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COMPARATIVE COCARCINOGENIC EFFECTS OF CROCIDOLITE ASBESTOS, HEMATITE, KAOLIN AND CARBON IN IMPLANTED TRACHEAL ORGAN CULTURES

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Abstract—The carcinogenicity of the polycyclic aromatic hydrocarbon (PAH), 3-methylcholanthrene (3 MC) in hamster tracheal tissues was examined after its adsorption on to four particulates—crocidolite asbestos, hematite, kaolin and carbon. After precipitation of 3 MC-coated particulates on the epithelial surface of the tracheal explants, the cultures were maintained for 4 weeks *in vitro* before either morphologic examination or implantation into syngeneic animals. Use of radiolabelled 3 MC made it possible to determine the relative amounts of the PAH adsorbed to and released from the particles with time. Tumours, the majority of which were carcinomas, appeared after exposure of organ cultures to 3 MC-coated particles, but not after exposure to particles alone. When total numbers of tumours (i.e. carcinomas, sarcomas and undifferentiated malignant tumours) were considered, the relative cocarcinogenicity of the dusts was crocidolite asbestos > hematite > kaolin > carbon. However, differences were not observed in the numbers of carcinomas induced by 3 MC-coated asbestos, hematite or kaolin. Relatively few carcinomas developed in animals exposed to carbon coated with 3 MC. Carcinogenicity was not related consistently to either the affinity of the particle for 3 MC or the elution of the hydrocarbon from the dust. For example, carbon adsorbed more 3 MC than the other particulates, but elution was minimal. Hematite released the greatest amount of PAH, whereas kaolin and asbestos eluted appreciably smaller quantities. Our data suggest that factors other than particle-enhanced availability of PAH are important in carcinogenesis in the respiratory tract. Alternative mechanisms of cocarcinogenesis by particulates include either cytotoxicity and the resultant augmentation of DNA synthesis or squamous metaplasia (or both).

INTRODUCTION

AIRBORNE particles act as sites for the condensation of chemical carcinogens in cigarette smoke and the urban environment (EPSTEIN *et al.*, 1979; FOX and OLIVE, 1979; NATUSCH and TOMKINS, 1978; NATUSCH and WALLACE, 1974). Experimental studies using animals indicate a synergistic effect of particulates on tumour induction by polycyclic aromatic hydrocarbons (PAH) (HENRY *et al.*, 1974; SAFFIOTTI *et al.*, 1968; SHABAD *et al.*, 1974; STENBACK and ROWLAND, 1979). For example, intratracheal instillation of PAH into rodents results in relatively few neoplasms unless the hydrocarbons are coated on asbestos (SHABAD *et al.*, 1974), hematite (SAFFIOTTI *et al.*, 1968), carbon (HENRY *et al.*, 1974) and other minerals (STENBACK and ROWLAND, 1979). Although the mechanism of cocarcinogenesis is not clear, adsorption of the PAH on to the particle, and retention of the carcinogen-coated particle in the respiratory tract, are believed to be important.

In the studies reported here, the cocarcinogenic effects of a fibrous (crocidolite asbestos) and three non-fibrous (hematite, kaolin and carbon) minerals were assessed after coating the particulates with the PAH, 3-methylcholanthrene (3 MC). Tracheal organ cultures exposed to the carcinogen-treated dusts *in vitro* were implanted

subsequently into syngeneic animals. The affinity of the particle for the hydrocarbon and uptake of the 3 MC by the tracheal epithelium were examined to determine the influence of these factors in carcinogenesis of the respiratory tract.

MATERIALS AND METHODS

Coating of particles with 3 MC

3 MC (Aldrich Chemical Co., Milwaukee, WI) was adsorbed to heat-sterilized preparations of crocidolite asbestos (UICC reference sample), the clay mineral, kaolin (3–5 μm dia.; Georgia Kaolin Co., Elizabeth, NJ), carbon and hematite (both obtained as sized preparations of 0.5–1 μm dia. from IIT Research Institute, Chicago, IL), using a modification of the low-temperature precipitation method as described by SAFFIOTTI (1970). A solution of ^{14}C-3 MC, 0.29 mg (specific activity = 60.2 mCi mmol^{-1}, New England Nuclear, Boston, MA) in acetone was combined with unlabelled 3 MC (0.29 g) at a ratio of 1:1000. An equal weight of each particulate (0.29 g) was added in cold acetone to the hydrocarbon, and the suspension agitated on a magnetic stirrer for 48 h. At this time, the carcinogen-coated dusts were pelleted by centrifugation and the residual acetone was removed under nitrogen. The preparations then were washed once with distilled water to remove unbound 3 MC (MOSSMAN and CRAIGHEAD, 1974). At this juncture, pellets of 3 MC-coated particles were resuspended in Hanks' Balanced Salt Solution (HBSS) at various concentrations (i.e. 4, 8 and 16 mg ml^{-1}). A drop from each suspension was pipetted onto a carbon stub, the liquid was allowed to evaporate at room temperature, and the 3 MC-coated materials were examined by scanning electron microscopy (SEM). At these concentrations, clumping of individual 3 MC-coated particles was minimal and crystals of insoluble 3 MC were not observed.

