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Murine lung response to kaolin conveyed by cigarette smoke

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Summary. The effects of chronic (3 to 8.5 months) smoke inhalation from cigarettes laced with 0.1, 1.0 and 10.0 mg kaolin (hydrated aluminum silicate) per gram of tobacco on the morphological integrity of lungs and the pulmonary macrophage population were evaluated in young and old male C57BL/6 mice. Lacing procedures, monitored by determining aluminum content in acid-digested aliquots of tobacco via atomic absorption spectrometry (AAS), proved to be uniform. Amounts of aluminum in right lungs of young mice evaluated by AAS and of kaolin assessed by electron diffraction and polarizing light microscopy were larger in mice which inhaled smoke from cigarettes laced with more kaolin. A more pronounced increase in lung parenchymal tissue and decrease of alveolar space was observed in old mice subjected to smoke from cigarettes containing higher doses of kaolin than in similarly treated young animals. Concomitant with the above, the lung macrophage population did not increase as markedly in response to smoke inhalation in old mice nor did it increase in as clear a dose-response fashion to kaolin as it did in young animals. Further, the degree of ultrastructural alteration of the phagocytes in the old mice suggested impaired cell function. Plate-like material resembling kaolin crystals was most conspicuous in lung macrophages of mice which inhaled largest amounts of kaolin. Manifestations of abnormal aggregates of lymphocytes and macrophages correlated with kaolin dose inhaled in old mice but not in young animals. The reported observations indicate that 1) kaolin gains access to lungs via cigarette smoke inhalation, 2) macrophages are important in maintaining pulmonary homeostasis and 3) the inorganic compound kaolin appears to impede macrophage function, resulting in potentiation of lung abnormalities.

Key words: Lung – Kaolin – Macrophages – Fibrosis – Cigarette smoke

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Introduction

Kaolin and kaolinite are clay minerals (hydrated aluminum silicate – $A1_2\ H_4\ O_9\ Si_2$) found in soils. They have a wide geographic distribution, including the tobacco belt in the United States. These minerals have been implicated in lung fibrosis (Lapenas and Gale 1983). Most investigations evaluating the relationship of kaolin and kaolinite to this disorder are epidemiological and have been conducted in kaolin industry workers who inhale substantial amounts of the material (Altekruse et al. 1984; Sepulveda et al. 1983; Lapenas and Gale 1983; Lapenas et al. 1984). Since these minerals are abundant in soils where tobacco is grown and accumulate on the surface of tobacco leaves during growth and harvest, they can gain access to the lungs via inhalation of tobacco smoke (Brody and Craighead 1975). For example, it was reported that kaolinite was present in lungs of a heavy smoker concomitant with systemic and diffuse fibrotic lesions (Choux et al. 1978). However, due to heterogeneity of the human population, the paucity of experimental subjects and understandable regulatory restrictions, it is difficult, if not impossible, to gather sufficient data from humans to show a definitive cause-effect relationship between kaolin conveyed to the lungs via smoke and pulmonary abnormalities. The significance of demonstrating the above is obvious to the tobacco and health

issue. Clearly, animal models would be useful in assessing this relationship. Previous studies using mice indirectly indicated that kaolin, conveyed by smoke, might contribute to the genesis of pulmonary disorders (Matulionis 1984a; Matulionis and Simmerman 1985). The mechanism by which this mineral potentiates or causes this condition is not known; however, kaolin appears to impede normal function of the lung macrophages (Matulionis and Simmerman 1985; Low et al. 1980), cells which play a crucial role in maintaining pulmonary integrity. In view of the above, the present study was designed to examine the contribution of kaolin delivered to the lungs by cigarette smoke inhalation and the relation of lung macrophages to development of pulmonary abnormalities.

Materials and methods

Lungs of 108 young (2 months old) and 126 old (8–10 months old) C57BL/6 male mice were assessed in the present study (Table 1). Animal housing and health monitoring were as described earlier (Matulionis 1984a). The mice were exposed to cigarette smoke or sham-treated and were maintained by the University of Kentucky Tobacco and Health Research Institute (THRI). Mainstream smoke exposure and sham treatment were accomplished with the Institute's mainstream-sidestream smoke exposure system as detailed elsewhere (Griffith and Hancock 1985; Griffith and Standafer 1985; Gairola 1986). Mice were exposed to smoke twice daily (once in the a.m. and once in the p.m.) for 3, 6 or 8.5 months. Sham treatment consisted of exposing animals (in machines used only for this purpose) to air instead of smoke. Absolute control mice were minimally handled but housed in close proximity of the other animals. Smoke inhalation by animals was monitored and verified by measuring blood carboxyhemoglobin (COHb) levels (Griffith and Standafer 1985; Gairola 1986; Matulionis 1984b). Daily estimates of total particulate matter (TPM) from cigarette combustion (Griffith and Hancock 1985) provided a means of assessing the day-to-day performance of the smoke exposure system.

Young and old animals were exposed to smoke from 2A1 cigarettes to which no kaolin was added (2A1-d_0) and to smoke from 2A1 cigarettes to which 0.1 (d_1), 1.0 (d_2) or 10.0 (d_3) mg kaolin (Sigma Chem. Co., St. Louis, MO) per gm tobacco was added (Table 1). Controls for smoke-treated animals included sham, absolute control and old mice which inhaled 2A1-d_3 cigarette smoke filtered through a Cambridge Filter (Phipps and Bird Inc., Richmond, VA) (inserted before the exposure chamber) (Griffith and Hancock 1985).

