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Biological Activity of Tobacco Smoke and Tobacco Smoke-Related Chemicals

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Exposure to whole cigarette smoke from reference cigarettes results in the prompt (peak activity is 6 hrs), but fairly weak (~2 fold), induction of murine pulmonary microsomal monooxygenase activity. This activity can be detected by using as substrates either benzo(a)pyrene or ethoxyresorufin, and can be inhibited by treatment with cycloheximide or actinomycin D. Unlike the induction of pulmonary monooxygenases following intratracheal administration of 3-methylcholanthrene, these cigarette smoke-induced increases were not unequivocally linked to the *Ah* locus.

Whole smoke condensate and fractions derived from these condensates can; a) induce pulmonary monooxygenase activity, b) inhibit benzo(a)pyrene metabolism *in vitro*, c) be metabolized to forms mutagenic to *Salmonella typhimurium* tester strains TA1538 or TA98, d) transform C3H 10T½ cells *in vitro*, and e) enhance the carcinogenicity of benzo(a)pyrene in murine pulmonary tissue. A potentially important observation is that whereas hepatic tissue is capable of activating whole cigarette smoke condensate to mutagenic forms *in vitro*, murine pulmonary tissue does not seem capable of such activation. Although these pulmonary-derived tissue homogenates have significant AHH activity and can metabolize Aflatoxin B_1, 2-aminofluorene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene to mutagenic forms, these homogenates fail to activate both cigarette smoke condensate and the pro-mutagen, 6-aminochrysene. These results are discussed with reference to the concept that whole cigarette smoke may be both a potential "initiator" and "promotor" of lung cancer in mice, and that this latter property may be the most important in determining cancer risk.

Introduction

Cancer is not randomly distributed among the human population (*1*), rather, both environmental (*2*) and genetic (*3–5*) factors strongly influence its occurrence. Lung cancer is a specific example of a neoplasia whose occurrence is nonrandomly distributed. Its incidence is often associated with cigarette smoking (*6*), but the actual role that tobacco smoke or smoke-related chemicals play in this association is unclear.

A potential role of tobacco smoke involves interaction with those microsomally-bound monooxygenases that seem important in determining susceptibility to chemically-induced cancer in model animal systems (*7, 8*). These enzymes are important because not only do they metabolize many environmental chemicals, but also naturally occurring variations in their steady-state levels are genetically linked with susceptibility to toxicity, mutagenesis, and carcinogenesis induced by many of the same chemicals (*7, 9–11*).

Three basic questions can be addressed concerning the role of tobacco smoke in cancer susceptibility in man: (a) Does tobacco smoke contain chemicals that interact with the microsomal monooxygenases? (b) If they do interact, what are some of the *in vivo* and *in vitro* effects? (c) Can certain individuals or tissues within an individual be at greater risk than others from the effects of tobacco-related chemicals?

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Does Tobacco Smoke Contain Chemicals Capable of Interacting with the Monooxygenase Systems?

Kinetics of increase of pulmonary AHH levels in C57BL/6 (B6), DBA/2J (D2), and C3H/fCum (C3) strains of mice exposed to 1A1 cigarettes are shown in Figure 1. Results suggested that there were rapid increases in pulmonary AHH activity that peaked by 6 hr post-exposure, and that these induced enzymes remained at this level for at least 24 hr. The D2 strain was virtually nonresponsive to this smoke exposure compared with B6 and C3 strains; this result paralleled that found when a polycyclic aromatic hydrocarbon (PAH), 3-methylcholanthrene (MC), was given intratracheally to the same strains (see Fig. 1). However, the kinetics of induction of pulmonary AHH by smoke are different from those obtained when MC was used as an inducer. The peak of activity in the smoke induced lungs occurred at 6 hr (vs. 24 hr for MC). Also the half-life of the smoke-induced enzyme was much longer (~24 hr vs. ~4 hr). Finally, these smoke exposure conditions did not induce the AHH activity in liver, colon, and kidney (data not shown) whereas MC-treatment did; moreover, filtered smoke (gas phase) did not induce enzyme activity in lung, liver, or kidney tissue (data not shown). Smoke-induced AHH in the lung was similar to MC-induced AHH, in that both are capable of *O*-deethylation of ethoxyresorufin (Table 1); see Burke and Mayer (*15*) for data on MC as an inducer. Data with ethoxyresorufin were particularly interesting because studies have suggested that this chemical is a specific substrate for the P-448 (or P_1-450) cytochrome (*16*). This is the cytochrome that is intimately associated with metabolizing benzo[a]pyrene (BP) and MC to cytotoxic (17–19), mutagenic (*2*), DNA-binding (*21, 22*), and carcinogenic (*9, 10*) forms. Similar to MC-induced AHH levels, the smoke-induced enzyme levels are dependent upon *in vivo* protein and RNA synthesis (see Table 1).