To determine the amount of 3 MC adsorbed to and released from the different types of particles, 200 mg of each particulate was coated with ^{14}C-3 MC as described above. After one wash with distilled water, centrifuged pellets of 3 MC-coated materials were resuspended in 10 ml Waymouth's medium. To assess the amounts of ^{14}C-3 MC adsorbed initially to the particles, 1 ml aliquots of resuspended dusts were collected on Millipore filters (0.22 μm pore dia.) that then were dissolved in Biofluor (New England Nuclear, Boston, MA). Other 1 ml aliquots in medium were either rotated for 24 h at 37°C or recentrifuged, the supernate removed and pellets of dusts dried at 80°C before their resuspension in benzene at room temperature for 1 week. After 24 h, dusts in medium were pelleted by centrifugation and 0.5 ml aliquots of the supernate added to 10 ml Biofluor for scintillation spectrometry. After one week, dusts in benzene were collected on individual Millipore filters after repeated washing of the extraction vessel with benzene. Filters were dissolved in Biofluor and the amount of ^{14}C-3 MC on equal weights of the materials determined. Data were analysed using analysis of linear regression.

Preparation of hamster tracheal organ cultures and addition of 3 MC-coated particles

Female 4–6-week old BIO 15.16 golden Syrian hamsters (Bio-Research Institute, Cambridge, MA) were used as donors and recipients of tracheas. These animals are pathogen-free and demonstrate increased susceptibility, in comparison with randomly

bred hamsters, to induction of tumours after intragastric or subcutaneous administration of PAH (HOMBURGER *et al.*, 1972).

The procedure for establishing tracheal organ cultures and their exposure to 3 MC-coated particulates has been described in detail (MOSSMAN and CRAIGHEAD, 1979). In brief, the trachea was excised, using sterile technique, from the larynx to the bronchial bifurcation and immersed in HBSS containing 100 μg ml^{-1} gentamicin and 25 U/ml mycostatin. After removal of extraneous tissue by dissection, the trachea was opened along the anatomical discontinuity in the cartilage and cut into hemisections. Organ cultures were grouped randomly in 35 mm Petri dishes that had been scored to allow adherence of the nonepithelial surface. Particles with and without adsorption of 3 MC were added to individual dishes at 4, 8 and 16 mg ml^{-1} HBSS. After 1 h, tissues were removed and transferred to new dishes.

In comparative studies, replicate cultures were maintained in two types of media: one that stimulates epithelial hyperplasia, i.e. Waymouth's MAB 87/3 (Gibco, Grand Island, NY) containing 5 μg ml^{-1} hydrocortisone and gentamicin (100 μg ml^{-1}); and a second that maintains the respiratory epithelium in a differentiated state, i.e. Eagle's minimum essential medium (MEM) (Gibco, Grand Island, NY) with gentamicin (100 μg ml^{-1}) (MOSSMAN and CRAIGHEAD, 1975). Tissues cultured in Eagle's MEM were evaluated in morphologic studies, whereas explants maintained in Waymouth's medium were implanted into syngeneic animals. Organ cultures were incubated at 35°C in a humidified 5% CO_2–95% air environment. Medium was changed twice on a weekly basis.

Implantation of organ cultures into hamsters

After 4 weeks *in vitro*, organ cultures were either examined morphologically or implanted subcutaneously into syngeneic weanling female hamsters. The epithelial surface of the tracheal segment was sutured along the midline at both ends to the underlying musculature of the recipient animal. Hamsters were palpated for tumours at 3-week intervals and masses excised at >5 mm dia. Animals not exhibiting tumours were sacrificed as they become moribund at 105–110 weeks of age. The tracheal implant was removed at this time.

Histologic techniques

Tracheal organ cultures were fixed *in situ* for light microscopy using Bouin's solution; 5 μm Paraplast sections were then prepared by standard techniques and stained with hematoxylin and eosin (H&E).

After removal from the host, tracheal implants were fixed in Bouin's solution before dehydration and embedding. When apparent, the sutures at either end were used as a reference to orient the trachea in Paraplast for sectioning. To determine the site of origin of tumours developing from the tracheal epithelium, the tissue was step-sectioned along the longitudinal axis. One of each 25, 5 μm serial tissue sections was prepared for histologic study. The ribbon, comprised of sections of the remaining tissue, was then stored for future reference. Tissue sections were examined routinely after staining with H&E. Alternate sections were stained selectively for mucin and reticulin fibres and by the Masson's trichrome and periodic acid-Schiff technique, with and without diastase (CRAIGHEAD and MOSSMAN, 1979). When lesions of interest were

observed, paraffin sections from the ribbon adjacent to the section under study were examined.

RESULTS

Adsorption to and elution of ^{14}C-3 MC from particles

The objective of our initial series of experiments was to determine whether the four types of particles adsorbed and released 3 MC in a comparable fashion. As can be seen in Table 1, carbon retained more ^{14}C-3 MC in comparison with the other particulates. However, less than 0.1% of the hydrocarbon was released by carbon into the culture medium after 24 h at 37°C and extraction with benzene for 1 week failed to remove additional 3 MC. Hematite and crocidolite adsorbed similar amounts of 3 MC, although substantially more was eluted into culture medium from hematite. Kaolin adsorbed minimal amounts of 3 MC in comparison with the other particles.

PAH uptake by tracheal organ cultures

To determine whether similar amounts (μg) of 3 MC were deposited on the tracheal epithelium at 24 h after exposure to the 3 MC-coated particulates, representative cultures were digested in tissue solubilizer and ^{14}C-3 MC determined by scintillation spectrometry. Comparable amounts of 3 MC remained on the epithelial surface of cultures exposed to lower concentrations (i.e. 4 and 8 mg ml^{-1} medium) of particles. At the highest concentrations (i.e. 16 mg ml^{-1} medium), the 3 MC on kaolin-exposed cultures was approximately 2-fold less than was found with carbon, hematite and crocidolite (Fig. 1).

