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A. L. Greife & D. Warshawsky

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INFLUENCE OF THE DOSE LEVELS OF COCARCINOGEN FERRIC OXIDE ON THE METABOLISM OF BENZO[a]PYRENE BY PULMONARY ALVEOLAR MACROPHAGES IN SUSPENSION CULTURE

A. L. Greife

National Institute for Occupational Safety and Health, Cincinnati, Ohio

D. Warshawsky

University of Cincinnati Medical Center, Department of Environmental Health, Cincinnati, Ohio

The concurrent administration of a cocarcinogenic carrier particle such as ferric oxide (Fe_2O_3) and the polycyclic aromatic hydrocarbon lung carcinogen benzo[a]pyrene (BaP) results in a decreased latency and an increased incidence in the production of lung tumors in hamsters compared to the administration of BaP alone. The pulmonary alveolar macrophage (AM), the primary lung defense cell, has been shown to endocytize BaP, metabolize BaP to a more biologically active form, and then release the metabolites. Therefore, a study was undertaken to determine in a dose-response manner the effect of AM phagocytosis of a carrier particle (Fe_2O_3) on the metabolism of a carcinogen (BaP) and on the production of reactive oxygen. The AM were lavaged from hamsters and cultured in suspension (2.5×10^6 cells/vial) with BaP (62.5 nmol, ^{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 high-performance liquid chromatography (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 with dose of Fe_2O_3. 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 7,8-dihydrodiol, which is considered to be the precursor of the ultimate carcinogenic metabolite of BaP, and superoxide anions, which have been shown to produce localized lipid peroxidation and edema in vivo, were significantly enhanced ($p = .05$, 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 production is indicative of increased endocytic capacity resulting in a greater amount of total metabolites being produced, in particular, the dihydrodiols of BaP, which are considered to be products of the active metabolic pathway of BaP.

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Requests for reprints should be sent to D. Warshawsky, University of Cincinnati Medical Center, Department of Environmental Health, Cincinnati, OH 45267-0056.

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INTRODUCTION

The National Institute for Occupational Safety and Health (NIOSH) lists occupational lung disease among the 10 leading occupational diseases or illnesses found in the workplace (Centers for Disease Control, 1983). Numerous epidemiologic and experimental studies indicate that various types of dusts and/or chemical carcinogens are important in the development of respiratory disease in the workplace (Falk, 1970; Nelson, 1970; Perera, 1981). An important aspect of the dust exposures is their cocarcinogenic potential when coexposure to other carcinogenic agents is present. Exposure to Fe_2O_3 in a mixture with potential carcinogens such as polyaromatic hydrocarbons (PAH) is associated with an increased incidence in various forms of cancer, particularly lung cancer, when compared to control populations (Axelson and Sjoberg, 1979; Teculescu and Albu, 1973), which suggests a synergy resulting from exposure to the mixture.

The hamster has been used as an in vivo animal model for studying the intratracheal administration of PAHs and a variety of particulate carriers (Saffiotti et al., 1985) because it gives rise to a carcinogenic response that closely resembles the human situation in terms of tumor pathology, histogenesis, and ultrastructural morphology (Becci et al., 1978; Saffiotti et al., 1968; Saffiotti, 1970). It also has a low incidence of spontaneous pulmonary tumors and is highly resistant to pulmonary infections and inflammatory reactions (Saffiotti et al., 1985).

It has been established experimentally in the hamster model that BaP in combination with ferric oxide produces an increased incidence of tumors of bronchiogenic origin following intratracheal administration (Becci et al., 1978; Saffiotti, 1970; Saffiotti et al., 1968, 1985). Similar increased respiratory cocarcinogenic responses have been reported for other particles, such as amorphous silica (Stenback and Rowland, 1979), titanium dioxide (Stenback et al., 1976), India ink (Pylev et al., 1969), carbon (Stenback et al., 1976), and iodine (Stenback and Rowland, 1979). Results with aluminum oxide (Saffiotti et al., 1985) and manganese oxide (Stenback and Rowland, 1979) did not show cocarcinogenic responses with BaP. Recently, it has been reported (Niemeier et al., 1986) that crystalline silica and foundry sands, in conjunction with BaP, caused an increased carcinogenic response in the range similar to that of ferric oxide and BaP. Animals receiving only the particles did not develop any tumors.

Benzo[a]pyrene (BaP), a well-characterized PAH, is a ubiquitous occupational and environmental pollutant and is the product of incomplete combustion associated with fossil fuels, cigarette smoke, and particles (Chrisp and Fisher, 1980; Hughes et al., 1980; National Academy of Sciences, 1972). It is known that the biological activity of BaP is influenced by its metabolic fate (Gelboin, 1980) such that the target tissue must be exposed to an appropriate level of reactive metabolites, which alter criti-

cal cell regulatory mechanisms. In the presence of particles in the lung, such as asbestos (Kandaswami et al., 1982), ferric oxide (Warshawsky et al., 1984), crude air particulate (Warshawsky et al., 1983), cigarette smoke (Lubawy and Isaac, 1980), and fly ash (Morgan et al., 1984), it has been demonstrated that the rate of metabolism of BaP is decreased, the metabolic pathways altered, and binding of BaP metabolites to macromolecules (Kandaswami et al., 1982) increased in comparison with exposures to BaP alone.

An important biological response to inhaled particles is ingestion by pulmonary alveolar macrophage (AM) and eventual clearance from the lung. This would be consistent with the hamster model in that BaP-containing AM were found in the tracheobronchial lumen; BaP either diffused from the luminal macrophages through the mucociliary layer into the tracheobronchial epithelium or diffused through the interstitial tissue from the lungs to the bronchi and trachea (Saffiotti et al., 1985; Schreiber et al., 1975).