Lacing of cigarette tobacco with different doses of kaolin was accomplished by spraying 2A1 research cigarette tobacco (THRI – University of Kentucky, Lexington, KY) with kaolin, suspended in distilled H_2O to add 0.1 (d_1), 1.0 (d_2) or 10.0 (d_3) mg kaolin/gm tobacco. 2A1 cigarette tobacco sprayed with H_2O and constituted into cigarettes (2A1-d_0) served as controls for the lacing procedures. Kaolin content in cigarettes was evaluated by determining the amount of aluminum. Six tobacco aliquots (approximately 90 mg) from each of the different cigarettes (2A1-d_0, -d_1, -d_2, -d_3) were acid digested as previously described (Yokel and Melograna 1983, Kline and Fogler 1981). The samples were analyzed by comparison to standard curves determined before and after each group of 6 to 10 samples in a Perkin-Elmer 400 atomic absorption spectrophotometer, equipped with an HGA 500 graphite furnace and programmer, an AS40 auto sampler and a GP-100 graphic printer (Yokel and Melograna 1983). The predicted aluminum concentration of kaolin-laced tobacco was calculated as the sum of the aluminum added (as kaolin) and the aluminum concentration determined in unlaced 2A1-d_0 tobacco. A substantial amount of aluminum was detected in 2A1 research cigarettes which were not laced with kaolin (d_0) (Table 2). The amount of aluminum in 2A1-d_1, $-d_2$ and -d_3 tobacco (to which 0.1, 1.0 and 10.0 mg kaolin per gm tobacco was added respectively) was similar to

Table 2. Amount of aluminum (μg/gm tobacco) in tobacco

Tobacco	Predicted	Obtained
2A1-d_0	–	343.5± 9.1[a]
2A1-d_1	364.4	378.8± 15.1
2A1-d_2	552.5	560.3± 19.8
2A1-d_3	2433.5	2158.2±127.1

[a] Mean±SEM, $N=6$

Table 1. Treatment and number (No.) of young (Y) and old (O) mice

Treatment	Total No.		No. and % deaths		No. used for m.a.[a]		Others[b]	
	Y	O	Y	O	Y	O	Y	O
2A1-d_0[c]	73	96	2 2.7%	66 68.8%	18	18	53	12
2A1-d_1	74	75	2 2.7%	41 54.7%	18	18	54	16
2A1-d_2	74	87	2 2.7%	56 64.4%	18	18	54	12
2A1-d_3	74	77	4 5.4%	49 63.6%	18	18	52	10
Sham	76	67	7 9.2%	35 52.2%	18	18	51	14
Control	68	32	2 2.9%	2 6.3%	18	18	58	12
CF[d]	–	72	– –	47 65.3%	–	18	–	7

[a] m.a. – morphological assessment
[b] Number of surviving animals used in other than the present study and to evaluate kaolin content in right lungs ($n=5$/per treatment group)
[c] 2A1 cigarettes to which no (d_0), 0.1 (d_1), 1.0 (d_2) or 10.0 (d_3) mg kaolin was added/gm tobacco
[d] Cambridge Filter, filtered 2A1-d_3 cigarette smoke

predicted values (Table 2). The aluminum content in mouse right lungs ($n=6$ per group) was assessed as above.

Kaolin was extracted from right lungs of 5 mice from each group by the method of Langer et al. (1971) and prepared for electron diffraction (Matulionis and Traurig 1977). Small amounts of kaolin (Sigma Chem. Co., St. Louis, MO USA), plated on coated grids and sealed with a carbon coating, served as standards for morphology of crystalline compound and electron diffraction patterns. The patterns were obtained in a Philips 400 electron microscope at 100 Kv. Thin sections from lung specimens processed for electron microscopy were also subjected to electron diffraction analysis. In addition, aliquots of the digestate samples used for diffraction were examined by polarizing light microscopy. All observations were rated qualitatively.

Lungs of 6 young and 6 old mice from each treatment group (Table 1) were assessed at 3, 6 and 8.5 months after onset of smoke exposure. Mice were anesthetized by intraperitoneal injections of sodium pentobarbital (50 mg/kg body weight), weighed and exsanguinated by severing the abdominal aorta. The lungs were dissected from the thoracic cavity with care. Right lungs were weighed, frozen at $-60°$ C and stored at $-20°$ for later determination of aluminum. Cephalic two-thirds of the left lungs was prepared for microscopy as detailed earlier (Matulionis 1984a). Slides, with adjacent sections, were stained with Van Gieson's reticular stain and periodic acid Schiff reagent-haematoxylin. All lung sections were cut from identical locations relative to the external pulmonary surface. The caudal one-third of the left lung was processed for electron microscopy using procedures described elsewhere (Matulionis 1984, 1975; Matulionis and Simmerman 1985).

Cross-sectional areas of 4 to 6 lung sections at the beginning, middle and end of a 360 μm sampling region, prepared for light microscopy, were calculated using area-perimeter analysis on the Zeiss Video Plan Image Analysis System (Carl Zeiss Inc., Thornwood, NY, USA). Lung section areas were divided by mouse body weight to normalize the influence of body size. Tissues prepared for light microscopy were used to evaluate the general morphology of lungs, the reticular fiber content and to quantify morphometrically the volume fraction (% volume) of alveolar space, parenchymal tissue (alveolar septa), other lung components (blood vessels, air passages, nonparenchymal connective tissue) and abnormal aggregates of lymphocytes and macrophages. For purposes of gaining a more critical insight into altered lung morphology, the parenchymal tissue and alveolar space were designated as normal or abnormal. Abnormal parenchyma was defined as markedly thickened (hypertrophied) alveolar septa measuring 53.50 ± 5.85 μm (range, 26.10–126.7 μm) that clearly deviated from the classically described normal state of these structures. Normal parenchyma was designated as lung septa which measured 10.37 ± 0.97 μm (range, 4.47–22.35 μm) and conformed to the classic descriptions. Alveolar space was classified as abnormal if it was encompassed by abnormal parenchyma and normal space as that surrounded by normal parenchyma. If assessment of parenchyma or space was problematic, it was scored as normal. Such scoring yields underestimates of abnormal variable which lends credibility to the values obtained in morphometric observations, since the quantification was intended to verify manifestations of anomalous conditions. Estimates of volume fractions of the variables identified were done with light microscopy on transverse lung sections as detailed earlier (Matulionis 1984a) by standard point counting procedures (Weibel 1979). From 534 to 801 independent test points intercepting various lung components (i.e., alveolar space, parenchyma, etc.) were scored per lung in the quantitative analysis. The above sections were also used to quantify the number of pulmonary macrophages (alveolar and septal) per unit area of lung tissue as detailed earlier (Matulionis and Simmerman 1985; Matulionis 1986).

Numerical data were evaluated statistically using the F test (analysis of variance) and Fisher's multiple comparison procedures. A P value of ≤ 0.05 was required for significance.