Morphologic changes in tracheal organ cultures

Explants exposed to hematite, kaolin and carbon exhibited a differentiated mucociliary epithelium for periods of several weeks. With the passage of time *in vitro*, the columnar mucosal cells acquired a cuboidal configuration. Foci of epithelial hyperplasia appeared at sites where microscopically-evident accumulations of particles were deposited on the tracheal epithelium. Keratinizing squamous metaplasia was seen

TABLE 1. ADSORPTION AND RELEASE OF ^{14}C-3 MC FROM PARTICLES*

Type of dust	^{14}C-3 MC (pg mg particle^{-1} ± S.E.)† Amount adsorbed initially	Amount eluted after 24 h in culture medium	Residual amount after 1 week extraction in benzene
Crocidolite	247 ± 16	1.6 ± 0.011 (0.6%)	35.7 ± 0.16 (14.0%)
Hematite	232 ± 18	4.9 ± 0.015 (2.0%)	2.7 ± 0.09 (1.2%)
Kaolin	26 ± 4	0.7 ± 0.009 (2.5%)	15.5 ± 0.32 (60.0%)
Carbon	1180 ± 69	1.2 ± 0.010 (0.1%)	1089.0 ± 5.9 (99.8%)

* 200 mg of each particulate was coated with ^{14}C-3 MC as described in Materials and Methods.

† N = 3–6 determinations from duplicate experiments.

‡ Numbers in parentheses represent the percentage of the amount of 3 MC adsorbed initially to each respective dust (i.e. the data presented in column 2).

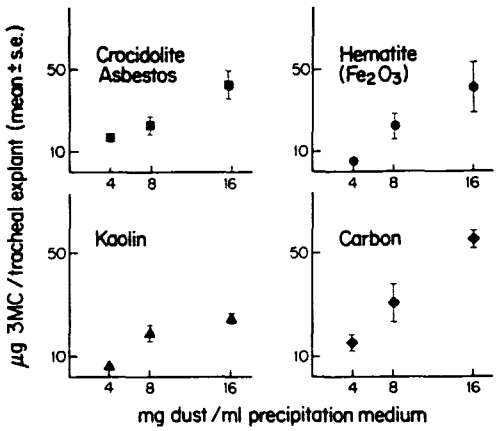


FIG. 1. Amounts of 3-methylcholanthrene (3 MC) on the tracheal epithelium at 24 h after exposure to 3 MC-coated particles. Dusts at various concentrations (i.e. 4, 8 and 16 mg ml^{-1} medium) were precipitated on tracheal organ cultures for 1 h. After transfer to fresh medium, 10 tissues were solubilized in NCS (New England Nuclear, Boston, MA) at each time period and the extracts counted in Biofluor. The amount of 3 MC in each sample was calculated by analysis of linear regression.

in organ cultures exposed to asbestos; this change was not present in tissues treated with the other dusts.

Tracheal explants exposed to 3 MC-coated particles exhibited squamous metaplasia and dysplastic changes. Neither *in situ* nor invasive neoplasia was apparent in the cultures, although atypical cells often were found exfoliating into the culture medium from the epithelial surface (Fig. 2).

Development of tumours

After implantation into syngeneic animals, neoplasms developed in the tracheal implants exposed to particulates coated with 3 MC. Tumours failed to appear in tissues treated with the mineral dusts alone. The development of tumours was dosage-dependent (Table 2). At the highest concentration of particles (i.e. 16 mg ml^{-1} medium), more neoplasms (sarcomas, carcinomas and undifferentiated malignant tumours) evolved in organ cultures exposed to crocidolite (50%). The tumour incidence with hematite, kaolin and carbon was somewhat lower, but the differences between these groups were not significant statistically. It should be noted that the number of carcinomas developing in cultures exposed to 3 MC-coated crocidolite, hematite and kaolin were similar (Table 2).

The survival time of animals implanted with tracheal grafts that had been exposed to 3 MC-coated particulates (16 mg ml^{-1} medium) is shown in Fig. 3. Since death of these animals was attributed to the malignant tumour, the measure is an indirect assessment of tumour induction time due to the 3 MC-coated particle concentrations. Analysis of the data established a significant difference between the animals in the groups implanted with crocidolite and carbon-treated explants.

The tracheal grafts were identified in the majority of lesions by sectioning the neoplasms serially. The origin of the tumour from the tracheal epithelium was apparent in about half of the specimens and transitions from *in situ* to invasive carcinoma often

were demonstrated (Fig. 4). Most of the tumours were poorly differentiated squamous carcinomas (Fig. 5). A common occurrence was a spindle cell carcinoma similar morphologically to the lesions described by LITTLE and O'TOOLE (1974) in hamsters after intratracheal instillation of benzo(a)pyrene and ^{210}Po (Fig. 6).

Six well differentiated squamous carcinomas were observed in our studies (Fig. 7). However, without exception they were found as focal lesions within a bulky mass of poorly differentiated carcinoma cells. As noted in Table 2, sarcomas and undifferentiated malignant tumours arose from a few explants exposed to each of the four different PAH-coated particulates.

A spectrum of epithelial alterations was noted in implants that failed to develop tumours. Squamous metaplasia and dysplasia commonly were observed (Fig. 8). The intensity and frequency of these changes could not be related specifically to either the particulate type or dosage of 3 MC. In contrast, a normal mucociliary epithelium was seen in implants exposed to the dusts in the absence of 3 MC.