Human, dog, and guinea pig AM (Autrup et al., 1978, 1979; Bond et al., 1984; Harris et al., 1978; Jaurand et al., 1981; Marshall et al., 1979) have been shown, in limited studies, to metabolize BaP and phagocytize BaP-coated particles, such as ferric oxide and diesel particles (Autrup et al., 1978; Bond et al., 1984), with subsequent release of BaP and its metabolites (Autrup et al., 1978, 1979; Bond et al., 1984) and binding to DNA and protein (Autrup et al., 1978). Coincubation of human AM with V79 cells showed that AM could metabolize BaP to induce ouabain-resistant mutations and sister chromatid exchanges in V79 cells (Hsu et al., 1979). Other studies with BaP-coated Fe_2O_3 and air samples have resulted in increased mutagenic responses (Bevan and Manger, 1985; Hubbard et al., 1986; Romert et al., 1985). It is apparent that the metabolism of BaP-coated particle by AM may play an important role in the overall development of lung disease.

Evidence suggests that exposure of AM to particles increases the endocytic rate and the accompanying release of oxygen radicals and/or hydrogen peroxide (Karnovsky, 1962; Rossi et al., 1972); however, a correlation between this enhanced endocytic rate and the effect that a carrier particle may have on the metabolism of a carcinogen in vitro remains undetermined (Kavet et al., 1978). Additionally, AM attached to a substrate appear to have fewer receptors available for endocytosis than macrophages in suspension because a layer of receptors generally is used in the attachment process (McKeever and Spicer, 1980). The AM do not attach to a substrate in vivo; thus suspension-cultured macrophages would more closely mimic macrophages in vivo. This article presents the results of studies that investigate the endocytosis of Fe_2O_3-coated BaP in a dose-response fashion, the total metabolic capacity of the AM as well as the production of the individual metabolite, and a measure of the production of reactive oxygen in a time-dependent manner in suspen-

sion culture. The results indicate that with increased endocytic capacity as measured by the increase in reactive oxygen metabolites a greater amount of total metabolite is produced, in particular the dihydrodiols of BaP, which are considered to be products of the active pathway of BaP.

MATERIALS AND METHODS

Chemicals

Labeled BaP was purchased from Amersham (Arlington Heights, Ill.) ([7,10-^{14}C]BaP, 29.7 mCi/mmol), unlabeled BaP was purchased from Aldrich (Milwaukee, Wis.), Brevital from E. Lilly Company (Indianapolis, Ind.), cytochrome *c* (Type VI), xanthine, xanthine oxide, and superoxide dismutase (SOD) from Sigma Chemical Company (St. Louis, Mo.), Fe_2O_3 and Scintiverse (for organic samples) from Fisher Scientific Company (Fair Lawn, N.J.), Aqualyte from J. T. Baker Chemical Company (Phillipsburg, N.J.) for aqueous samples, and Metrizamide from Sigma. The BaP metabolite standards were obtained from the NCI Chemical Repository: 9,10-dihydrodiol (9,10-diol), 7,8-dihydrodiol (7,8-diol), 4,5-dihydrodiol (4,5-diol), 9-hydroxy (9-OH), 3-hydroxy (3-OH), and the 3,6-quinone. A 5 m*M* stock solution of ^{14}C-BaP and cold BaP was prepared such that a 12.5-μl aliquot contained 1 μCi and 62.5 nmol BaP.

Animals and Feeding Protocol

Male Syrian golden hamsters, 6–8 wk old, 100–150 g, were purchased from Harlan Co., Harlan, Ind. All animals were kept on a standard light cycle (12/12) with the room temperature at 74 ± 2°F and relative humidity at 45 ± 1%, and were fed single-batch, standard laboratory hamster feed ad libitum up to the time of sacrifice. Animals were maintained in the animal care facility, which was kept free of any pesticides, for 2 wk prior to use in any study.

Cell Preparation

The AM were harvested by tracheal lavage with cold sterile saline from hamsters anesthetized with Brevital Sodium ip (0.4 ml, 1% solution) followed by exsanguination (Castranova et al., 1980; Sweeny et al., 1981). The AM were separated from the lavage fluid by centrifugation at 1500 × g for 5 min at 2°C (Castranova et al., 1980). The erythrocytes were separated from the AM by hemolysis by resuspending the pellet in 3.0 ml of cold 0.83% NH_4Cl for 5 min. The cell suspension was then recentrifuged and the cell pellet washed using Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS) containing HEPES buffer. The cells were cultured in suspension in NaOH-washed siliconized 20-ml culture vials (2.5×10^6 cells/vial) using 2.5 ml Ca^{2+}- and Mg^{2+}-free HBSS containing HEPES buffer, glucose, and cytochrome *c* (0.12 m*M*) (pH 7.4), without serum, at 37°C in a shaking water bath (Sweeny et al., 1981). Before use, the culture vials

were prewashed with NaOH and treated with silicon by using a dilute solution of Prosil (Prosil-28, SCM Chemical Corporation) and rinsing three times with deionized water. Short-term culture viability was assessed by erythrosine-B exclusion staining. A minimum of 100 cells was counted with a hemocytometer and a Leitz Wetzlar optical microscope with a 10× objective. The percentage of viable cells was equal to nonstained cells/(stained + nonstained cells) × 100. Exclusion staining was also used to determine the cytotoxicity of Fe_2O_3, BaP, and dimethyl sulfoxide (DMSO). Cytotoxicity was defined as a decrease in cell viability of greater than 5% as indicated by exclusion staining and a depression in basal metabolism greater than 10% as indicated by cytochrome *c* reduction. These evaluations were done in triplicate at each incubation period (0, 1, 2, 3, and 6 h).

Cytochrome *c* Assay

Basal metabolic activity was examined by measuring the reduction of cytochrome *c* by superoxide anions that were released by the AM (Castranova et al., 1980). At the beginning of each incubation period, 2.97 mg cytochrome *c* (final concentration of 0.12 m*M*) was added to each culture containing 2.5×10^6 cells in 2.5 ml of culture medium (Kaplan and Keogh, 1982) and incubated for 0, 1, 2, 3, or 6 h. Controls without cells were incubated over the same time points. At the end of the incubation period, the cells or cells and Fe_2O_3 were separated from the medium by centrifuging at 1500 × g for 5 min at 2°C (Kaplan and Keogh, 1982). The optical density of a 1-ml aliquot of the supernatant was monitored at 550 nm with a Beckman DB-GT grating spectrophotometer.