Results

The health condition of young and old mice at the onset of the smoke exposure period was good. However, during the study significant attrition of old smoke-exposed (including Cambridge Filter-filtered smoke, CF) and sham-treated mice occurred (Table 1). Similar animal losses were reported and discussed in an earlier study (Matulionis 1984a). Animal deaths occurred uniformly over the 8.5-month study. Attrition of all young and old control mice was minimal (Table 1) and attributed to natural causes.

Animal body weights, weight of right lungs and left lung sectional areas (lung size) of young and old mice are shown at the time of sacrifice (3, 6 and 8.5 mo) in Table 3. Body weights reflected a stress effect related to manipulation of animals during smoke and sham treatments. Right lung weights indicated a smoke and suggested a kaolin dose effect. The size of lungs increased in response to cigarette smoke inhalation but no clear kaolin effect was observed.

Lung morphology of young and old sham and control animals was similar and normal in appearance over time (Fig. 1). Lungs of young smoke-exposed mice at 3 and 6 months of treatment were, for the most part, similar to those of shams and controls. However, in some regions of lungs, especially in those exposed to 2A1-d_2 and -d_3 cigarette smoke for 8.5 months, the septa were thickened and alveolar size was reduced (Fig. 2). Lungs of old mice inhaling 2A1-d_2 and -d_3 cigarette smoke for 3 and 6 months were likewise nearly normal, although some thickening of alveolar septa was noted. However, at 8.5 months of smoke exposure, lungs of old animals differed considerably from the norm (Fig. 3). Many of the air spaces were noticeably reduced in size and the parenchymal components (septa) were thick and cellular in nature (Fig. 3). Reticular fibers were clearly most prominent in lungs of 2A1-d_2 and -d_3 smoke-exposed old mice. Often large, vacuolated macrophages were noted in septal regions (Fig. 3).

Lungs of both young and old mice exposed to smoke from the different cigarettes contained abnormal aggregations of vacuolated macrophages and lymphocytes (Fig. 4). These abnormalities were in close proximity to airways and blood vessels in all smoke-exposed animals but were largest

Table 3. Treatments (Trt.), body weights, lung weights, and lung sectional area

Trt.	Body wt. (gm)			Rt. lung wt. (mg/gm b. wt.)			Lung section area (mm^2/gm b. wt.)		
	3 mo	6 mo	8.5 mo	3 mo	6 mo	8.5 mo	3 mo	6 mo	8.5 mo
Young animals									
2A1-d_0	24.1±0.3[a]	24.6±0.6	25.8±0.6	4.7[b]	4.5[b]	4.5[b]	0.262±0.006	0.213±0.009	0.194±0.004
2A1-d_1	24.1±0.4	26.0±0.5	24.8±0.5	4.7	4.5	4.9	0.254±0.014	0.229±0.006	0.216±0.010
2A1-d_2	23.0±0.5	24.7±0.3	24.1±0.8	4.9	4.7	5.1	0.269±0.017	0.244±0.006	0.255±0.007
2A1-d_3	22.8±0.7	23.7±0.6	23.7±0.3	4.8	5.0	5.0	0.246±0.004	0.261±0.009	0.234±0.007
Sham	24.6±0.5	25.0±0.4	26.2±0.8	3.8	3.7	3.5	0.199±0.004	0.185±0.006	0.170±0.006
Control	27.2±0.8	28.9±0.8	28.3±0.8	3.5	3.3	3.4	0.186±0.005	0.164±0.008	0.165±0.004
Old animals									
2A1-d_0	25.5±1.2	28.3±0.3	28.4±0.3	4.9	4.9	5.0	0.252±0.011	0.207±0.010	0.225±0.007
2A1-d_1	25.9±0.9	27.9±0.5	30.5±1.5	4.9±0.3	4.9	5.1	0.241±0.011	0.208±0.004	0.204±0.003
2A1-d_2	26.2±0.3	27.4±0.4	28.9±0.5	5.2±0.2	5.3	5.3	0.241±0.008	0.203±0.003	0.220±0.006
2A1-d_3	25.3±0.5	27.8±0.6	28.5±0.5	5.2±0.2	5.0	5.3	0.262±0.009	0.215±0.006	0.217±0.007
Sham	26.3±0.4	27.9±0.9	28.6±0.4	3.9	3.8	3.8	0.202±0.009	0.177±0.008	0.188±0.010
Control	29.9±0.5	31.4±0.3	33.0±0.5	3.3	3.3	3.1	0.175±0.007	0.170±0.007	0.162±0.007
CF	28.4±0.6	28.7±0.6	28.6±0.2	3.6	3.6	3.4	0.201±0.006	0.160±0.004	0.178±0.003

[a] Mean ± one standard error of the mean (SEM)
[b] SEM = 0.1 unless otherwise indicated

Abbreviations: 2A1-d_0, -d_1, -d_2, -d_3 tobacco to which 0.0, 0.1, 1.0, 10.0 mg kaolin was added to 2A1 cigarette tobacco, respectively. CF, Cambridge Filter-filtered 2A1-d_3 cigarette smoke

and most frequent in lungs of old mice exposed to 2A1-d_3 cigarette smoke for 8.5 months. They were never observed in lungs of any sham, control or CF-treated animal. Ultrastructural evaluation of lung tissue confirmed light microscopic observations.

Following morphological assessment, the volume fraction (% volume) (V_v) of alveolar space (normal and abnormal), parenchymal tissue (normal and abnormal), airways, blood vessels, non-parenchymal connective tissue and lymphocyte-macrophage aggregates was estimated morphometrically. With the exception of the latter, V_v of all variables quantified at 3 months were similar over treatment and age. Abnormal lymphocyte-macrophage aggregates (Fig. 4) occupied a significantly larger volume fraction of lungs in smoke-treated young ($\bar{x}$ ± SEM over treatments 2.3±0.91% volume) than old ($\bar{x}$ ± SEM over treatments 0.63±0.28% volume). However, no kaolin effect was detected.