DISCUSSION

Our system allowed a comparative evaluation of the cocarcinogenicity of four different types of mineral particulates in the hamster trachea. In this study, the affinity of each particle for the PAH and the amount deposited on the epithelium were uncontrollable variables. Although both the adsorption and elution of 3 MC by the various dusts differed (Table 1), the amounts of 3 MC retained on the tracheal epithelium after 24 h of incubation *in vitro* were similar (Fig. 1). It seems likely that the physical properties (i.e. size, structure and flocculence) of the four particles influenced sedimentation on and clearance from the epithelial surface. We have not addressed this question in detail.

The physicochemical properties of each dust appear to affect its interaction with the epithelium of the respiratory tract. For example, hematite (Fe_2O_3) and carbon are often considered relatively 'inert', since the surfaces are not charged ionically. These substances are relatively non-toxic *in vitro* (MOSSMAN *et al.*, 1978; MOSSMAN *et al.*, 1980a) and they do not interact avidly with cell membranes (WOODWORTH *et al.*, 1981). When instilled intratracheally or inhaled, the dusts induce infrequent, non-specific bronchial epithelial alterations and a few granulomatous changes in the lungs of experimental animals (STENBACK and ROWLAND, 1979). Although an excessive incidence of lung cancer has been reported in hematite miners (MORGAN and SEATON, 1975) and rodents instilled with iron ore from an open-hearth furnace (ISHINISHI *et al.*, 1976), some investigators argue that pure iron and its compounds are not carcinogenic (BOYD *et al.*, 1970; HUEPER, 1966). The increased mortality in hematite miners in the U.S.A. and Britain appears to be associated with ionizing metal contaminants (MORGAN and SEATON, 1975).

The increased risk of bronchial carcinoma and mesotheliomas of the pleura and peritoneum after exposure to crocidolite asbestos is well established (reviewed in BECKLAKE, 1976). Asbestos is cytotoxic to cultured cells (reviewed in HARINGTON *et al.*, 1975) and induces proliferative and metaplastic changes in the tracheobronchial epithelium (MOSSMAN *et al.*, 1980b). Kaolin, a hydrated aluminosilicate, has been less well characterized biologically. The hemolytic capability (MANYAI *et al.*, 1969) and fibrotic potential of the dust in man (SHEERS, 1964) and laboratory animals

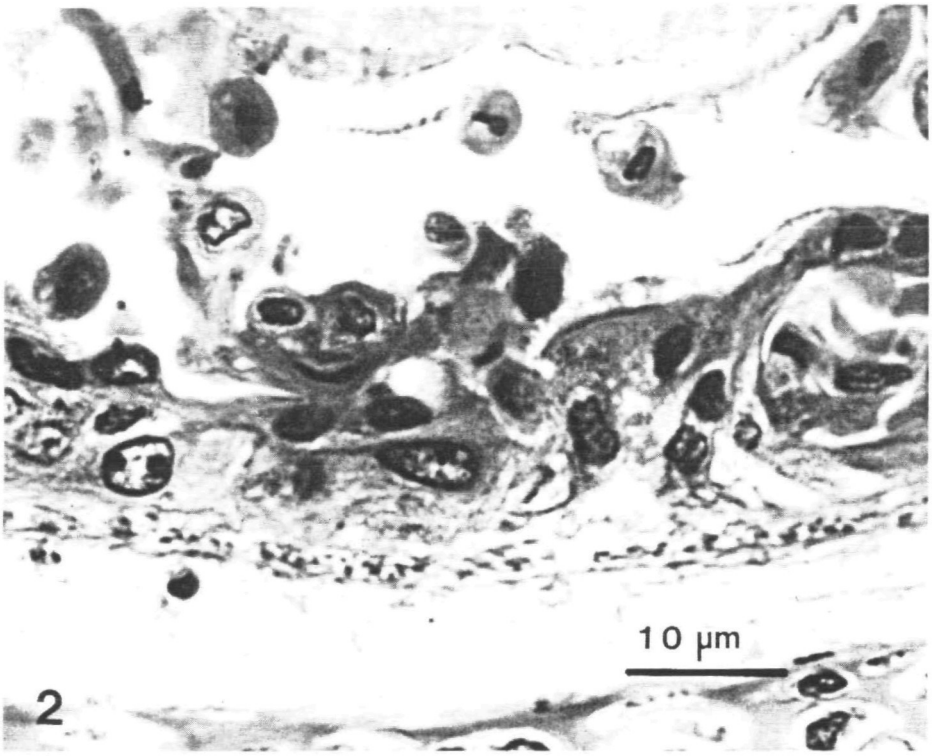


FIG. 2. Dysplastic changes in a tracheal organ culture after exposure to 3 MC-coated crocidolite asbestos (16 mg dust). The explant was examined after 4 weeks *in vitro* (H&E).

