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Mouse Skin Tumor Initiation-Promotion and Complete Carcinogenesis Bioassays: Mechanisms and Biological Activities of Emission Samples

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Extracts of soots obtained from various sources were applied to the skin of mice in an effort to identify carcinogens in these mixtures and to link these materials to the etiology of human cancer. Samples of coal chimney soot, coke oven materials, industrial carbon black, oil shale soot, and gasoline vehicle exhaust materials have been examined by this method.

The studies reported here have been constructed to compare the carcinogenic and tumorigenic potency of extracts from various particulate emissions: coke ovens, diesel and gasoline vehicles and a roofing tar pot. Automobile emission samples were obtained by collecting the diluted and cooled exhaust on Teflon-coated glass fiber filters. Coke oven and roofing tar samples were particulate emission samples collected by impaction and filtration. The organic components associated with each of the particles were extracted with dichloromethane and dermally applied to SENCAR mice. All agents were applied as tumor initiators by using a five-dose protocol. Selected extracts were also applied as complete carcinogens and as tumor promotors. Statistical analyses of the resulting tumor data were performed by using nonlinear Poisson and probit models. The results from these experiments provide a suitable data base for comparative potency estimation of complex mixtures.

Introduction

Experimental animal studies and epidemiological studies provide evidence that certain chemicals present in the environment either as pure chemicals or in complex mixtures are a major contributing factor in the etiology of some human cancers. For example, polycyclic aromatic hydrocarbons (PAHs) are widespread contaminants in our environment, occurring primarily as the result of combustion and pyrolysis of organic materials (e.g., the burning of cigarettes, operation of coke ovens, operation of automobiles, industrial combustion processes, etc.). Concern over the prevalence of PAHs has increased as evidence establishing the carcinogenicity of a significant number of these compounds has accumulated. Besides PAHs, a wide variety of other structurally diverse environmental chemicals (aromatic amines, nitrosamines, nitrosamides, metals, aflatoxins, and many others) are known to be carcinogenic (*1*).

Current information suggests that chemical carcinogenesis is a multistage process; in this regard, one of the best studied models is the two-stage carcinogenesis system in mouse skin. Skin tumors can be induced by the sequential application of a subthreshold dose of a carcinogen (initiation phase) followed by repetitive treatment with a noncarcinogenic tumor promoter (promotion phase). The initiation phase requires only a single application of either a direct or indirect carcinogen at a subthreshold dose and is essentially irreversible. The promotion phase is brought about by repetitive treatments after initiation and is initially reversible but later irreversible. If an agent is given repeat-

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edly by itself, the mouse skin system can also be used to determine if the agent is a complete carcinogen (i.e., if it has both tumor initiating and promoting activities). In addition, if an agent is given concurrently with a known complete carcinogen or tumor initiator, one can determine if the agent as cocarcinogenic or cotumor-initiating activity or possibly even anticarcinogenic activity. Similarly, if an agent is given concurrently with a known tumor promoter, one can determine if the agent has copromoting or antipromoting activity. Furthermore, as in most carcinogenesis systems, skin carcinogens may display additive or synergistic effects. The skin system is an important model not only for studying carcinogenesis and for the bioassay of carcinogenic agents but also for studying modifiers of carcinogenesis. The major disadvantage of the skin system (as of many other carcinogenesis systems) is that some carcinogens are tissue specific.

Recently the generality of two-stage tumor induction has been demonstrated in a number of experimental carcinogenesis systems (e.g., liver, bladder, lung, colon, esophagus, stomach, mammary gland, pancreas, lung cells in culture) (*2*). A wide variety of agents (e.g., diet, bile acids, hormones, saccharin, L-tryptophan, phenobarbital, polychlorinated biphenyls, polybrominated biphenyls, butylated hydroxytoluene) have been used successfully as promoters (*2*).

Among rodent species, mice are generally more sensitive than rats and hamsters to skin carcinogenesis by either the complete carcinogenesis protocol or the initiation-promotion protocol (Table 1) (*3,4*). In mice, the complete carcinogenesis protocol gives rise to a low number of papillomas followed by a high incidence of squamous cell carcinomas, whereas the initiation-promotion protocol gives rise to a large number of papillomas followed by a moderate incidence of squamous cell carcinomas. In rats, both the complete carcinogenesis and initiation-promotion protocols give rise to basal cell carcinomas and very few papillomas and squamous cell carcinomas. In hamsters, the complete carcinogenesis protocol produces mainly squamous cell carcinomas and some melanomas, whereas the initiation-promotion protocol produces mainly melanomas.

