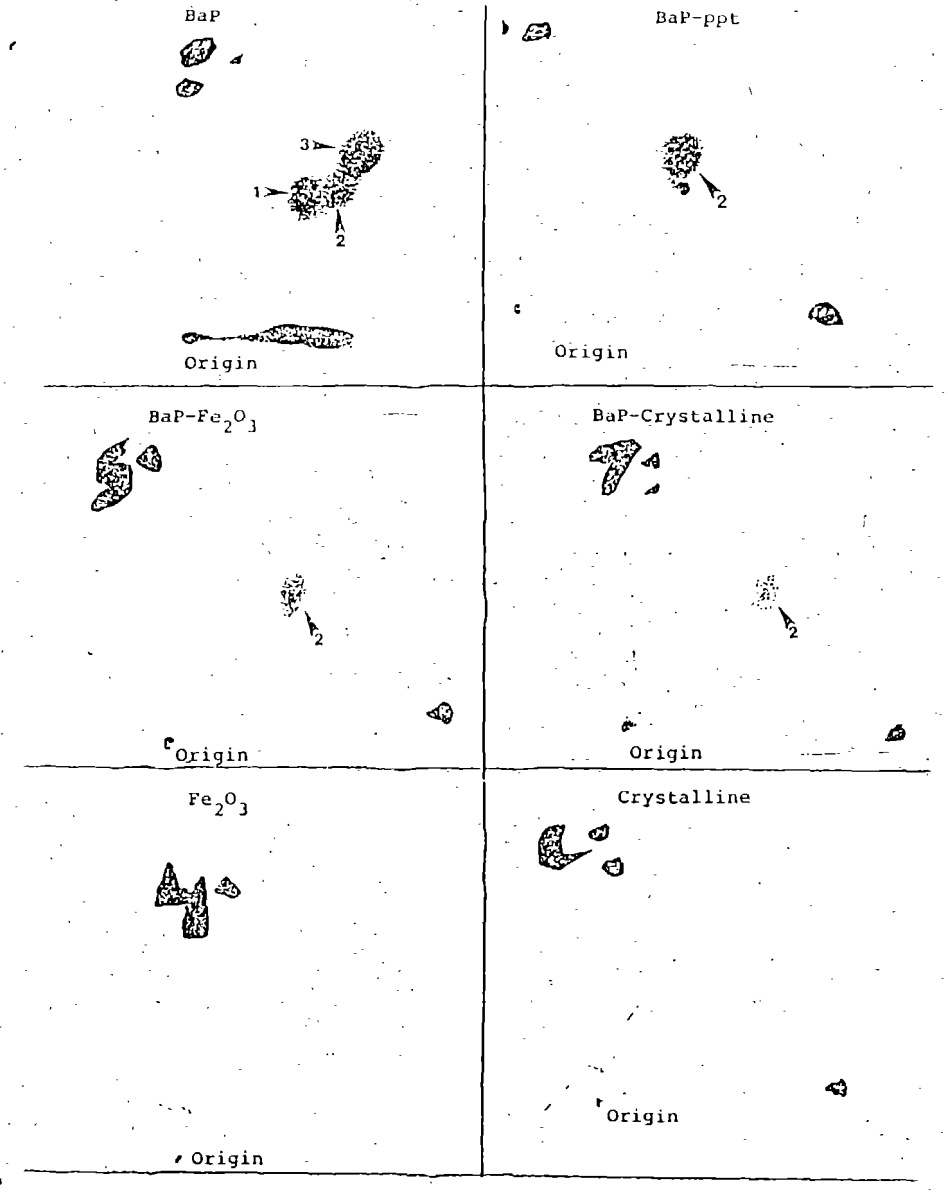
BaP
Origin
BaP-ppt
Origin
BaP-Fe2O3
Origin
BaP-Crystalline
Origin
Fe2O3
Origin
Crystalline
Origin

FIGURE 2

Figure 2 have been normalized for the total DNA content in Table 4. These data are consistent with the metabolic data previously reported in that particles appear to alter both the metabolic pattern in the media and adduct pattern in AM. Adduct #1 cochromatographs with the (+)anti-diolepoxide of BaP bound to dG which is considered to be the ultimate P450 metabolic pathway. Adduct #2 appears to be formed through a noncytochrome P450 metabolic pathway (-anti). This increase in the level of binding (adduct #2) is correlated with the increased formation of the active forms of the dihydrodiols, in particular the 7,8-diol. What is evident is that particles with the larger surface areas appear to have altered the formation of BaP active metabolic intermediates.