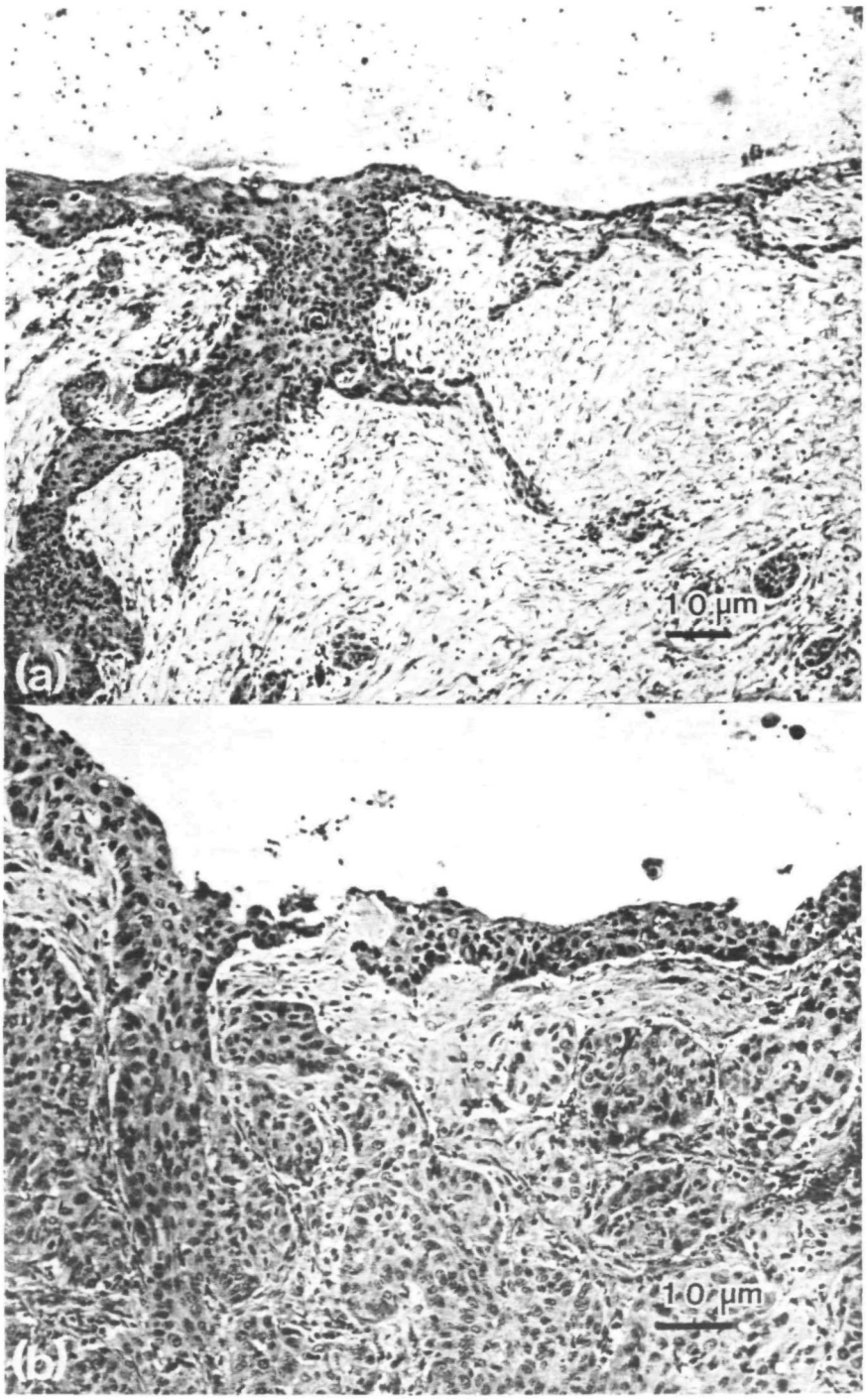
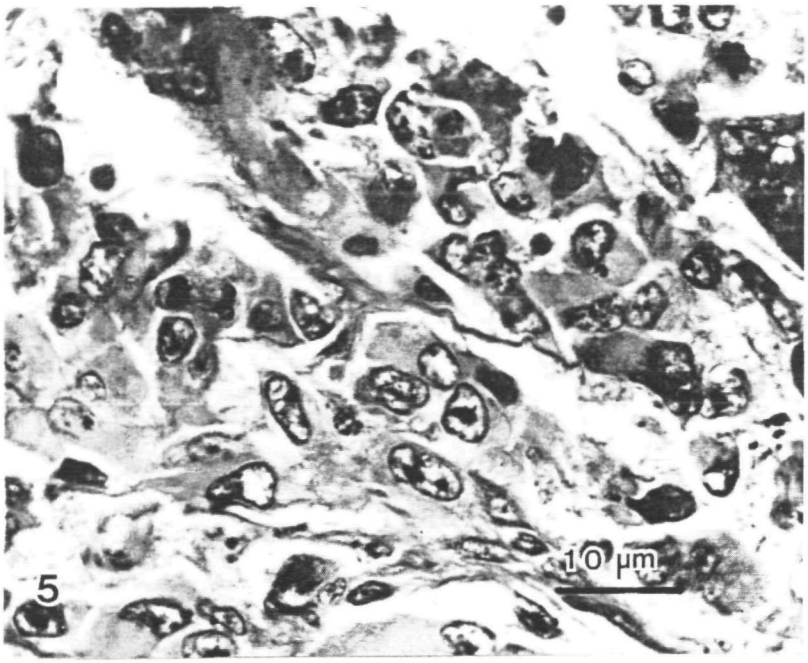


FIG. 4. (a) A well differentiated squamous cell carcinoma developing from a tracheal organ culture exposed to 3 MC-coated carbon (4 mg dust). The tumour appeared at 51 weeks after implantation of the culture; (b) a moderately well differentiated squamous cell carcinoma developing from a tracheal organ culture exposed to 3 MC-coated kaolin (16 mg dust). The tumour appeared at 30 weeks after implantation of the culture (H&E).