To ensure that the observed reduction of cytochrome *c* was due to superoxide anions and not other reactive oxygen species released by the AM, experiments were conducted in the presence and in the absence of superoxide dismutase (SOD). The AM (2.5×10^6 cells) were added to NaOH-prewashed siliconized culture vials that contained 2.5 ml culture medium, 0.12 m*M* cytochrome *c*, and SOD (0.4 mg/ml) and were incubated for 6 h. The culture medium was centrifuged and assayed for the reduction of cytochrome *c*. The activity of the SOD was determined by a modified method of McCord and Fridovich (1969). The assay was performed in 3 ml of 0.05 *M* potassium phosphate buffer containing 10^{-4} *M* EDTA in a 1.0-cm cuvette at ambient temperature (pH 7.8). Cytochrome *c* (0.12 m*M*), xanthine (5×10^{-5} *M*), and xanthine oxidase (6×10^{-9} *M*) were added to the cuvette. The reduction of cytochrome *c* was monitored at 550 nm with a Beckman DB-GT grating spectrophotometer. These conditions produced a steady rate of cytochrome *c* reduction. The SOD was then added to the cuvette until a maximum reduction in the amount of cytochrome *c* reduced was observed, which required 0.4 mg/ml of SOD.

The following experiments were conducted to ensure that the observed reduction of cytochrome *c* was not due to an experimental vari-

able. Each of the following variables were incubated for 6 h with culture medium (2.5 ml) and cytochrome *c* (0.12 m*M*): BaP alone (62.5 nmol; 25 μM), Fe_2O_3 alone (2.0 mg), BaP metabolites (4,5-, 7,8-, 9,10-dihydrodiol, 3-, 7-, 9-hydroxy, 3,6-, 4,5-quinone, 4,5-epoxide, at a total of 0.362 μM), DMSO alone (25 μl), BaP/Fe_2O_3 alone, and BaP/Fe_2O_3, BaP metabolites, and DMSO together. The contents were centrifuged at 1500 × g for 5 min at 2°C and the supernatant was decanted and assayed for the reduction of cytochrome *c*. To ensure that the reduction of cytochrome *c* was not due to photooxidation, cytochrome *c* (0.12 m*M*) was added to 2.5 ml of culture medium and incubated for 6 h in one of three shaking water baths (37°C): covered, exposed to unfiltered light, or exposed to lights equipped with yellow filters. All vials were incubated for 6 h, the contents were centrifuged, and the supernatant was assayed for the reduction of cytochrome *c*.

BaP/Particle Preparation

The desired dose of particles (0.0, 0.5, 1.0, or 2.0 mg Fe_2O_3), which had been prescreened (using a cyclone separator with a flow rate of 13.5 ft^3/min rotor speed, 1250 rotations/min; Donaldson Classifier model C-532, Donaldson Co.) so that 87% of the particles were 2.5 μm or less, was placed in a 20-ml glass culture vial just before use. An aliquot of the ^{14}C-BaP stock solution (62.5 nmol dissolved in benzene) was added directly on top of the 0.0, 0.5, 1.0, or 2.0 mg Fe_2O_3. The solution was evaporated to dryness under a gentle stream of nitrogen at 37°C to remove the benzene solvent. All handling of BaP solutions was done under lights equipped with yellow filters (Warshawsky et al., 1984) to avoid photooxidation.

BaP Metabolism

One milliliter of media containing 0.12 m*M* cytochrome *c* and 25 μl DMSO was added to a series of culture vials containing ^{14}C-BaP (62.5 nmol) adsorbed onto previously prepared doses of Fe_2O_3 [0.0, 0.5, 1.0, or 2.0 mg; the Fe_2O_3 doses were based on work by Saffiotti et al. (1968, 1985; Saffiotti, 1970), and these doses were also noncytotoxic for the AM]. The vials were vortexed for 30 s and then 0.5 ml of media containing 2.5 × 10^6 cells (checked for viability) was added to each culture vial and the mixture incubated for either 0, 1.0, 2.0, 3.0, or 6.0 h. At the end of the appropriate incubation period, cultures were centrifuged and the amount of cytochrome *c* reduced in the supernatant (medium) was quantified. The cell pellets were resuspended in 5 ml cold HBSS and transferred to centrifuge tubes that contained a subphase of Metrizamide. The tubes were then centrifuged for 20 min at 400 × g at 2°C (Kavet et al., 1978). Cells were collected at the interface while the cell-free particles formed a pellet. A sample of the cells was analyzed for viability by exclusion staining. The remaining AM were rinsed twice to remove any radiolabel. The

washes were returned to the media. An aliquot of both media and macrophages was then extracted three times with ethyl acetate. These ethyl-acetate-extractable metabolites were evaporated under a gentle stream of nitrogen at 40°C and resuspended in chloroform. The metabolites were then separated using Waters HPLC equipped with a WISP unit fitted with a 25 × 4.8 mm, 10-μm particle size Whatman ODS Partisil C-18 reverse-phase column. A methanol/water gradient elution program, which began with an initial concentration of 80% methanol and increased 1%/min for 3 min, 3%/min for 3 min, 0%/min for 3 min, and 8%/min for a final concentration of 100% methanol, was used at room temperature with a flow rate of 1 ml/min (Warshawsky et al., 1984). Fractions were monitored at 254 nm, collected over time, and compared to a standard mixture of metabolite. Each fraction was quantified by liquid scintillation spectrometry, which was run daily. The AM pellet was solubilized with NaOH, decolorized with *tert*-butyl hydroperoxide, and quantified by liquid scintillation spectrometry. The ethyl acetate-nonextractable metabolites were also quantified. The radiolabel associated with the particles was determined by placing the particles in 2 ml benzene and counted.