At six months % volume of alveolar space was marginally decreased ($p = 0.08$) in lungs of 2A1-d_1, -d_2 and -d_3 smoke-treated old mice when compared with that of sham, control, CF and 2A1-d_0 animal lungs. The volume fraction of lymphocyte-macrophage aggregates in lungs of young ($\bar{x}$ over treatments 3.45±0.65% volume) and old ($\bar{x}$ over treatments 2.4±0.64% volume) smoke-treated animals increased compared to 3-month values. In old mice, though not in young, smoke from cigarettes laced with increasing amounts of kaolin generally elevated the V_v of these abnormalities (2A1-d_0, -d_1, -d_2, -d_3: 0.93±0.18, 1.98±0.37, 2.0±1.04, 4.5±0.97% volume of aggregations in old mice, respectively). Highly significant ($p \leq 0.0046$) increase of the aggregation V_v was detected in old mice exposed to 2A1-d_3 smoke at 6 months when compared with the other smoke treatments.

At 8.5 months volume fraction of alveolar space surrounded by normal septa (normal space) was significantly ($p < 0.05$) lower in lungs of all smoke-treated mice compared with control values (Fig. 5). Lowest values of normal space were detected in lungs of old mice exposed to 2A1-d_2 and -d_3 cigarette smoke. Converse was true in regard to air space surrounded by thickened alveolar septa (abnormal space) (Fig. 6). A similar condition was noted concerning parenchymal tissue. Volume fraction of normal-appearing septa was reduced and that of thickened septa increased over control values with more reduction and increase (respectively) noted in lungs of 2A1-d_2 and -d_3 cigarette smoke-exposed old animals (Fig. 6). Lymphocyte-macrophage aggregations occupied 5.5±0.87% (mean ± SEM over treatments) of lung unit volume in young smoke-treated mice. No effect of kaolin was noted in the young animals. Mean volume

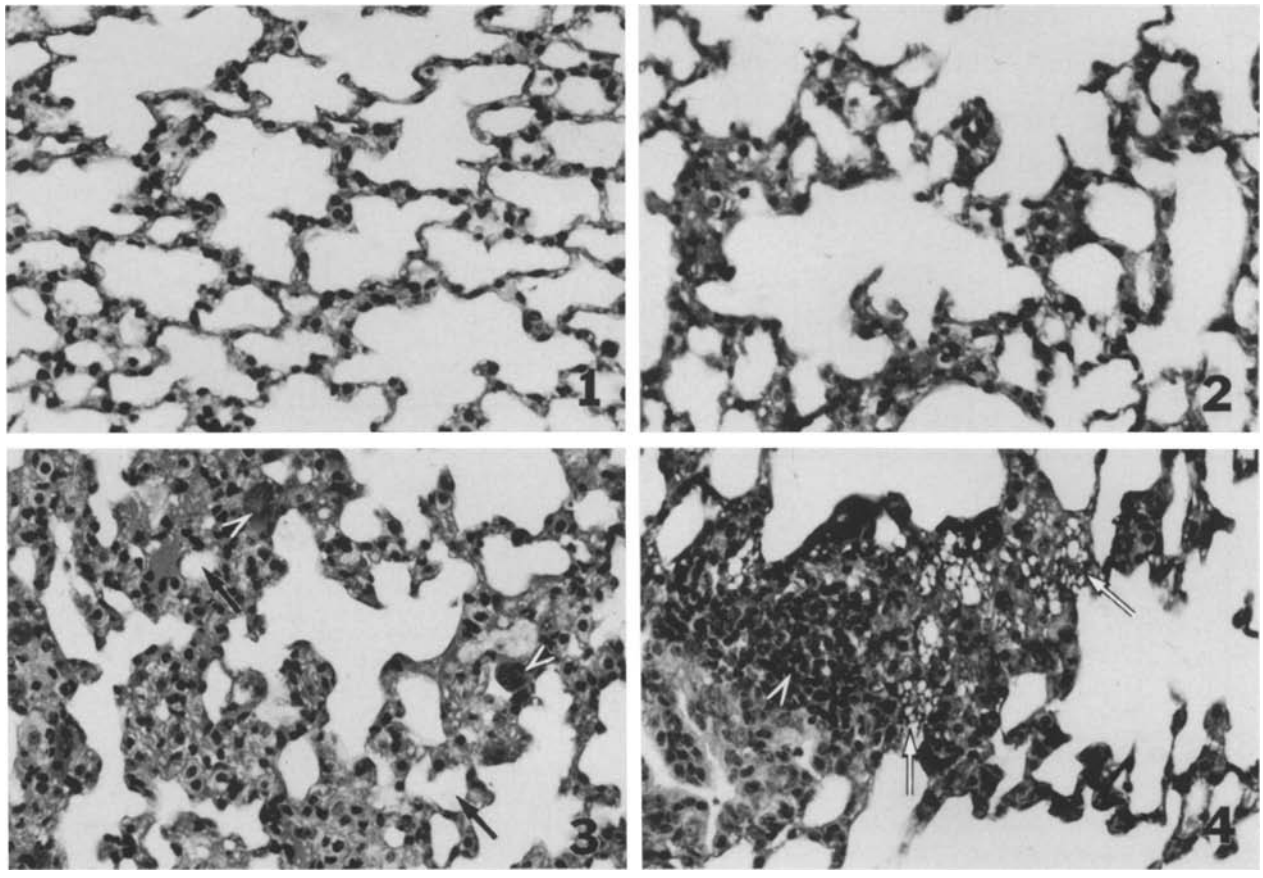


Fig. 1. Light micrograph of lung tissue from an old control mouse at the 8.5 month sampling point depicting normal lung morphology. × 500

Fig. 2. Light micrograph of lung tissue from a young 2A1-d_2 smoke-exposed mouse for 8.5 months. × 500

Fig. 3. Light micrograph of lungs from an old 2A1-d_3 smoke-exposed mouse for 8.5 months. Note the thickened cellular alveolar septa (*S*) and small air spaces (*arrow*). Septal macrophages, *arrowhead*. × 500

Fig. 4. Light micrograph of lung tissue from a young 2A1-d_3 smoke-exposed mouse depicting an abnormal aggregation of lymphocytes (*arrowhead*) and vacuolated macrophages (*arrow*). × 500

fraction ($4.6 \pm 1.22\%$ volume) of these abnormalities in old mice was similar to that of the young. However, in old mice which inhaled 2A1-d_2 and -d_3 cigarette smoke, their V_v was significantly ($p < 0.05$) higher than in those inhaling 2A1-d_0 and -d_1 smoke, indicating a kaolin effect.