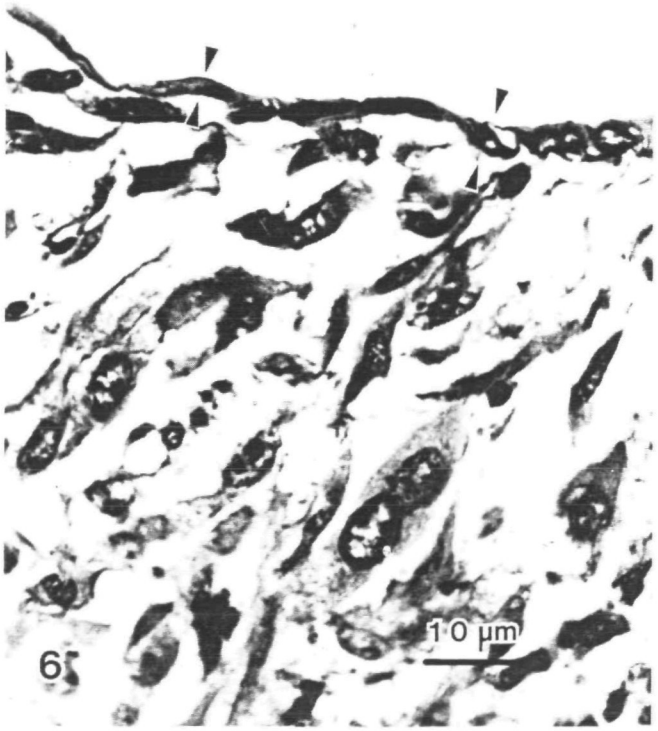


FIG. 5. A poorly differentiated carcinoma developing from a tracheal organ culture exposed to 3 MC-coated hematite (16 mg dust). The tumour appeared at 21 weeks after implantation of the culture (H&E).

FIG. 6. A spindle cell carcinoma originating from the tracheal epithelium (arrowheads) of a tissue exposed to 3 MC-coated hematite (16 mg dust). The tumour appeared 25 weeks after implantation of the organ culture (H&E).

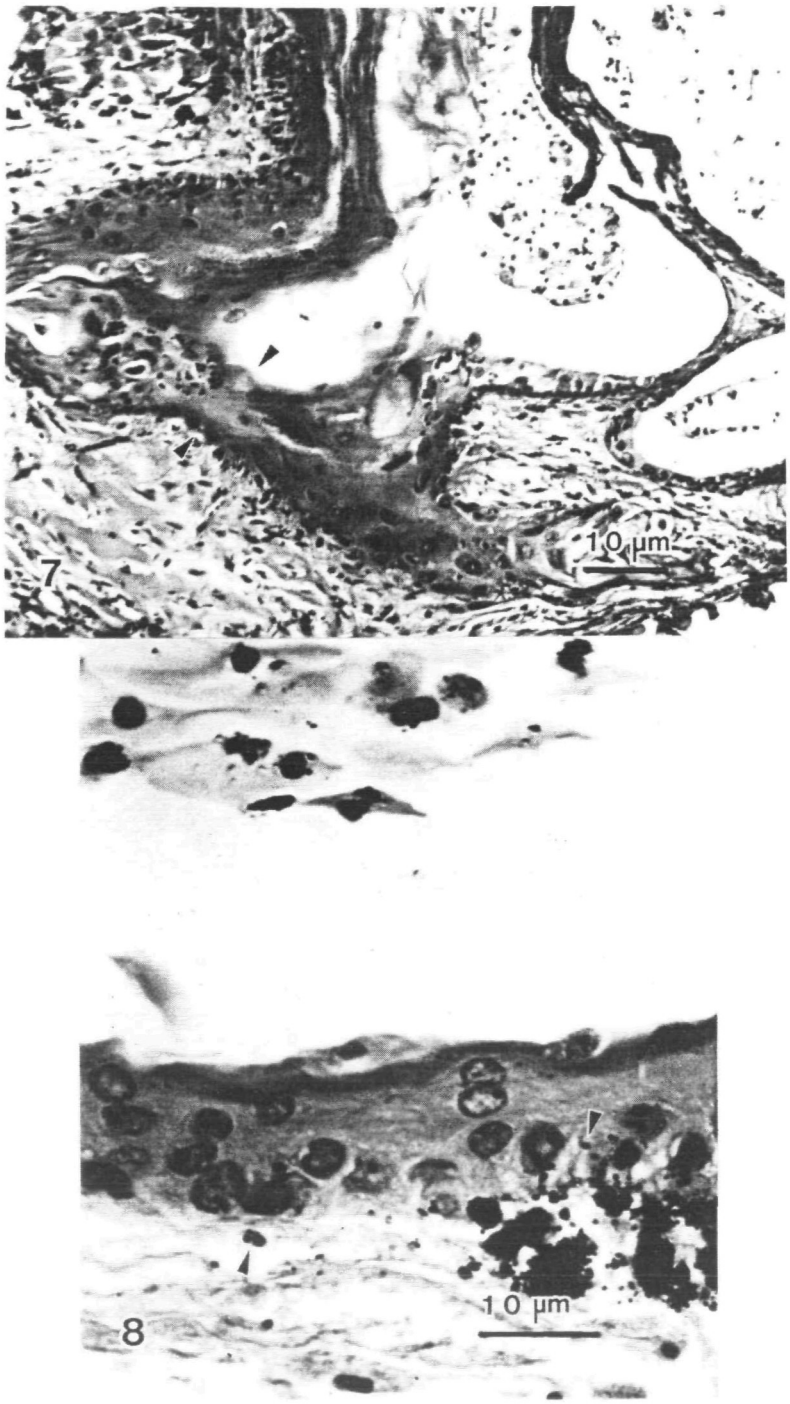


FIG. 7. A well differentiated squamous cell carcinoma originating from the tracheal epithelium (arrowheads) of a tissue exposed to 3 MC-coated hematite (8 mg dust). The tumour appeared 25 weeks after implantation of the culture (H&E).