Statistics

The initial statistical analysis of the data consisted of analysis of variance in a general linear model to determine the existence of pair-wise or multiple interactions. This was followed by Duncan's multiple comparison test as performed by a statistical analysis system program. Significance was determined at the $p = .05$ level unless otherwise indicated.

RESULTS

Short-Term Culture Viability

Viability of AM was greater than 95%, as determined by exclusion staining, in short-term suspension cultures in HBSS without serum for the first 6 h, regardless of treatment. The viability of all cultures with or without particles declined over the next 3 h. The degree of decline, to a minimum of 36% viability at the highest particle dose (2 mg), appeared to be dependent on the dose of BaP-coated particles present.

Cytochrome *c* Reduction

With cells or BaP plus cells, the amount of cytochrome *c* reduced increased from 0 to 6 h and appeared to reach a plateau after that time (Table 1). The addition of BaP-coated Fe_2O_3 (Table 1) or Fe_2O_3 alone (not shown) resulted in a significant increase ($p = .05$ when compared to cells alone) in the amount of cytochrome *c* reduced in a dose-dependent manner. The addition of the BaP-coated Fe_2O_3 or Fe_2O_3 alone did not affect the 6-h plateau (Table 2). It should be noted that the addition of

TABLE 1. Alveolar Macrophage Cytochrome *c* Reduction[a] in the Presence of BaP/Fe_2O_3

Time (h)	nmol Cytochrome *c* reduced/10^6 cells				
			BaP/Fe_2O_3[b]		
	Cells alone	BaP plus cells[c]	0.5 mg	1.0 mg	2.0 mg
0	0.0	0.0	0.0	0.0	0.00
1	0.3 ± 0.1[d]	1.6 ± 0.5[e]	4.4 ± 0.3[e]	4.7 ± 0.4[e]	4.4 ± 0.9[e]
2	0.7 ± 0.1	3.3 ± 0.9[e]	7.1 ± 1.0[e]	7.8 ± 0.9[e]	9.2 ± 0.9[e]
3	0.7 ± 0.1	5.0 ± 0.8[e]	10.8 ± 1.5[e]	12.5 ± 1.1[e]	15.3 ± 1.1[e]
6	1.4 ± 0.4	11.8 ± 1.0[e]	14.6 ± 1.9[e]	19.5 ± 2.3[e]	22.4 ± 2.4[e]
9	1.4 ± 0.5	12.8 ± 1.2[e]	16.3 ± 1.8[e]	21.0 ± 2.9[e]	23.7 ± 3.0[e]

[a]Measured by absorbance at 550 nm, $n = 3$.
[b]BaP (62.5 nmol) adsorbed to Fe_2O_3.
[c]BaP (62.5 nmol).
[d]± Standard deviation.
[e]$p = .05$ Compared to 0.0 mg (cells alone), Duncan's multiple comparison test.

larger particle amounts did not result in a proportional increase in cytochrome c reduction.

Endocytosis of BaP

The highest noncytotoxic concentration of BaP tolerated by the AM in this system was 25 μM (62.5 nmol/2.5 ml). The AM did phagocytize BaP-coated particles as evidenced by quantitation of BaP in the ethyl acetate-extractable fraction of AM by HPLC analysis and liquid scintillation spectrometry (Fig. 1). The highest concentration of BaP phagocytized was found in cells exposed to BaP-coated (2.0 mg) Fe_2O_3 at 3 h. For the first hour, the amount of BaP phagocytized by the AM appeared to be dependent on the concentration of Fe_2O_3 given.

Metabolism of BaP

Ethyl acetate-extractable intracellular BaP metabolites indicated that AM did metabolize the phagocytized BaP (Fig. 2). The total amount of

TABLE 2. Cytochrome *c* Reduction[a] at 6 Hours

Cells alone	Cells + BaP[b]	Cells + Fe_2O_3[c]	Cells + BaP/Fe_2O_3[d]
1.4 ± 0.4[e]	11.8 ± 1.0[f]	21.6 ± 4.6[f]	22.4 ± 2.4[f]

[a]Measured by absorbance at 550 nm, $n = 3$, and given as nmol cytochrome *c* reduced/10^6 cells.
[b]BaP (62.5 nmol).
[c]Fe_2O_3 alone (2.0 mg).
[d]±BaP (62.5 nmol) adsorbed to Fe_2O_3 (2.0 mg).
[e]± Standard deviation.
[f]$p = .05$ Compared to 0.0 mg (cells alone), Duncan's multiple comparison test.