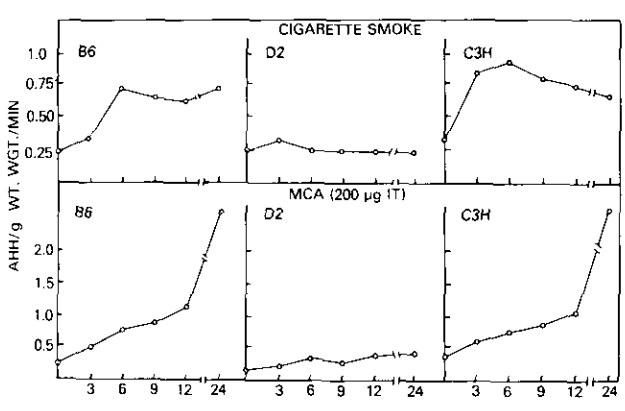

FIGURE 1. Pulmonary response of C3H/fCum, C57BL/6Cum, and DBA/2J inbred strains of mice to MC and whole cigarette smoke. Mice were either treated with 200 μg MCA/0.2% gelatin-saline solution intratracheally (IT) as described previously (*12*) and sacrificed at the time periods specified or exposed to four cigarettes from a Walton smoking machine employing a 2-sec puff, 10% smoke, 30-sec exposure, and 30-sec purge (*13*). Mice were given a 2-min rest period before exposure to the next cigarette and were sacrificed at varying times after the fourth cigarette. Lungs were excised and frozen at −70°C until assayed. AHH units are expressed as nmole 3-OH-BP formed/min/g wet weight tissue (*12*). Average activity of three animals is given.

Although tobacco smoke induced AHH activity in the B6 strain preferentially to the D2 strain, the genetic basis for this difference may not be the same as that for the difference in AHH activity induced by MC. Figure 2 shows that, whereas MC given intratracheally to (B6D2) F_1 × D2 progeny induced pulmonary AHH activity in approximately 50% (13/29) of the mice (*12, 13*), no clearcut discrimination was observed in the smoke-exposed animals. However, it may be that the inducing capacity of cigarette smoke for pulmonary tissue is just too low to easily evaluate its genetic regulation.

Whole tobacco smoke can be collected by condensation with the use of Dry Ice, and this cigarette smoke condensate (CSC) or fractions derived therefrom (*23*) can be evaluated for potential biological effects. The fractionation scheme and

Table 1. Effect of cycloheximide and actinomycin D on smoke-induced BC3F$_1$ lung AHH and ethoxyresorufin *O*-deethylase activity.[a]

Treatment	AHH activity, nmole/min-g tissue[b]	*O*-Deethylase activity nmole/min-g tissue[c]
Machine controls	0.35	<0.04
Smoke	0.70	0.61
Smoke + saline	0.67	0.83
Smoke + propylene glycol	0.53	0.83
Smoke + cycloheximide, 500 μg/g	0.32	0.14
Smoke + actinomycin D, 1 μg/g	0.42	0.15

[a] Cycloheximide and actinomycin D were injected intraperitoneally (IP) in saline or in propylene glycol respectively, immediately before exposure to one 2A1 cigarette on a Walton smoking machine. Methods given in legend to Fig. 1. Mice were sacrificed 6 hr after smoke exposure. Procedures were as described by Van Cantfort and Gielen (*14*).

[b] AHH units are expressed as nmole 3-OH-BP formed/min/g wet weight of tissue. Average activity of three animals.

[c] *O*-Deethylase units are expressed as nmoles resorufin formed/min/g wet weight of tissue according to the procedures of Burke and Mayer (*15*).