FIG. 8. Squamous metaplasia occurring in the tracheal epithelium of an implant exposed to 3 MC-coated carbon (16 mg). The tissue was removed from the animal at 100 weeks after implantation. Note the carbon in the tracheal epithelium and the submucosa (arrowheads) (H&E).

TABLE 2. NEOPLASMS DEVELOPING FROM IMPLANTED TRACHEAL ORGAN CULTURES AFTER EXPOSURE *in vitro* TO 3 MC-COATED PARTICLES

Particle	Dose* (mg dust/ ml medium)	Histologic classification† WDC	PDC	PDS	MCS	UM	Nos. carcinomas (%)‡	Nos. sarcomas (%)‡	Total tumours (%)‡	Latency period (wks) (mean ± S.E.)
Crocidolite	16		4	3		1	4/16 (25%)	3/16 (19%)	8/16 (50%)	45 ± 5
	8	1				1	1/16 (6%)	—	2/18 (11%)	60 ± 30
	4					1	—	—	1/17 (6%)	19
Hematite	16	1	3	2			4/16 (25%)	2/16 (13%)	6/16 (37.5%)	48 ± 10
	8	1					1/12 (8%)	—	1/12 (8%)	25
	4	1					1/17 (6%)	—	1/17 (6%)	31
Kaolin	16	1	5		1		6/25 (24%)	—	7/25 (28%)	43 ± 6
	8		1		1	1	1/27 (4%)	—	3/27 (12%)	51 ± 15
	4		2			1	2/27 (8%)	—	3/26 (12%)	50 ± 14
Carbon	16		2	1		1	2/29 (6%)	1/29 (3%)	4/29 (14%)	69 ± 6
	8		2				2/27 (7%)	—	2/27 (7%)	36 ± 15
	4	1	3				4/27 (15%)	—	4/27 (15%)	50 ± 8

* Amounts of 3 MC (μg) on individual tissues were assessed at 24 h after exposure to each amount of dust. This information is recorded in Fig. 1.

† WDC = well differentiated carcinoma; PDC = poorly differentiated carcinoma; PDS = poorly differentiated sarcoma; MCS = mixed carcinomatous and sarcomatous elements; UM = undifferentiated malignancy.

‡ Numbers of tumours/numbers of hamsters (%).

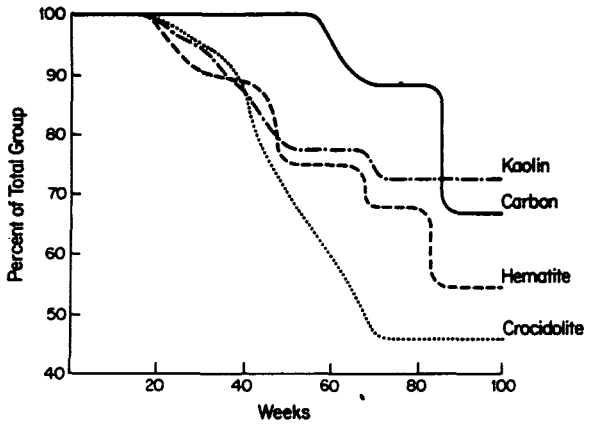


FIG. 3. Survival curves for hamsters implanted with tracheal organ cultures exposed to the highest concentration (16 mg ml^{-1}) of 3 MC-coated particles. The mortality of animals exposed to 3 MC-coated crocidolite is significant statistically ($P < 0.02$, overall Chi square test) from deaths in the group exposed to 3 MC-carbon.

(WOODWORTH *et al.*, 1981; KING *et al.*, 1948) have been reported. This clay mineral occurs in soil and is found in the pulmonary macrophages of cigarette smokers (BRODY and CRAIGHEAD, 1975).

In the classical experimental models of respiratory carcinogenesis, particles with adsorbed PAH are instilled intratracheally into rodents. Since the dusts are non-carcinogenic and the hydrocarbons only weakly so, the importance of the 'carrier' effect of the dust and its retention in the respiratory tract has been stressed. KENNEDY and LITTLE (1975) reported that tracheobronchial epithelial cells 'adsorb' benzo(a)pyrene (BP) after intratracheal administration of BP-coated hematite. Since human pulmonary macrophages ingest BP-coated hematite *in vitro* and release the metabolites (AUTRUP *et al.*, 1979), transfer of activated forms of hydrocarbons by phagocytes to the respiratory epithelium might be important. We have documented phagocytosis of asbestos by superficial tracheal epithelial cells which transport the material to basal cells (MOSSMAN *et al.*, 1977).

Although the mechanisms described above might be intrinsic to carcinogenesis, the results of our experiments suggest that relative binding affinity and elution of PAH from different types of particles might relate to cocarcinogenic potential. In our studies, carbon was a weak cocarcinogen. Although it adsorbed 3 MC avidly, it did not elute the compound. A dosage-dependent effect of 3 MC on tumour induction was not observed with use of this particle, presumably because the dust released only limited amounts of the hydrocarbon at both high and low concentrations of material. For reasons that probably relate to its structural features, kaolin neither retained nor released appreciable amounts of the chemical carcinogen.