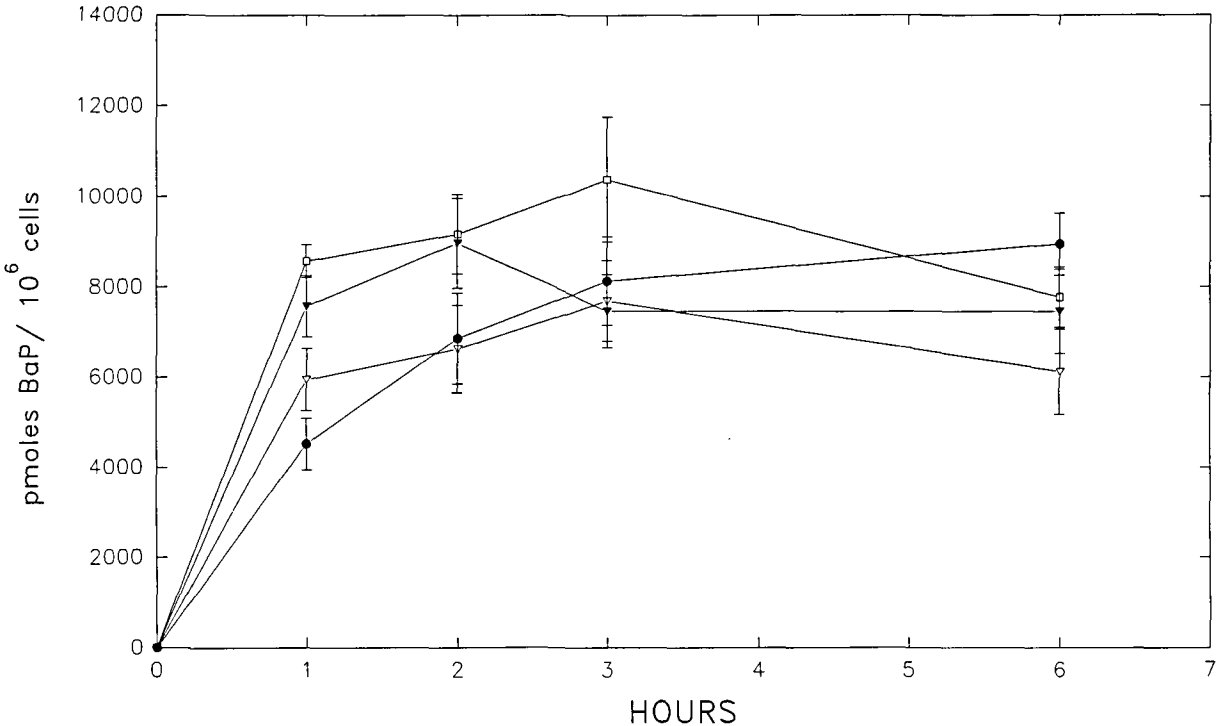

FIGURE 1. Phagocytosis of BaP by AM following exposure to BaP-coated Fe_2O_3 particles over time, BaP (62.5 nmol), $n = 3$: ● 0.0 mg Fe_2O_3 (BaP alone), ▽ BaP coated on 0.5 mg Fe_2O_3, ▼ BaP coated on 1.0 mg Fe_2O_3, □ BaP coated on 2.0 mg Fe_2O_3.

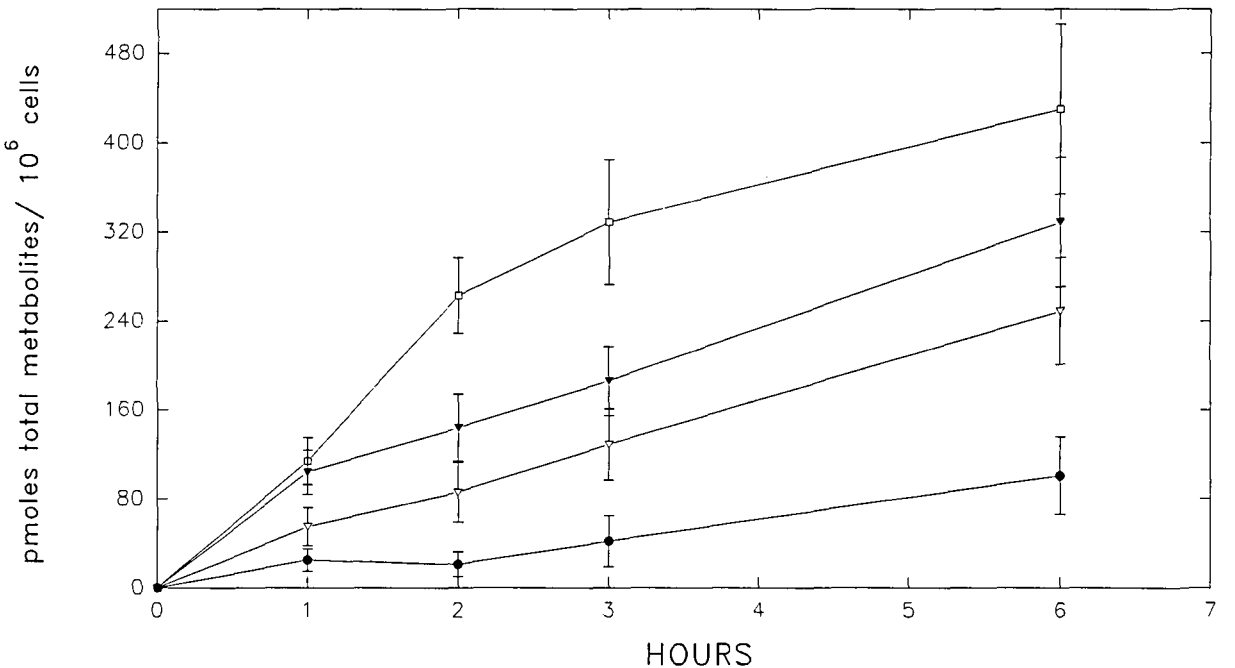

FIGURE 2. Appearance of total intracellular BaP metabolites in AM following exposure to BaP-coated Fe_2O_3 particles over time, BaP (62.5 nmol), $n = 3$: ● 0.0 mg Fe_2O_3 (BaP alone), ▽ BaP coated on 0.5 mg Fe_2O_3, ▼ BaP coated on 1.0 mg Fe_2O_3, □ BaP coated on 2.0 mg Fe_2O_3. The total BaP metabolites found in cells exposed to BaP-coated Fe_2O_3 were significantly different from levels found in cells exposed to BaP alone at $p = .001$ for all Fe_2O_3 concentrations at all time points.

metabolites produced by the AM was dependent on the concentration of Fe_2O_3 given. The total amount of intracellular metabolites produced increased in a near linear fashion during the entire course of the experiment. In all cases, more metabolites were found in the cells exposed to BaP-coated Fe_2O_3 than in the cells exposed to BaP alone. This was significant for all concentrations of Fe_2O_3 at all time points when compared to controls ($p = .001$). The rate of appearance of the intracellular metabolites up to 6 h was directly related to the concentration of Fe_2O_3.