The pulmonary macrophage response to added kaolin burden in young and old mice was markedly different (Fig. 7). A significantly ($p = 0.0001$) greater number of macrophages was present in lungs of all smoke-exposed young animals than in similarly treated old mice at 3 and 6 months of exposure (Fig. 7). Macrophage response was greatest in young and old mice inhaling smoke from 2A1-d_2 and d_3 cigarettes at these times. At 8.5 months of smoke exposure phagocytes in lungs of old animals failed to respond to the two highest doses of kaolin conveyed by smoke while the response was maintained in young animals (Fig. 7). Macrophage population in lungs of young 2A1-d_1 smoke-exposed animals at 8.5 months declined ($p < 0.05$) compared with that at 6 months to values of similarly treated old mice (Fig. 7). Number of macrophages was markedly lower ($p = 0.0001$) in all control groups compared with values of the smoke-treated mice. At 6 and 8.5 months significantly ($p \leq 0.004$) fewer phagocytes were present in lungs of old sham, control and CF mice than in corresponding young animals (Fig. 7) indicating an age effect.

As was previously observed, ultrastructure of lung macrophages from young and old sham and control (and old CF) mice was similar and appeared normal (Fig. 8) over time (Matulionis and

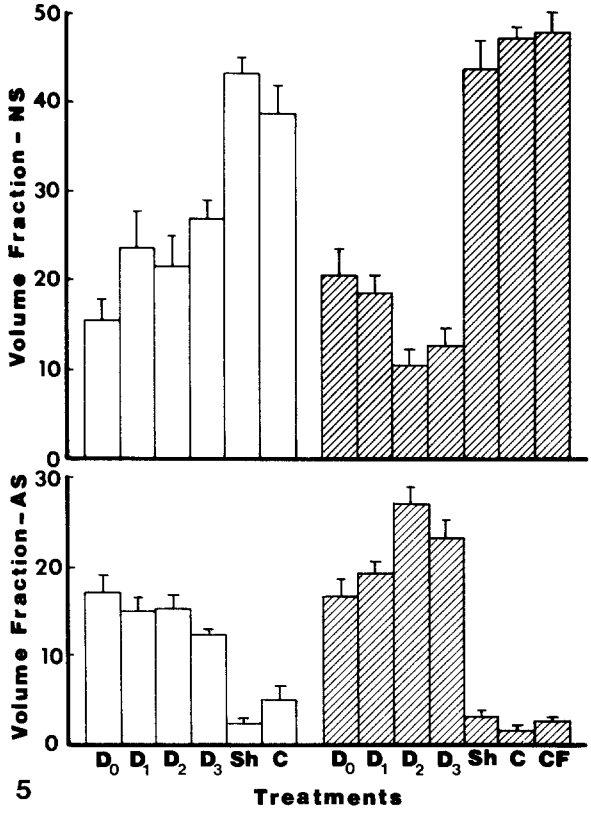


Fig. 5. Mean (±SEM) volume fraction of normal (NS) and abnormal (AS) air space at the 8.5 month sampling point in lungs of young (*clear bars*) and old (*hatched bars*) mice. Treatments: smoke from 2A1 cigarettes to which 0.00 (d_0), 0.1 (d_1), 1.0 (d_2), 10.0 (d_3) mg kaolin was added per gm tobacco; sham-treated animals, Sh; untreated controls, C; Cambridge Filter-filtered cigarette smoke, CF

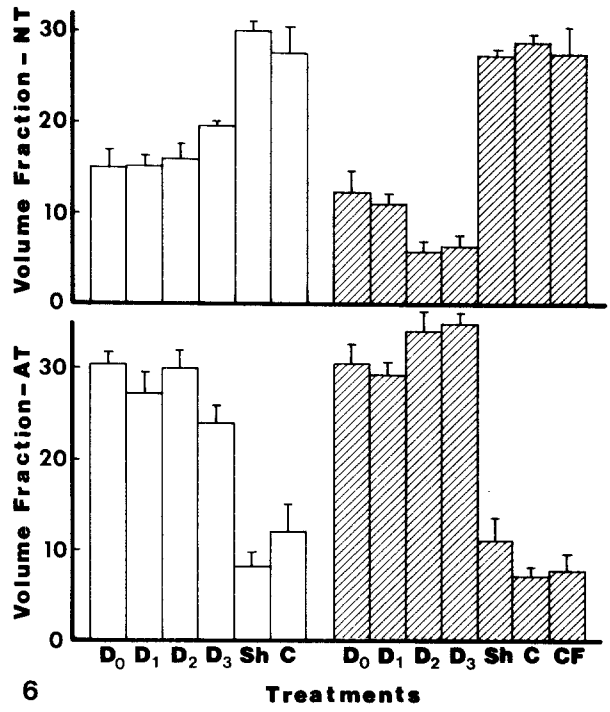


Fig. 6. Mean (±SEM) volume fraction of normal (NT) and abnormal (AT) parenchymal tissue at the 8.5 month sampling point in lungs of young (*clear bars*) and old (*hatched bars*) mice. See Fig. 5 for abbreviations

Simmerman 1985). Macrophage ultrastructure of smoke-exposed young and old animals was highly variable. Since a detailed description of macrophage structure has been reported elsewhere (Matulionis and Simmerman 1985) only the major differences and features of the phagocytes will be related presently. Some of these cells, especially from young mice exposed to 2A1-d_0 and -d_1 smoke for 3 and 6 months, were similar in appearance to those of controls. However, most phagocytes of these young animals contained more lysosome-like bodies (lysosomes) (Fig. 9, 10) than noted in controls (Fig. 8). Other macrophages in young animals, especially at 8.5 months of 2A1-d_2 and -d_3 cigarette smoke inhalation, were characterized by a cytoplasm replete with crystalline inclusions (Fig. 11). Crystal-like inclusions were also observed in macrophages of 3- and 6-month smoke-exposed mice although to a lesser degree (Fig. 10).