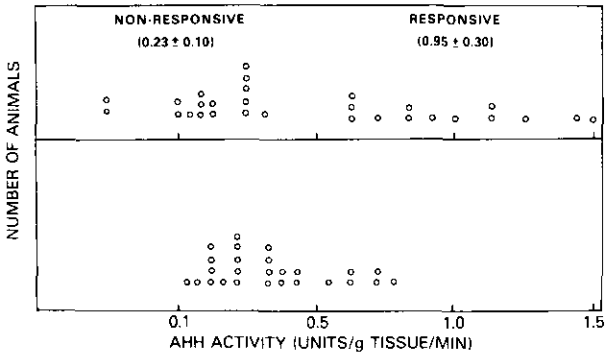

FIGURE 2. Pulmonary response of B6D2F$_1$ × D2 progeny to (top) MC and (bottom) whole cigarette smoke. Animals were generated and housed as described before (*12*). Inoculation of MC and smoke exposure was given as described in legend to Figure 1. AHH activity was determined 24 hr after IT instillation of MC and 6 hr after exposure to cigarette smoke. Mean and standard deviation is given for the MC-treated populations that are representative of the nonresponsive (0.23 ± 0.10) and responsive (0.95 ± 0.30) populations.

nomenclature were as described by Swain et al. (*26*). Table 2 summarizes some of these effects. Intratracheal administration of the whole CSC and reconstituted fractions induced a small, but significant, increase in pulmonary AHH activity. The CSC fractions varied in their capacity to induce AHH activity with the fractions B_Ia, B_Ib, N_{MeOH}, and N_{NM} the best inducers, fractions B_E and WA_I weaker inducers, and fractions B_W, SA_I, SA_E and SA_W, actually inhibiting AHH levels in the lungs. The fractions that induced AHH activity (e.g. B_Ia or B_Ib) contained chemicals that could metabolically compete with BP in an *in vitro* assay (see Table 2). This implies that CSC contains not only chemicals that induce pulmonary AHH, but also chemicals that are potential substrates for these enzymes.

What Are Some *in Vitro* and *in Vivo* Effects of Tobacco-Related Chemicals?

In Vitro Assays

The preceding section showed that tobacco smoke contains chemicals which interact with the monooxygenase system. This interaction can be beneficial if it produces nonbiologically active, polar metabolites which can be excreted from the body, or it can be detrimental if it results in intermediates with exceptional biological potency. Table 2 shows that 1A1 CSC fractions are metabolized by enzyme fractions from rat liver to forms mutagenic to tester strains TA1538 (*25, 27*) of *Salmonella typhimurium*, and also by endogenous cellular enzymes to forms capable of transforming the C3H 10T½ cells in culture. This table also depicts the levels of nicotine, phenols, and BP in fractions B_E, WA_E, and N_{NM}—the fractions which contain virtu-

Table 2. Effects of fractions of 1A1 cigarette smoke condensate (CSC) in various model systems.

Fraction[a]	Content, mg/cig	AHH inducibility[b]	(X)/BP to give 50% inhibition[c]	Mutagenesis[d]	Transformation[e]
Whole CSC	23.50	1.7	5.0	+++	+
Reconstituted CSC	23.00	1.8	5.2	+++	+
B_Ia	0.81	3.6	0.8	++	–
B_Ib	0.29	2.5	0.5	++	+
B_E	0.95	1.5	3.0	+	–
B_W	0.36	0.5	>10.0	–	–
WA_I	2.27	1.6	5.0	++	+
WA_E	1.98	1.1	2.0	±	–
SA_I	0.39	0.5	>10.0	±	–
SA_E	0.78	0.3	>10.0	–	–
SA_W	8.69	0.4	>10.0	–	–
N_{MeOH}	1.19	2.5	3.0	±	–
N_{CH}	4.58	1.2	ND	–	–
N_{NM}	0.70	3.2	1.0	±	–

[a] Whole CSC has 21.0 mg nicotine/g, 5.70 mg phenols/g, and 0.98 μg BP/g. Reconstituted CSC has 22.0 mg nicotine/g, 5.5 mg phenols/g, and 0.90 μg BP/g. B_E has 31.0 mg nicotine/g, WA_E has 41 mg phenols/g, and N_{NM} has 31.1 μg BP/g. Details given by Patel et al. (*23*).