Equivalent amounts of 3 MC were adsorbed by comparable amounts of crocidolite and hematite. Since hematite eluted more of the PAH than the other dusts, this could explain its increased cocarcinogenicity in comparison with kaolin and carbon. However, since more tumours were observed in trachea after their exposure to asbestos, factors other than release of 3 MC appear to be important in asbestos-induced carcinogenesis. In comparison with carbon, hematite and kaolin, asbestos is

more cytotoxic to tracheal epithelial cells (MOSSMAN *et al.*, 1977; MOSSMAN *et al.*, 1978; MOSSMAN and CRAIGHEAD, 1979). Sloughing of superficial cells and proliferation of basal cells is observed within 1 week after exposure of tracheal organ cultures to the mineral (MOSSMAN *et al.*, 1977). Since both trauma and scarification increased the probability of tumour induction in experimental animals (BERENBLUM, 1944; DEELMAN, 1972), the increased cocarcinogenicity of asbestos could relate directly to its ability to damage the tracheal epithelial cell.

Regeneration of basal cells and augmentation of DNA synthesis also might be critical preneoplastic events. In this regard, we have documented previously increased uptake of ^{3}H-thymidine, basal cell hyperplasia and squamous metaplasia in the tracheal mucosa after exposure to crocidolite (MOSSMAN *et al.*, 1980b). Similar metaplastic changes are not observed in cultures treated with carbon, kaolin and hematite. Furthermore, biosynthesis of polyamines is enhanced significantly in asbestos-exposed tracheal epithelial cells (MOSSMAN *et al.*, 1980c). Undoubtedly these cytologic and proliferative changes are important in tumour induction, as rapidly dividing cells are more susceptible to transformation by chemical carcinogens (MARQUARDT, 1974).

The methodology described in this paper provides a novel and convenient means to evaluate the cocarcinogenic properties in the respiratory tract of known amounts of minerals. Use of radioactively tagged PAH allows determination of relative amounts adsorbed to particles and quantitation of exposure to the tracheal epithelium. We suggest that these properties might be of significance in particle-enhanced carcinogenesis; however, contributing factors such as cytotoxic and hyperplastic changes induced by minerals such as asbestos (MOSSMAN *et al.*, 1980b) also must be considered.

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DISCUSSION

P. C. ELMES: Did the dust remain in the tracheal implant when you put it into the animal? How did it get from the surface underneath and was it inside macrophages during this time? Finally, was there any difference in clearance rates between the four dusts you used?

Dr MOSSMAN: After precipitation on the explant, the majority of dust is cleared *in vitro*. However, particles are able to penetrate through the mucociliary barrier and to land on the tracheal epithelium. We have documented previously that this material is transported from the tracheal epithelium to the submucosa of the explant. This process occurs not only in organ culture but after implantation of the organ culture into animals. Dusts are removed from the epithelial surface and submucosa not only by the macrophages in the organ culture but also by those of the recipient. However, depending on the dosage of mineral, particles can be found in the tracheal epithelium at several months after exposure. We did not measure differences in clearance rates between dusts.

P. A. VALBERG: If you leave out the tracheal explant from the subcutaneous implantation and give a subcutaneous injection of particles coated with 3 MC, what is the outcome over the lifetime of the hamster?

Dr MOSSMAN: We have not injected minerals subcutaneously in animals. Under these circumstances, fibrosarcomas probably would develop. In contrast, we are interested in the development of epithelial tumours, as these are representative of neoplasms occurring in man. We invert the epithelium of the trachea so that it is against the musculature of the animal before suturing. This procedure prevents exposure of the skin of the animal to the dusts and discourages the development of fibrosarcomas.

M. LIPPMANN: Your particle preparations differed greatly in particle size and morphology. Thus, if there had been any marked effect of the particles on the 3 MC carcinogenesis, the results would have been very difficult to interpret. Would an effect have been ascribed to the material's composition, mean size, specific surface, or other particle parameter? In experiments of this type, it would be highly desirable to use particle suspensions having equivalent specific surfaces, rather than equivalent total masses.

Dr MOSSMAN: We realized that, regardless of controlling for equivalent mass or surface area, additional variables such as mean size, number and composition of particles would exist. The easiest controllable variable to standardize was mass. More important variables in relationship to co-carcinogenic potential are adsorbance and release of 3 MC from equal masses of individual particles. In addition, use of radiolabelled 3 MC allowed us to determine how much hydrocarbon remained on the tracheal epithelium after exposure to each preparation. I believe that the amounts of 3 MC on the tracheal epithelium were comparable with use of each material with the exception of kaolin. Although this dust adsorbed less 3 MC than other dusts at highest concentrations used in our studies, there were no differences statistically between groups.

S. F. MCCULLAGH: You recently reported an *in vitro* study which suggested that retinoids, vitamin A analogues, modify beneficially the response of tissue to asbestos. Do you intend to continue these animal studies?

Dr MOSSMAN: We have studies in progress in which we have cultured explants, during the 1-month period prior to implantation, in media with or without the addition of vitamin A analogues. At present, 22 weeks after implantation, tumours have not appeared in either group.

O. G. RAABE: In your studies, the particles are merely inert carriers of carcinogenic material. Is it desirable to use the term 'co-carcinogenic' to describe the passive roles of the particles in this case in view of the fact that they apparently have no independent carcinogenic potential?

Dr MOSSMAN: The term 'co-carcinogenic' generally refers to something which augments carcinogenesis but is not generally carcinogenic by itself. That is the frame in which I use the term in the paper. Use of the term 'carcinogen' would be inappropriate to describe the non-carcinogenic particles in these studies. I should emphasize that crocidolite asbestos appears to play more than a passive role in co-carcinogenesis. In contrast, the proliferative and metaplastic alterations observed in the tracheal epithelium after exposure to crocidolite might be intrinsic to asbestos-induced co-carcinogenesis.