The above macrophage morphology also typified many lung phagocytes of old smoke-exposed mice over similar times and treatments. Others, however, were much larger (twice or more) in size than those noted in young animals (compare Figs. 9 and 12). Further, lysosomes become quite numerous, in some cases almost completely filling the cell (Fig. 12). The lysosomes were highly heterogeneous in appearance ranging from finely granular electron opaque to electron dense to highly vacuolated entities. Many of the phagocytes appeared to be degenerating. Bi- and multinucleated macrophages were noted more frequently in lungs of old animals, especially those that inhaled 2A1-d_2 and -d_3 cigarette smoke for 6 and 8.5 months (Fig. 13), than in similarly treated young mice. Crystalline inclusions were present in phagocytes of all old smoke- exposed mice (Fig. 12). However, in many macrophages these structures were curiously clumped (Fig. 14), an arrangement not seen in young animals.

Attempt at quantification of the aluminum content in lungs met with only partial success. The problem encountered was that the amount of aluminum disassociated from kaolin was at or below limits of detection of the atomic absorption spec-

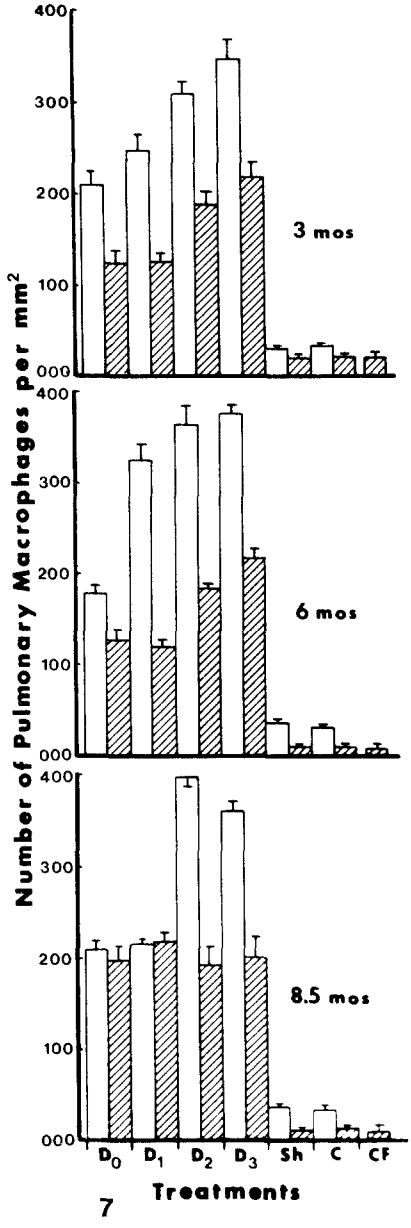
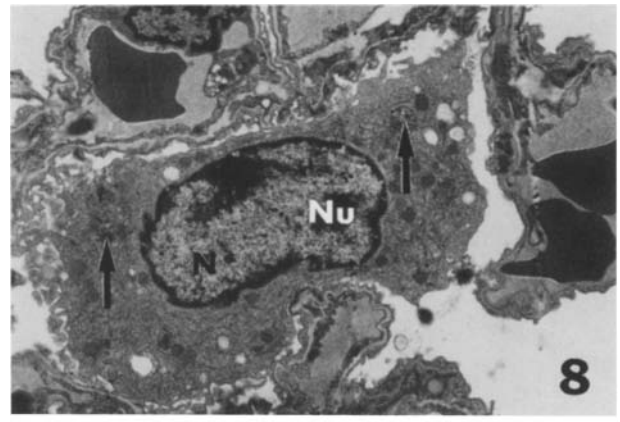
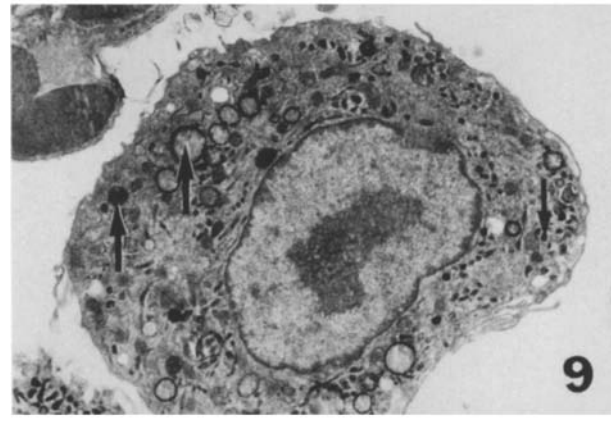


Fig. 7. Number of pulmonary macrophages per mm^2 (±SEM) of lung tissue of young (*clear bars*) and old (*hatched bars*) mice at 3, 6 and 8.5 month (mos.) sampling points subjected to various treatments. Abbreviation as in Fig. 5





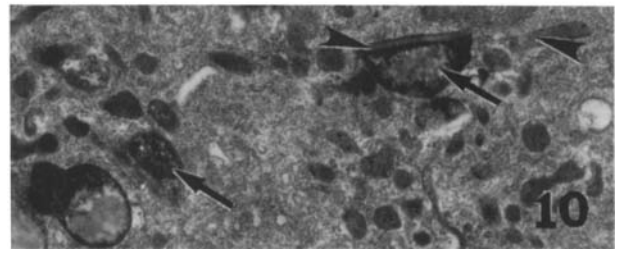


Fig. 8. Electron micrograph of an alveolar macrophage from a young control mouse at the 3 month sampling point which typifies macrophages in all control, sham and CF mice over time. Nucleus, N; nucleolus, Nu; lysosomes, arrow. ×4600

Fig. 9. Electron micrograph of an alveolar macrophage from lungs of a young animal which inhaled smoke from 2A1-d_3 cigarettes for 6 months. Note the increase in number of lysosomes (*arrows*) when compared with macrophages of controls (Fig. 8) ×4600

Fig. 10. Crystal-like inclusions (*arrow head*) and lysosomes (*arrow*) in cytoplasm of septal macrophage from lungs of a 2A1-d_2 smoke-exposed young mouse for 3 months. ×15100

trophotometer. The consequence was large variations in aluminum in a number of lung samples of several groups. In view of this problem, the data were rated qualitatively and relative amounts of aluminum present in lungs are reported (Table 4). Small amounts of aluminum were detected in lungs of 2A1-d_0 and -d_1 smoke-exposed mice while in those inhaling 2A1-d_2 and -d_3 smoke considerably more was found (Table 4). Most sham and control animal lungs were negative for this element (Table 4).