[b] AHH inducibility = effect of fractions of 1A1 CSC fractions on pulmonary AHH activity of C57BL/6 Cum mice relative to a corn oil control (*12*).

[c] BP inhibition = competitive *in vitro* effect of CSC fractions of BP metabolism by hepatic microsomes from 3-methylcholanthrene-treated C57BL/6 mice (*13*).

[d] Mutagenesis = mutagenic activity of 1A1 CSC fractions in *Salmonella typhimurium* TA 1538 in the presence of liver microsomal S-9 mix (*24*).

[e] Transformation = malignant transformation frequency in C3H 10T1/2 Cl. 8 cells treated with CSC fractions (*25*).

ally all of these three chemicals. The most biologically active fractions are not those that contain the BP or phenols. The B_Ia and B_Ib fractions have not been analyzed for their chemical content. However, because of the nature of the fractionation scheme (B_Ia = bases insoluble after ether, B_Ib = bases insoluble before ether), these fractions could contain some aromatic amine-like chemicals.

A similar distribution of mutagenic activity was found with CSC fractions from the 2A1 reference cigarette (Table 3), and such activity was observed to be stable even if the fractions were stored frozen for one year. Thus, for at least two different types of cigarettes, certain fractions of their condensates can be metabolized to forms that are active in an *in vitro* mutagenesis test system.

However, subsequent studies (Table 4) revealed a potentially important hindrance to the extrapolation of the foregoing studies to an *in vivo* situation. Mouse pulmonary tissue preparations, under conditions where BP is significantly metabolized to 3-OH-BP (see AHH levels), failed to significantly activate either 2A1 condensate or 6-aminochrysene (6-AC) to forms mutagenic to the TA98 tester strain.

Table 3. Mutagenesis of TA 98 with 2A1 cigarette smoke condensate (CSC) fractions.[a]

Fraction	Content, mg/cig	Mutants per plate per 250 μg of sample		Calculated mutants per plate per cigarette		Activity, %	
		Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Whole CSC	40.00	133	122	21,280	19,520		
Reconstituted CSC	39.50	159	148	25,122	23,284		
BI[a]	0.60	987	1,149	2,369	2,758	11	10
BI[b]	0.24	3,510	5,266	3,370	5,055	16	18
B_E	1.04	1,258	2,065	5,233	8,590	25	30
B_W	0.52	102	151	212	314	1	1
WA_I	3.63	289	394	4,196	5,721	20	20
WA_E	2.66	141	206	1,500	2,192	7	8
SA_I	1.44	53	43	305	248	2	1
SA_E	0.88	8	14	28	49	0	0
SA_W	15.60	8	20	499	1,248	2	4
N_{MeOH}	2.16	65	93	562	804	3	3
N_{CH}	9.54	57	24	2,175	916	10	3
N_{NM}	1.24	80	139	397	689	2	2

[a] The 2A1 (low nicotine) CSC fractions were generated by Meloy Laboratories according to the methods of Patel et al. (*23*) and the pour plate incorporation mutagenesis assay was performed according to the methods of Kier et al. (*24*).

Table 4. Capacity of TCDD-induced mouse hepatic and pulmonary S-9, and Aroclor 1254-induced rat hepatic S-9 to activate whole cigarette smoke condensate to form(s) mutagenic to *S. typhimurium* TA 98.[a]

Source of S-9	Compound		Protein, mg	AHH, pmole/tube[b]	Survival, %[c]	Mutants/plate (minus background)
	Type	μg/plate				
C57BL/6 hepatic						
Pour plate	2A1	650	3.14	4929		115
	AFB_1	1	3.14	4929		170
Suspension	2A1	260	3.14	4929	109	61
	6-AC	5	3.14	4929	102	115
C57BL/6 pulmonary						
Pour plate	2A1	1300	11.5	8800		8
	AFB_1	25	11.5	8800		250
Suspension	2A1	1300	1.44[d]	1071	105	5
	6-AC	125	6.90	3208	83	3
Rat hepatic						
Pour plate	2A1	1300	4.43	9625		185
	AFB_1	1	4.43	9625		760
Suspension	2A1	650	2.95	7371	106	83
	6-AC	0.5	0.03	190	82	315

[a] Pour plate incorporation mutagenesis assays were performed according to the method of Kier et al. (*24*). Suspension assays consisted of a 35 min incubation of a mixture of bacteria, S-9, and test chemicals in a buffer composed of 3.6 mM NADPH, 4.2 mM NADH, 3 mM $MgCl_2$, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl and 137 mM NaCl.