TABLE 4. Relative Adduct Levels ($x10^9$) in Hamster Alveolar Macrophage

| | Adduct #1 | Adduct #2 | Adduct #3 |
|---|---|---|---|
| BaP | 19.4 | 14.9 | 16.2 |
| BaP-ppt Silica | 1.6 | 37.6 | 2.75 |
| BaP-$Fe_2O_3$ | ND | 23.0 | ND |
| BaP-Crystalline Silica | ND | 9.4 | ND |

Corrected for DNA content of sample
ND - Not detected (Below detection limit ($1x10^9$)

The Mutagenicity of BaP Metabolite Mixtures. The Ames Salmonella test is an in vitro assay used to detect mutagenic compounds. In the assay, histidine-requiring bacteria strains with specific types of mutations are plated with the test chemicals in the presence of growth-limiting concentrations of histidine; only bacteria which can revert to histidine independence are capable of producing colonies. With the addition of mammalian S9 microsomal preparation, compounds may be enzyme activated compounds and detected as mutagens. After 48 hours incubation at 37°C the revertant colonies on the test and control plates are counted and the presence of auxotrophic bacteria is confirmed. Compounds producing revertant counts more than 2 to 3 times the spontaneous reversion counts are considered positive mutagens. The Ames assay was used to determine the mutagenicity of BaP metabolite standards (from Midwest Research Incorporated) and the organic extracts of several macrophage media.

BaP trans-7,8-diol was mutagenic in the presence of 3-MC induced S9 for TA 98 and TA 100 at 0.25, 0.5, and 1.0 $\mu$g per plate, with the revertants showing a dose response. Higher doses plated with S9 were toxic. The mutagenic response was more than 3 times spontaneous reversion. BaP trans-9,10-diol was mutagenic only in the presence of 3-MC induced S9, for TA 98 at 5, 10, 25, and 50 $\mu$g per plate and for TA 100 at 10 and 50 $\mu$g per plate with a dose response relationship. The mutagenic response was more than 3 times the spontaneous rate, except for TA 98 at 5 $\mu$g and TA 100 at 10 $\mu$g per plate where the response was > 2 times spontaneous. BaP trans-4,5-diol at 0.4, 4, 8, and 20 $\mu$g per plate was not mutagenic for either strain, with or without S9 activation.

None of the extracts from the macrophage media were mutagenic with or without BaP. BaP had been isolated from the extracts by HPLC. The data indicate that

the 7,8 and 9,10-diols are mutagenic as expected but that the levels of metabolites are too low to be detected in the assay, although, we have detected mutagenic metabolites from rabbit macrophage using this assay (73). Two other assays were attempted, the T97 micro suspension assay and the TM677 forward mutation assay both of which have the capacity to detect smaller amounts of metabolites. We have used TM677 to detect mixtures of metabolites (97). We had to obtain new clones of TM677 due to the fact that the clones lost their plasmids. These latter assays did not show any positive results.

## Conclusions

This laboratory has had a long-term interest in the effects of particle on the metabolism and binding in the lung of xenobiotics, such as BaP, dibenz(c,g)carbazole and dibenz(a,j)acridine (31,33,73,74). Using an isolated perfused lung preparation of BaP in the lung, we have shown ferric oxide particulates as a major component alters the metabolism of BaP in the lung; whole animal exposure to a particle enhances dihydrodiol formation and depresses water soluble conjugates (31). Because dihydrodiol formation is involved in the active pathway of BaP metabolism (6), these data suggest that pulmonary exposure to a known cocarcinogen, ferric oxide, in the presence of BaP results in increased production of dihydrodiols which may be further metabolized to the ultimate carcinogenic form(s) of BaP. Therefore, ferric oxide can enhance the metabolic activation of BaP by the lung as well as act as a carrier for penetration and retention in the lung.