Kaolin present in KOH lung digestates was also assessed qualitatively by electron diffraction and polarizing light microscopy. Few patterns were obtained from lungs of 2A1-d_0 and -d_1 smoke-exposed mice (Table 4). A few lung samples of 2A1-d_0 and -d_1 smoke-treated and all sham and

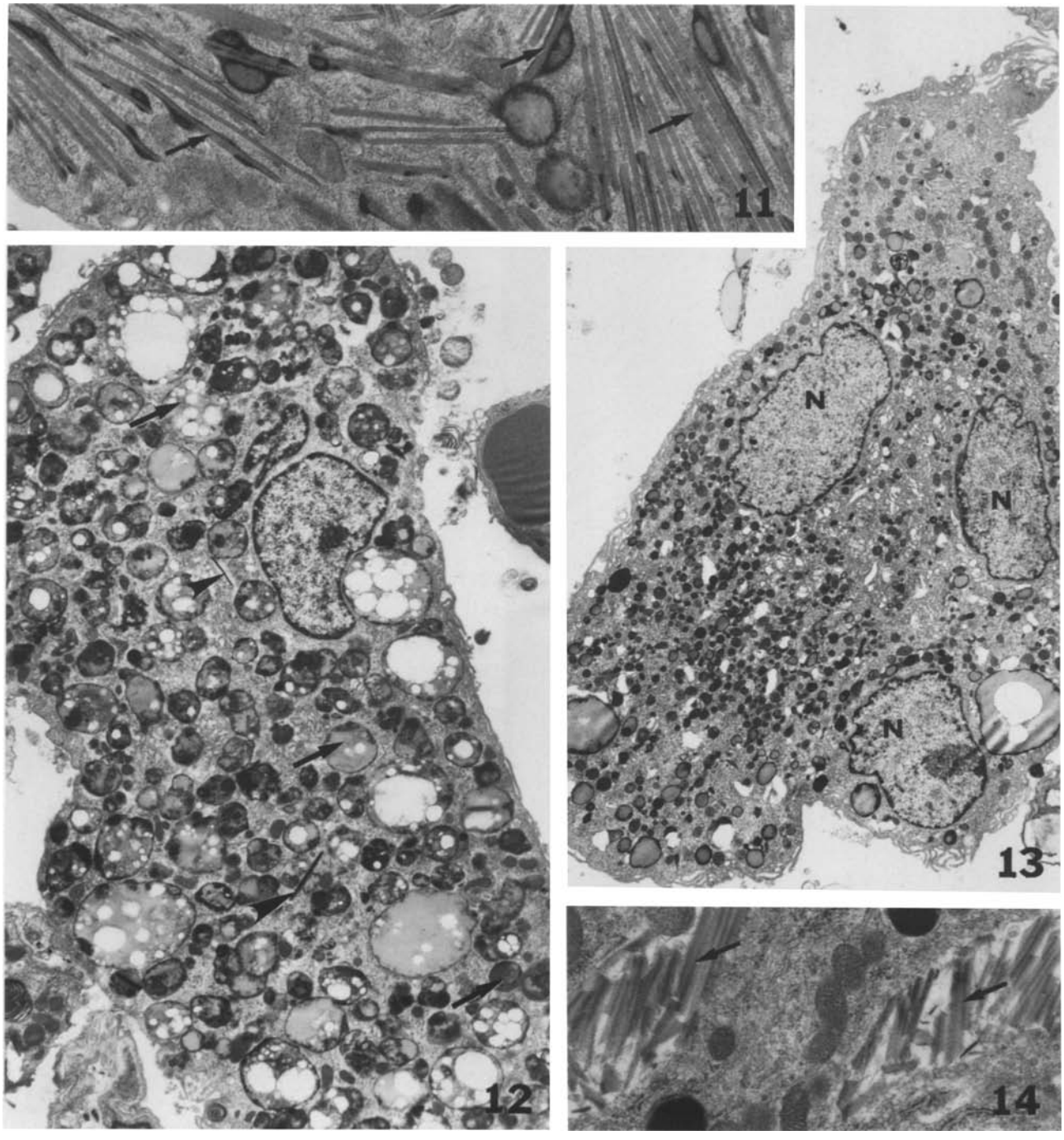


Fig. 11. Electron micrograph of an alveolar macrophage cytoplasm from a 2A1-d_3 smoke-exposed young mouse for 8.5 months. Note the numerous crystal-like inclusions (*arrows*). ×15000

Fig. 12. Electron micrograph of an alveolar macrophage from an old mouse which inhaled 2A1-d_3 cigarette smoke for 6 months. Note the numerous heterogeneous lysosomes (*arrows*). Crystalline inclusions, arrowhead; nucleus, N. ×4600

Fig. 13. Electron micrograph of a multinucleate alveolar macrophage from lungs of a 2A1-d_2 smoke exposed old mouse for 8.5 months. Nuclei, N. ×4600

Fig. 14. Electron micrograph of clumped crystalline material (*arrows*) in the cytoplasm of a septal phagocyte from an old mouse which inhaled 2A1-d_2 cigarette smoke for 8.5 months. ×14500

Table 4. Aluminum and kaolin in right lungs of young animals

Treatment	Aluminum via Atomic Absorption	Kaolin via Electron Diff.	Kaolin via Polarizing LM
d_0	+[a]	+ −	+ −
d_1	+	+	+ −
d_2	+ + + +	+ +	+ + +
d_3	+ + + +	+ + + +	+ + + +
Sham	− +	−	−
Control	− +	−	−

[a] Pooled observations over time. +, small amount; + +, moderate amount; + + +, large amount; + + + +, largest amount observed; + −, small amount in most sample but few with none detected; − +, most samples negative but few with small amounts; −, none detected in all samples

control samples were negative for kaolin (Table 4). Patterns were most frequently encountered in lung preparations of animals inhaling 2A1-d_3 smoke (Table 4). Diffraction patterns similar to commercial kaolin were also obtained in favorable thin plastic sections which had numerous crystalline inclusions, indicating *in situ* kaolin material. Polarizing light microscopic assessment of aliquots from the above lung digestates supported electron diffraction observations in regard to amount of kaolin present in lungs of differentially treated mice (Table 4).