PAHs are one of the major classes of chemical carcinogens that have skin tumor initiating and/or complete carcinogenic activity on mouse skin and have been studied extensively in this system. Over 100 PAHs, PAH derivatives, and PAH metabolites are known to be mouse skin tumor initiators and/or complete carcinogens (*5,6*). Among these, moderate to strong initiators and/or complete carcinogens include *7,12*-dimethyl benz(a)anthracene, DMBA, 3-methylcholanthrene, benzo(a)pyrene [B(a)P], 7-methylbenz(a)anthracene, 5-methylchrysene, dibenz(a,h)anthracene, dibenzo(a,h)pyrene, dibenzo(a,i)pyrene, dibenzo(a,e)pyrene, benzo(a)phenanthrene, dibenzo(a,j)anthracene, benzo(c)chrysene, benzo(g,h,i)perylene, dibenzo(a,c)naphthacene, and 11-methylcyclopenta(a)phenanthren-17-one (*5, 6*). Besides PAHs, many other chemicals and chemical classes are known to be tumor initiators and/or complete carcinogens on mouse skin (Table 2) (*5,6*).

Tumor Initiation

When appropriately tested, known complete carcinogens in skin show skin tumor-initiating activity (*7*). In the two-stage mouse skin system, initiation is the only stage that requires the presence of the complete carcinogen, and the measured complete carcinogenic potency of a chemical reflects its capacity for tumor initiation. There is both a good qualitative and quantitative correlation between the complete carcinogenic and tumor initiating activities of several chemical carcinogens in mouse skin (*7*). This relationship holds when one considers the number of papillomas per mouse at early times (10 to 20 weeks) or the final incidence of carcinomas after tumor initiation (*7*).

It is possible that a carcinogen lacking promoting ability would not be detected when tested as a complete carcinogen. It has been reported that a number of chemical compounds, e.g., benz(a)anthra-

Table 1. Comparison of complete carcinogenesis and initiation-promotion in the skin of various species.

Species	Treatment	Tumor histology			
		Basal cell carcinomas	Carcinomas	Papillomas	Melanomas
Mouse	Complete		+ +	+	
	Two-stage		+	+ + + +	
Rat	Complete	+			
	Two-stage	+			
Hamster	Complete		+		+
	Two-stage				+

Table 2. Chemicals other than polycyclic aromatic hydrocarbons that are positive as skin tumor initiators and/or complete carcinogens.[a]

Class	Chemical(s)
Aldehyde	Malonaldehyde
Aziridine	β-Hydroxy-1-ethylaziridine
Carbamate	Urethane
	Vinyl carbamate
	N-Butyl-*N*-nitrosourethane
Epoxide, diepoxide	Glycidaldehyde
	1,2,3,4-Diepoxybutane
	1,2,4,5-Diepoxypentane
	1,2,6,7-Diepoxyhexane
	Chloroethylene oxide
	1,2-Epoxybutyronitrile
Haloalkyl ether	Bis(chloromethyl) ether
	α,α-Dichloromethyl methyl ether
	Chloromethyl methyl ether
Haloaromatic	2,3,4,5-Tetrachloronitrobenzene
	2,3,4,6-Tetrachloronitrobenzene
	2,3,5,6-Tetrachloronitrobenzene
	Pentachloronitrobenzene
Haloalkyl ketone, acid	Chloroacetone
	3-Bromopropionic acid
Hydroxylamine	*N*-Acetoxy-4-acetamidobiphenyl
	N-Acetoxy-2-acetamidofluorene
	N-Hydroxy-2-aminonaphthalene
	N-Acetoxy-2-acetamidophenanthrene
	N-(4-Methoxy)benzoyloxypiperadine
	N-(4-Nitro)benzoyloxypiperadine
	N-Acetoxy-2-acetamidostilbene
Lactone	Propiolactone
Multifunctional	Triethylenemelamine
	4-Nitroquinoline-*N*-oxide
Natural product	Aflatoxin B1
	Sterigmatocystin
Nitrosamide	*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine
Sulfonate	Allyl methylsulfonate
Sultone	1,3-Propanesultone
Urea	*N*-Nitrosomethylurea
	N-Nitrosoethylurea