[b] As pmole 3-OH-BP formed per assay tube; total time was 35 min; 25 μg BP/ml was substrate.

[c] Percentage of bacteria surviving 35 min suspension relative to TA 98 alone.

[d] Subsequent assays with 11.5 mg protein, 8800 units AHH, and 1300 μg 2A1 still showed no induced mutants.

Under similar conditions both rat and mouse hepatic tissue could activate both 6-AC and the 2A1 CSC to mutagenic intermediates. However, the pulmonary S-9 preparations were capable of activating aflatoxin B_1 (AFB_1) (see Table 4), 7,8-dihydro-7,8-dihydroxybenzo(a)-8-diol pyrene-7, and 2-aminofluorene (2-AF) to mutagenic forms (Table 5). Thus, the lung preparations were metabolically active by some measures, but did not activate 6-AC or CSC. That the tissue hypothetically at risk to tobacco-associated carcinogenesis seems unable to activate the tobacco smoke condensate allows many interesting interpretations which will be discussed in the last section of this report.

In Vivo Studies

Tobacco smoke condensate fractions have been shown to "promote" carcinogen-initiated skin tumors in mice (*28, 29*). Table 6 demonstrates that at least some fractions from the 2A1 CSC are capable of acting synergistically with intratracheally instilled BP (with or without Fe_2O_3), resulting in a much higher incidence of lung cancers in BC3Fl mice; see Saffiotti (*30*) for discussion on use of Fe_2O_3 in pulmonary cancer model systems. BP alone (given every other week for 6 weeks) resulted in only one malignant tumor by 39 weeks after treatment out of a total of 82 treated animals (combining animals given 0.6 mg and 1.2 mg BP per treatment).

Table 5. Activation of 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene (7,8-diol) and 2-aminofluorene (2-AF) by pulmonary S-9.[a]

Pulmonary S-9, mg protein	2-AF, μg/tube	7,8-diol, μg/tube	AHH, pmole/tube[b]	Survival, %[c]	Mutants/ plate
6.57	25		1904	48	536
	10		1925	81	668
	10		1904	61	521
3.28	25		1452	52	458
	10		1452	52	540
	5		1680	101	627
0	25		—	82	39
	10		—	84	52
6.57		0.5	1928	57	544
3.28		0.5	1680	18	338
0		1.0	—	88	76
0		0.1	—	99	84
TA 98 alone	—	—	—	100	20

[a] Suspension assays performed according to procedures given in Table 4.
[b] AHH = pmoles 3-OH-BP formed per 35 min incubation in separate tubes containing 25 μg BP/ml as substrate.
[c] Percentage of viable bacteria after 35 minute assay relative to TA 98 alone.

Table 6. Evaluation of cocarcinogenic effects of 2A1 CSC and selected fractions of the condensates on BP-initiated lung cancers in $BC3F_1$ mice.[a]

Treatment group	Gel saline[b]	CSC fraction[b]					
		B_1a	B_1b	WA_1	N_{MeOH}	RF[c]	SM[d]
No other treatment	0/118	0/37	1/47	0/28	1/40	0/68	0/50
0.6 mg Fe_2O_3	0/48	1/47	0/71	0/48	0/46	2/65	0/51
0.6 mg BP	1/43	10/64	5/74	14/59	19/82	4/60	13/58
1.2 mg BP	0/39	9/62	3/35	12/46	34/79	10/58	9/66
0.6 mg BP + mg Fe_2O_3	3/60	7/64	4/40	2/40	3/50	3/42	1/56
1.2 mg BP + 0.6 mg Fe_2O_3	7/69	9/71	13/57	6/55	10/74	5/47	11/50

[a] Groups of $BC3F_1$/Cum female mice (8–14 weeks old) were intratracheally (IT) inoculated once every 2 weeks for a total of 6 weeks with 0.02 ml gelatin–saline solutions of BP, Fe_2O_3, BP:Fe_2O_3 alone or in combinations with the cigarette smoke condensate (CSC) fractions (10 μg each). At the end of the "carcinogen treatment" period, IT treatment with the appropriate CSC fractions was continued once every 2 weeks until 4 weeks prior to sacrifice. Data presented represents tumor incidence for mice randomly sacrificed after 26 and 39 weeks on test. The tumor types diagnosed histopathologically include alveologenic adenocarcinomas, adenosquamous carcinomas, squamous cell carcinomas, and squamous neoplasms. A more complete description of these lung cancers is given elsewhere (*33*).
[b] Data given in terms of the number of mice with lung carcinomas per the total number of animals at risk.
[c] RF = reconstituted fractions.
[d] SM = starting material.