Metabolites identified intracellularly were the dihydrodiols (4,5-, 7,8-, and 9,10-), the phenols (3-OH and 9-OH), and the 3,6-quinone (Fig. 3). In general, the amount of the individual metabolites increased with time and the dose of BaP-coated Fe_2O_3 given. With a given concentration of BaP-coated Fe_2O_3 for a given time, the amount of the 7,8-dihydrodiol produced was always greater than that of any other metabolite. However, the amount of 7,8-dihydrodiol produced was significantly greater than the 9,10-dihydrodiol at 6 h only for cells exposed to 2.0 mg Fe_2O_3 ($p = .05$). The production of 7,8-dihydrodiol was only occasionally significantly greater than the 4,5-dihydrodiol or the 3- or 9-hydroxy, and was always significantly greater than the 3,6-quinone ($p = .05$). The amount of the 7,8-dihydrodiol was significantly greater for cells exposed to all concentrations of Fe_2O_3 when compared to controls for a given time point ($p = .05$). It should be noted that the ferric oxide alone (without AM) did not chemically modify or oxidize BaP.

Ethyl acetate-extractable extracellular BaP metabolites also indicated that the AM metabolized BaP and subsequently released metabolites into the media (Fig. 4). The total amount of metabolites present extracellularly was related to the concentration of BaP-coated Fe_2O_3 given. This release was significant ($p = .05$) for all concentrations of BaP-coated Fe_2O_3 given when compared to BaP alone. The total amount of extracellular metabolites present increased during the entire course of the incubation of AM.

The rate of appearance of the extracellular metabolites was dependent on the concentration of BaP-coated Fe_2O_3 given. The same metabolites found intracellularly were also found extracellularly (Fig. 5). Within a given concentration of Fe_2O_3 and for a given time the amount of the 7,8-dihydrodiol produced was always more than any other metabolite. However, the amount of 7,8-dihydrodiol produced was never significantly greater than the 9,10-dihydrodiol, occasionally significantly greater than the 4,5-dihydrodiol or the 3- or 9-hydroxy, and nearly always significantly greater than the 3,6-quinone ($p = .05$). The amount of the 7,8-dihydrodiol was significantly greater for cells exposed to all concentrations of Fe_2O_3 when compared to controls for a given time point except for cells exposed to 0.5 mg at 2 h ($p = .05$).

There was an extremely small amount of ethyl acetate-nonextractable metabolites in both media and cells produced by the AM (less than 1% in

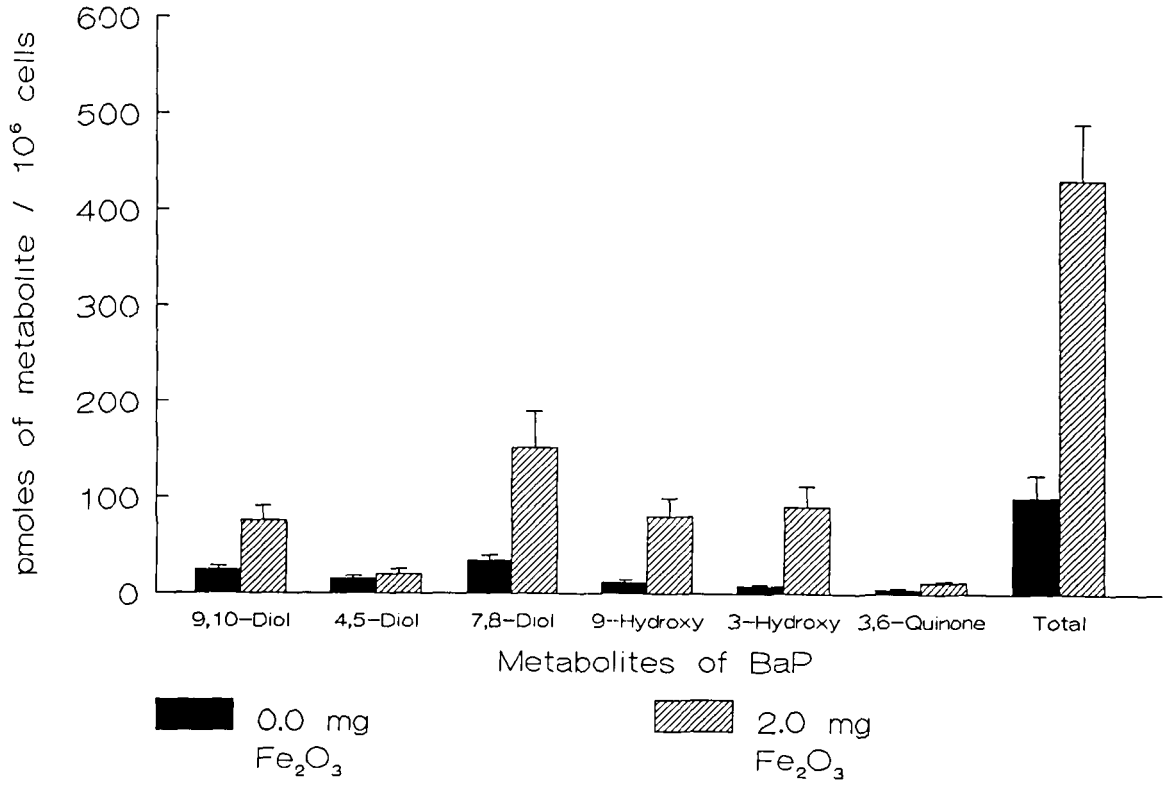

FIGURE 3. BaP metabolites in AM at 6 h following AM exposure to BaP-coated Fe_2O_3, $n = 3$: 0.0 mg, BaP alone (62.5 nmol); 2.0 mg, BaP coated on 2.0 mg Fe_2O_3. Each of the BaP metabolites and the total BaP metabolites produced with 2.0 mg Fe_2O_3 were significantly different from BaP alone at $p = .05$ except for the 4,5-dihydrodiol.