In related studies (73), the Salmonella/microsome test was applied as an assay of mutagenicity in lung tissue and blood extracts. Lung, trachea-bronchi and macrophage extracts from the isolated perfused lung (IPL) receiving BaP were found to be mutagenic. A part of this activity was attributed to BaP metabolites rather than to the parent compound remaining in extracts. When the lungs were exposed to BaP plus ferric oxide or fly ash, only the AM extracts were consistently mutagenic. It appeared in the latter cases that the AM accumulated BaP, but did not metabolize it appreciably during the course of the perfusion. It was not possible under conditions of BaP pretreatment of the whole animal to determine whether the macrophages from BaP-plus-particulate-treated lungs were unable to readily metabolize BaP, or if metabolism was delayed in some way such that it required longer times than 180 minutes during which the IPL functions. AM could retain the compounds for extended periods of time beyond the time frame of the IPL before metabolizing or releasing them to lung tissue for possible metabolism at a later time, or another site, such as the trachea and bronchi.

A logical extension of this work was to assess the influence of phagocytosis of BaP-coated ferric oxide on BaP metabolism in AM (8). The AM were lavaged from hamsters and cultured in suspension ($2.5 \times 10^6$ cells) with BaP (62.5 nmoles, $^{14}C$ labeled) alone or adsorbed onto 0.5, 1.0 or 2.0 mg $Fe_2O_3$ in the presence of cytochrome C. Following separate ethyl acetate extractions of the AM and medium, the metabolites were isolated by HPLC and quantified by liquid scintillation spectrometry. The production of superoxide anions was monitored by the reduction of cytochrome C.

Concurrent exposure of AM to BaP coated $Fe_2O_3$ resulted in a significant increase

in the amount of BaP metabolites and superoxide anions produced. The following metabolites were identified in both the medium and the AM: 9,10-dihydrodiol; 7,8-dihydrodiol; 4,5-dihydrodiol; 9-hydroxy; 3-hydroxy; and 3,6-quinone. In general, the amount of 7,8-dihydrodiol (a precursor of the ultimate carcinogenic metabolite of BaP) and superoxide anion (produce localized lipid peroxidation and edema in vivo) formation was significantly enhanced ($p = 0.05$, Duncan's multiple comparison test) in AM exposed to all doses of $Fe_2O_3$ when compared to AM exposed to BaP alone. This $Fe_2O_3$ dose related enhancement of superoxide anion was indicative of increased endocytic capacity resulting in a greater amount of total metabolites being produced, in particular, the dihydrodiols of BaP (see reference 8, in appendix).

In an effort to assess metabolism and DNA binding of BaP-coated particles such as silica relative to $Fe_2O_3$ we assessed 1) the ability of AM to phagocytize BaP coated forms of silicas relative to $Fe_2O_3$ and $Al_2O_3$, 2) the pattern of metabolites derived from BaP coated particulates incubated with AM and 3) BaP binding to DNA from BaP-coated particles.

Summary of data. An in vitro AM culture system was used for assessing both phagocytosis and cytotoxicity of particles as well as metabolism, DNA-binding. The physical characteristics of the particles indicated that crystalline silica had a smaller surface area and a larger count median diameter than any of the amorphous silicas and that gelled had the largest surface area of all the particles tested including ferric oxide or aluminum oxide. Gelled silica based on equivalent doses given to AM was the most cytotoxic, consistent with its large surface area. All forms of amorphous silica had smaller particle sizes and were cytotoxic suggesting that more of the dose for the amorphous silicas entered the AM. The toxicity also appears related to the surface properties; those particles having more silanol groups were more cytotoxic. This may explain the fact that ferric oxide and aluminum oxide were not cytotoxic while silicas were. The dose of the silica is important in that more silanol groups are probably available on the crystalline silica surface and therefore a larger dose of amorphous silica must be incorporated into the AM in order to observe the same toxic effect as crystalline silica. Therefore dose, surface properties and particle size each affect cytotoxicity of silica to AM (see appendix for paper in press).