Discussion

The present study was designed to evaluate the contribution of kaolin from cigarette smoke to the development of pulmonary abnormalities in mice. Assessment of lung tissue and pulmonary macrophage population from young and old mice inhaling smoke from cigarette tobacco with different amounts of kaolin revealed that more aluminum and kaolin were present in lungs of mice subjected to smoke from cigarettes containing greater amounts of kaolin and that a greater increase of parenchymal tissue and a decrease of air space occurred in old than in young mice subjected to smoke from cigarettes containing higher doses of kaolin. Qualitative and quantitative differential alterations of lung macrophage ultrastructure, phagocyte population size and abnormal lymphocyte-macrophage aggregations were seen in old versus young mice inhaling higher amounts of kaolin.

Since the objective of the present study was to determine whether kaolin conveyed to the lungs via cigarette smoke inhalation contributes to development of lung abnormalities, it is crucial to demonstrate that this material gains access to the lungs by the proposed route. Data reported in the present study indicate that aluminum and kaolin content in lung samples was greater in mice that inhaled smoke from cigarettes laced with larger amounts of kaolin. The above observation suggests that the compound is conveyed into the lungs via smoke. This suggestion is supported by evidence which demonstrates that various metallic components of cigarettes, including aluminum, are transferred from tobacco to mainstream smoke during pyrolysis (Cogbill and Hobbs 1957). Other studies show increase in aluminum and silicon in lungs of human smokers vs. nonsmokers (Vallyathan and Hahn 1985; Choux et al. 1978). Brody and Craighead (1975) reported kaolinite in lung macrophages of human smokers. Likewise, kaolin has been identified previously in lungs of smoke-exposed mice (Matulionis and Traurig 1977) and dogs (Frasca et al. 1971). The compound was not detected in control nonsmoking animals. Thus, kaolin is a component of tobacco and is conveyed into lungs by smoking.

The consequence of inhaled kaolin is not fully understood or documented at present. However, evidence exists in the literature to suggest that this compound is mildly fibrogenic (Low et al. 1980; Ruttner et al. 1973; Goldstein 1959; King et al. 1948). Thickened alveolar septa, increased cellularity and decreased air space are features of pulmonary fibrosis (Hopewell 1980). Such conditions, accompanied by increase of reticular fibers, were noted in the present study most clearly in old mice that inhaled smoke from tobacco laced with higher amounts of kaolin for 8.5 months. In previous studies (Matulionis 1984a; Matulionis et al. 1985) when such morphological features were present, total lung and carbon monoxide diffusing capacities were reduced in mice. These alterations of pulmonary function are correlated with lung fibrosis (Salonim and Hamilton 1981). However, the earlier studies (Matulionis 1984a; Matulionis et al. 1985) related kaolin to fibrosis indirectly by the presence of crystal-like inclusions which morphologically resembled kaolin material in lung macrophages. The present study substantiates the above observations and implicates kaolin in the development of lung abnormalities which resembles fibrotic-like lesions more directly since higher doses of kaolin delivered via smoke produced a more marked manifestation of deviant condition. However, it is acknowledged that increased lung cellularity suggests chronic interstitial pneumonia. Conceivably the increase in tissue and reduction of air space is a result of both pathologic conditions.

The mechanism by which kaolin potentiates the noted abnormalities is not clear. However, data presented and reports in literature suggest that lung macrophages play a pivotal role. Macrophage response to cigarette smoke was depressed in old smoke-exposed mice. It has been suggested that the decreased macrophage population in these animals (Matulionis and Simmerman 1985) results from elevated corticosterone serum level, which normally occurs with advancing age. Elevated steroid levels markedly reduce the influx and efflux of lung macrophages resulting in increased longevity of the resident phagocytes (Van Furth and Blusse Van Oud Alblas 1982). Phagocytic activity of macrophages is not impeded by increased steroid levels (Slonecker and Lim 1972) or the aging process (Weeks 1979; Finger et al. 1982). Under these conditions, individual macrophages in lungs of old smoke-exposed animals would phagocytose more smoke particulates, including kaolin material, over time in attempts to maintain a low level of contaminants. Subsequently, synthetic activities and degradation processes of the phagocytes are depressed (Matulionis and Simmerman 1985) resulting in altered homeostasis of the lung. White and Kuhn (1980) have shown that aluminum silicate depresses elastase secretion; therefore, the remodeling of connective tissue in the lungs would be altered, a condition conducive to the development of fibrosis. Other investigators have reported that activated alveolar macrophages produce factors which inhibit fibroblast growth and synthetic activity (Elias et al. 1984; 1985). Since kaolin is cytotoxic to macrophages (Low et al. 1980) and appears to alter macrophage synthetic activities (Matulionis and Simmerman 1985) it is possible to suggest that the fibroblast inhibitory factor synthesis could have been curtailed. In this event, favorable conditions would exist for increased synthesis of reticular fibers since fibroblast proliferation and biosynthesis would be unchecked. A relation between abnormal function of macrophages and manifestation of fibrotic-like lesions is suggested.

Appearance of lymphocyte and macrophage aggregations, subsequent to smoke inhalation, has been observed and discussed in detail previously (Matulionis 1984a). Of particular interest in this study was the degree to which these lesions showed a kaolin dose effect in old but not young mice. A greater alteration of macrophage function in lungs of old animals by the higher burden of kaolin might evoke the higher incidence of these lesions. Thus, again a kaolin-macrophage-pathology relationship is indicated.

The development of fibrotic-like and pneumonia-type lesions, though mild in nature, in young 2A1-d_0 smoke-exposed animals is of concern and warrants comment, since in an earlier study they were not observed in similarly treated mice (Matulionis 1984a). A possible explanation for the discrepancy is the use of a new smoke delivery system to expose animals by the University of Kentucky Tobacco and Health Institute. The system was changed to refine and update smoke exposure of animals to the state-of-the-art status (Griffith and Standafer 1985; Griffith and Hancock 1985). It has been demonstrated that smoke generated by different smoking machines elicits differential macrophage responses (Matulionis 1984b). The observed inconsistency stresses the importance of careful control and evaluation of the smoke delivery systems used in animal research dealing with tobacco smoke inhalation.

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