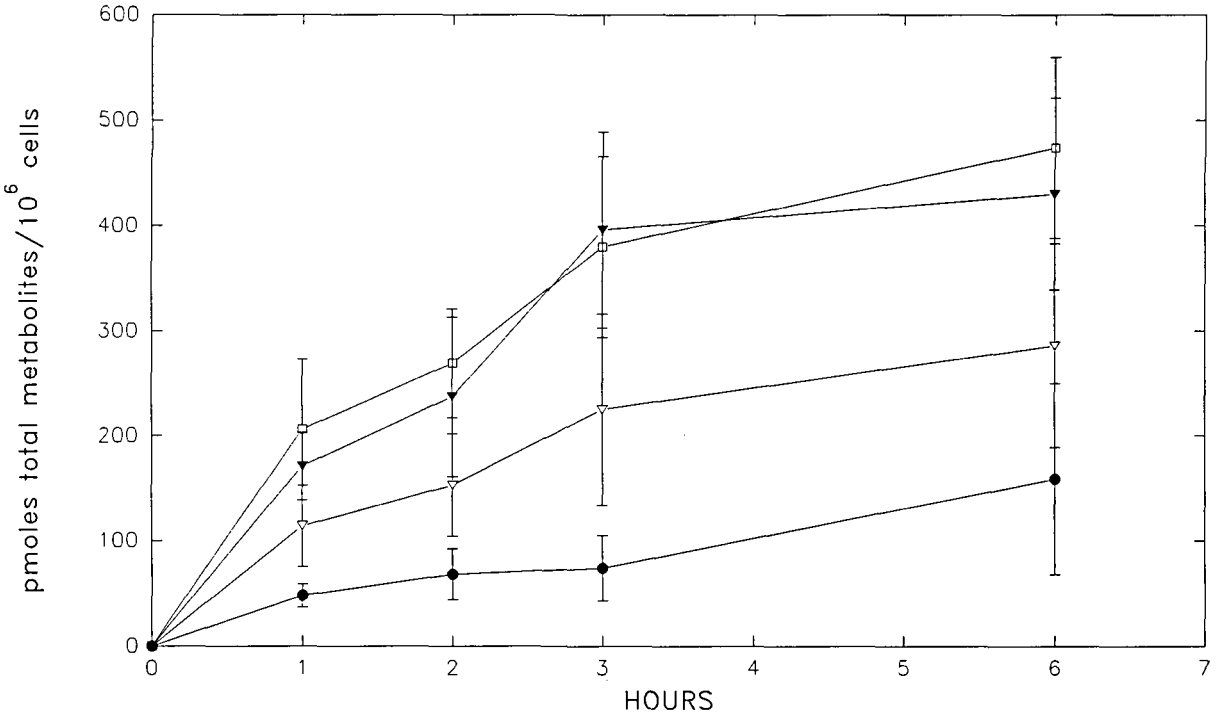

FIGURE 4. Appearance of total extracellular BaP metabolites in media following exposure to BaP-coated Fe_2O_3 particles over time, BaP (62.5 nmol), $n = 3$: ● 0.0 mg Fe_2O_3 (BaP alone), ▽ BaP coated on 0.5 mg Fe_2O_3, ▼ BaP coated on 1.0 mg Fe_2O_3, □ BaP coated on 2.0 mg Fe_2O_3. The total BaP metabolites present extracellularly as a function of BaP-coated Fe_2O_3 were significantly different from BaP alone at all concentrations of Fe_2O_3 at all time points at $p = .05$.