These same properties appear to be important in the metabolic activation of BaP by AM. At a similar dose of BaP and particle, all of the BaP-coated particles produced more BaP dihydrodiols than BaP alone. The three amorphous forms of BaP-coated silica produced more 7,8-diol of BaP (in pmol/$10^6$ cells) than the BaP-coated $Fe_2O_3$ and BaP-coated crystalline silica. The 7,8-diol is considered to be the precursor to the ultimate metabolite of BaP. Although these metabolites were produced in low levels, it was sufficient to produce BaP-DNA adducts. In all three cases which were studied, BaP/ppt silica, BaP-crystalline silica and BaP-$Fe_2O_3$ in comparison to BaP alone, the BaP adduct patterns were altered such that adduct #2 was increased relative to adducts 1 and 3. Of these three the relative adduct levels for the ppt form were greater than that of $Fe_2O_3$ or crystalline silica which is consistent with the greater surface area of ppt silica. Adduct #1 is the +anti-diolepoxide of BaP bound to dG from P450 cytochrome metabolism while adduct 2 and 3 (have been found to be - -anti-diolepoxide of BaP bound to dG and -anti-diolepoxide of BaP bound to dA,

respectively) could involve non P450 cytochrome metabolism.

Morphometrically, we have shown that AM exposed to particle coated with BaP, responded differently than either BaP or particles alone at noncytotoxic doses. This approach would give a good estimate of AM activation as a result of coexposure which could be correlated with the biochemical parameters. Based on the preliminary data, BaP-coated particles at a single dose in comparison with BaP alone have an impact on the metabolism, DNA binding, the extent of adduction through P450 cytochrome metabolism, and AM activation and perturbation. Additionally for metabolism, DNA binding and cytotoxicity parameters, the surface area of the particle appears to play a major role.

## 10. ACKNOWLEDGEMENTS, REFERENCES, AND APPENDICES

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## 11. LIST OF PRESENT AND FUTURE PUBLICATIONS

Relevant Publications

Griefe, A. and Warshawsky, D. (1993) Influence of dose levels of the cocarcinogen ferric oxide on the metabolism of benzo(a)pyrene by hamster pulmonary alveolar macrophages in suspension culture. J. Toxicol. Environ. Health 38:399-417.

Warshawsky, D., Reilman, R., Cheu, J., Radike, M. and Rice, C. (1994) Influence of particle dose on the cytotoxicity of hamster and rat pulmonary alveolar macrophage in vitro. J. Toxicol.Environ. Health, in press

Warshawsky, D., Reilman, R., Radike, M., Rice, C., Jaeger, M. and Talaska, G. (1994). Metabolism and DNA binding of BaP in hamster alveolar macrophage in vitro. To be submitted

Abstracts

Warshawsky, D., Reilman, R., Cheu, J. and Radike, M. (1992). Influence of particles on benzo(a)pyrene metabolism. Society of Toxicology, February 23-27, Seattle, WA.

Warshawsky, D., Reilman, R., Cheu, J., Radike, M. and Talaska, G. (1993). Metabolism of benzo(a)pyrene coated particles by hamster pulmonary alveolar macrophage. Society of Toxicology, March 14-18, New Orleans, LA.

Warshawsky, D., Reilman, R., Collins, T., Jaeger, M., Cheu, J., Radike, M. and Talaska, G. (1994). Metabolism and DNA binding of BaP-coated particles by hamster pulmonary alveolar macrophage. Society of Toxicology, March 13-17, Dallas, TX.