Addition of ferric oxide (Fe_2O_3) with BP resulted in 8% malignant tumors (*30*). However, treatment with selected 2A1 condensate fractions every other week for the duration of the study (39 weeks), resulted in significantly increased malignant lung lesions in animals treated with both BP alone or with BP plus Fe_2O_3. The fractions alone, or with Fe_2O_3, resulted in few malignant tumors (5 out of a total of 764 treated animals). Thus, in an *in vivo* lung tumor model system, the fractions seemed to have mainly "promotor-like" activity.

Can Individuals or Tissues within an Individual Differ in Risk to the Effects of Tobacco-Related Chemicals?

The answers to this question are at best complex. The results of studies discussed in the previous sections can be somewhat difficult to reconcile. On the one hand, tobacco smoke contains chemicals that interact with hepatic mixed-function oxidases such as AHH, and this metabolism generates intermediates that are mutagenic and transforming in *in vitro* test systems. On the other hand, CSC is not activated to mutagenic forms by mouse-derived pulmonary tissues *in vitro*. Also, CSC material does not seem to efficiently cause lung tumors *in vivo*. Many interpretations are feasible. Perhaps the lack of activation is characteristic of mouse pulmonary tissue alone and cannot be generalized to other species. In point of fact, rat pulmonary tissue has been reported to activate certain smoke condensates to mutagenic forms (*24*). However, both rat and human lung tissues have also failed to activate CSC material (*24, 31*), and so the generality of the activation (or lack thereof) is still not understood. Second, the fact that hepatic tissue can activate these condensates suggests that tobacco material could be activated by this organ and, following that, the tissues at risk (e.g., lung) would be exposed to these active forms. Or third, the lung is at risk only for the effects of those chemicals in cigarette smoke capable of "promoting" the carcinogenic event that may be initiated by a myriad of environmental pollutants.

The first interpretation is difficult to assess because no lung cancer model animal system directly applicable to man is currently available. However, the kind of pulmonary lesions observed in mice following exposure to known chemical carcinogens, that is, bronchogenic squamous cell carcinomas, alveolar adenocarcinomas, and adenosquamous carcinomas, resemble lesions found in man (*32, 33*).

Some data suggest that the liver plays a major role in the eventual susceptibility of other organs to chemically-induced cancers (*34*). However, no evidence is available to suggest that lung tissue is at risk from liver-metabolized chemicals. Evaluation of this alternative *in vivo* will be difficult because of the close liver/lung relationship.

The third interpretation is particularly intriguing and suggests that "promotion" represents the real risk of cigarette smoke to lung tissue. This idea is consistent with the following facts: (a) tobacco smoke condensate is capable of "promoting" mouse skin carcinogenesis; (b) tobacco-smoke condensate can "promote" lung carcinogenesis in model animal systems (see Table 6); (c) the conditions of cigarette-smoke exposure that result in the highest risk of human lung cancer are quite similar to those that are most promotive in animal test systems; that is, frequent and relatively prolonged treatment; (d) the chemicals in cigarette smoke (particulate phase) that are known initiators of carcinogenesis may be too low in concentration [total = 400 ng/cigarette (*35*)] to initiate significant transformation *in vivo*. The evaluation of whole cigarette smoke as a potential initiator and promoter of lung cancer in the inbred strains of mice is now being studied in our laboratory.

NOTE ADDED IN PROOF: We have recently found that a pulmonary S-9 preparation from Aroclor 1254-induced mice is capable of weakly activating 6-AC to a bacterial mutagen with the use of a pour plate assay (2–3-fold over background). We have still not observed an increase in bacterial mutations using this S-9 preparation and 2A1 cigarette smoke condensate.

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