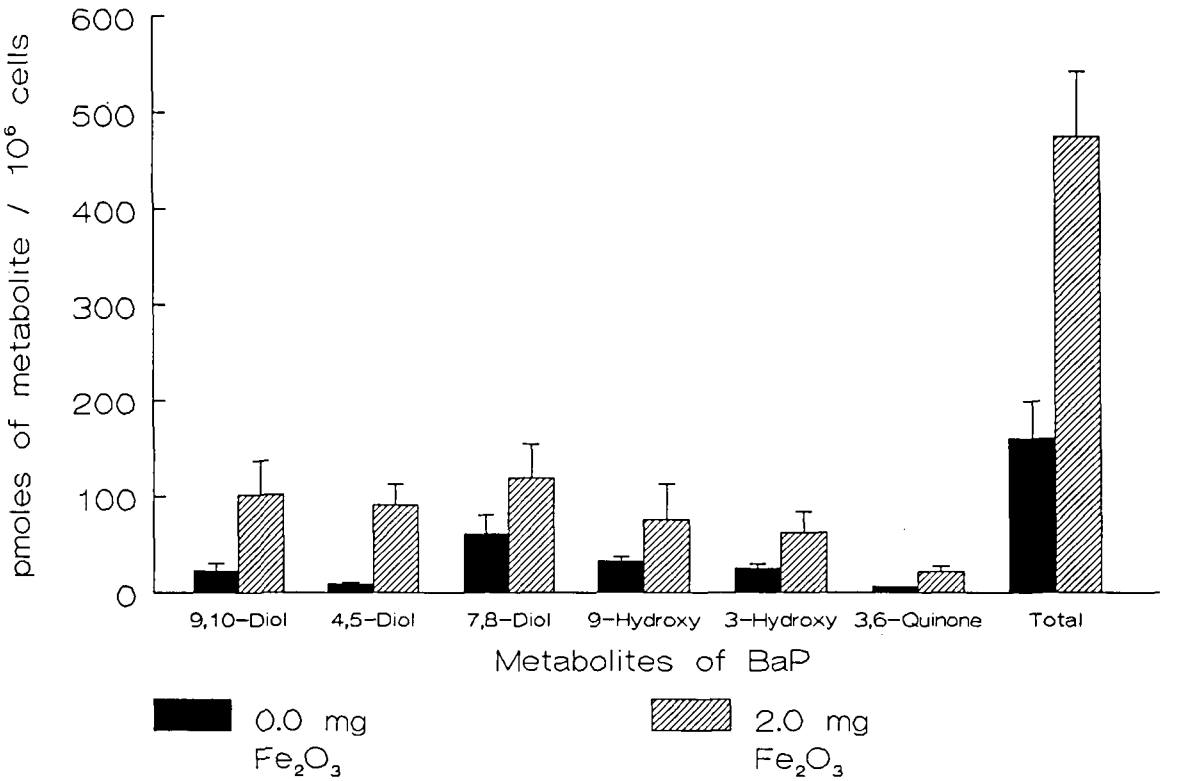

FIGURE 5. BaP metabolites in media at 6 h following AM exposure to BaP-coated Fe_2O_3, $n = 3$: 0.0 mg, BaP alone (62.5 nmol); 2.0 mg, BaP coated on 2.0 mg Fe_2O_3. Each of the BaP metabolites and the total BaP metabolite produced with 2.0 mg Fe_2O_3 were significantly different from BaP alone at $p = .05$ except for 3- and 9-hydroxy BaP.

the cells and media). It was assumed that these metabolites represented conjugation of BaP by the AM. Sulfate conjugates were not extracted by ethyl acetate extraction as verified by HPLC.

DISCUSSION

This laboratory has had a long-term interest in the effects of particles on the metabolism and binding in the lung of xenobiotics such as BaP (Morgan et al., 1984; Warshawsky et al., 1984). Our laboratory has reported that ferric oxide particles altered the metabolism of BaP in the rabbit isolated perfused lung (IPL) preparations; BaP intratracheal administration to the IPL with and without ferric oxide particles enhanced dihydrodiol formation appearing in the blood over 3 h and depressed nonextractable water-soluble conjugates (Warshawsky et al., 1984). These data suggested that pulmonary exposure to a known cocarcinogen, ferric oxide, in the presence of BaP resulted in increased production of dihydrodiols, which could be further metabolized to the ultimate carcinogenic form(s) of BaP.

A logical extension of this work was to assess the influence of phagocytosis of BaP-coated ferric oxide on BaP metabolism in AM (Kaplan and Keogh, 1982; Greife et al., 1988). It was demonstrated that AM released superoxide anions as a result of basal metabolic activity (Castranova et al., 1980). Challenge of the AM by foreign material, including particles and bacteria, resulted in phagocytosis of the foreign material. This phagocytosis was accompanied by an increase in the basal metabolic activity of the AM, and a subsequent increase in the release of superoxide anions (Castranova et al., 1980). It was thought that this release was associated with the defense system of the AM. There were some in vitro studies examining the association between reduction of cytochrome *c* by superoxide anions released from AM and the presence of particles. One such study challenged rat AM with opsonized particles. This resulted in AM release of superoxide anions in a particle dose-dependent manner. The anions released reduced cytochrome *c* (Castranova et al., 1980).

The investigation presented here also examined the release of superoxide anion with and without particles (Fe_2O_3). It was observed that the presence of particles resulted in an increased release of superoxide anion by the cells and subsequent reduction of cytochrome *c* for the first 6 h when compared to cells alone. This increase, which was significant at all dose levels, was indicative of phagocytosis. The observed decline in the amount of superoxide anion produced by the cells after 6 h was indicative of the loss of AM viability.

The data indicate that the AM can endocytize and metabolize BaP in this culture system. The addition of Fe_2O_3 enhanced the AM metabolism of BaP, and the rate of metabolism and enhancement was dose dependent. The particles appeared, therefore, to facilitate uptake of BaP by the

AM by acting as a carrier of BaP into the cell. This increased amount of BaP in AM led to increased production of BaP metabolites by the AM in the presence of particles. These results, therefore, indicate that AM had more BaP to metabolize or that the AM may simply have metabolized the BaP that was endocytized in the presence of the particles faster than the BaP that was endocytized without particles. It should be noted that the cells not only have more BaP to metabolize, but the potential for a preferential production of precarcinogenic metabolites also exists.

The research presented here indicates that there is a preferential production of precarcinogenic metabolites. Specifically, the dihydrodiols are produced with the 7,8-dihydrodiol (which is presumed to be the precursor to the ultimate carcinogenic metabolite of BaP) being found in the largest quantity, with the amount of this metabolite produced being dependent on the dose of Fe_2O_3 given. The presence of particles enhances the production of dihydrodiols rather than shifting production to another metabolic form, such as phenols or quinones. These data are supported by other studies in which BaP was metabolized by human and dog AM in the presence of particles (Autrup et al., 1978; Bond et al., 1984) with increased production of metabolites, in particular the 7,8-dihydrodiol (Bond et al., 1984).

The enhanced production of the 7,8-dihydrodiol and superoxide anions observed in this research may partially explain the enhanced production of tumors observed in vivo following concurrent administration of carcinogen-coated particles. Following an intratracheal administration of BaP-coated Fe_2O_3 to hamsters (Henry et al., 1975), AM in vivo that contain phagocytized carcinogen-coated particles were observed to have migrated to the tracheobronchial bifurcation, forming clusters of quiescent AM. The most severe changes in the lung tissue were observed adjacent to the AM clusters. These changes could be attributed to the release of superoxide anions and BaP metabolites, including the 7,8-dihydrodiol. The metabolites could either be endocytized a second time by other macrophages and metabolized, or metabolized further to ultimate carcinogenic products by the surrounding lung tissue. Such an involvement by the AM would serve to compromise the ability of this cell type to serve as part of the lung's defense mechanism. An increased localized release of superoxide anions (as a function of dose of Fe_2O_3) could cause localized damage to the surrounding tissue (i.e., hypertrophy and inflammation), predisposing the tissue to further injury by the increased production of the biologically active BaP metabolites.

Similar studies are underway at the present time with BaP-coated crystalline silica. Preliminary studies indicate so far that BaP-coated crystalline silica incubated with AM produces more dihydrodiols of BaP relative to BaP-coated ferric oxide. These data may have important implications in the overall carcinogenic process of BaP-coated particles. Further metabolism and DNA binding studies are in progress.

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