[a]Data of Pereira (*5*) and Nesnow (*6*).

cene, dibenz(a,c)anthracene, chrysene, triethylenemelamine, urethane, B(a)P-7,8-dihydrodiol-9,10-epoxide, benz(a)anthracene-3,4-dihydrodiol-1,2-epoxide, have mouse skin tumor-initiating activity but either lack or have very weak complete carcinogenic activity (*6,7*).

Many carcinogens produce good dose-response relationships when bioassayed as tumor initiators using SENCAR mice. For example, results for DMBA and B(a)P (Table 3) (*7*) show good correlations between the number of papillomas per mouse at 15 weeks and the final carcinoma incidence at 50 weeks. For each agent the percentage of mice wth papillomas also shows a reasonable correlation, but the dose response is very narrow. The SENCAR mouse was derived by crossing Charles River CD-1 mice with skin tumor sensitive mice (originally derived from Rockland mice) and selecting for sensitivity to DMBA–12-*O*-tetradecanoylphorbol-13-acetate (TPA) two-stage carcinogenesis for 8 generations starting with the F_1 cross as described by Boutwell (*7,8*). The mice developing the most papillomas with the shortest latency period after initiation-promotion treatment were selected for each breeding.

Experiments with B(a)P and DMBA using different stocks and strains of mice (*9*) suggest the following ranking for sensitivity to two-stage (initiation-promotion) carcinogenesis: SENCAR >> CD-1 > ICR/Ha Swiss > BALB/c ≥ C57BL/6 ≥ C3H ≥ DBA/2. It is important to emphasize the limitations of this subjective ranking. Firstly, only responses to B(a)P and DMBA were considered. Secondly, dose-response data for both the carcinogen and/or promoter were not available for many of the mouse strains and stocks. Despite these limitations, however, the differences between mice at the extremes of the ranking are significant.

SENCAR mice are between 10 and 20 times more sensitive than CD-1 mice to DMBA tumor initiation (*10*). However, SENCAR mice are only between 3 and 5 times as sensitive as CD-1 mice to B(a)P tumor initiation (*10*). SENCAR mice are 2 to 3 times as sensitive as CD-1 mice to TPA promotion (*9*).

Between SENCAR and C57BL/6 mice there is an even greater difference in sensitivity to two-stage skin carcinogenesis. C57BL/6 mice are very refractory to two-stage skin carcinogenesis by B(a)P-TPA. Even high initiating doses of B(a)P (1600 nmoles) and high promoting doses of TPA (10 μg) are very ineffective in causing skin tumors (Slaga and Nesnow, unpublished data). However, C57BL/6 mice do respond to complete carcinogenesis by B(a)P (*9*). Such unequal susceptibility to complete and two-stage carcinogenesis within a stock or strain of mice strongly suggests that the promotional phases of complete and two-stage carcinogenesis are dissimilar. In addition, differences in sensitivity to initiation and promotion between mice may be due to alterations in the promotional phase of two-stage carcinogenesis. In this regard, we recently found that benzoyl peroxide is an effective promoter in C57BL/6 and SENCAR mice (Slaga et al., unpublished data). For some reason, TPA is not an effective promoter in C57BL/6 mice.

Tumor Promotion

An agent that induces tumors when given repeatedly after a subthreshold dose of a carcinogen is referred to as a tumor promoter. Although the phorbol esters are the most potent mouse skin

Table 3. Dose-response studies on the ability of DMBA and B(a)P to initiate skin tumors in SENCAR mice.[a,b]

Initiator	Dose, nmole	Mean papillomas per mouse at 15 weeks	Mice with papillomas at 15 weeks, %	Mice with carcinomas at 50 weeks, %
DMBA	100	22	100	100
DMBA	10	6.8	100	40
DMBA	1	3.2	93	22
DMBA	0.1	0.5	20	5
B(a)P	200	7.5	100	55
B(a)P	100	3.2	78	30
B(a)P	50	1.4	60	18

[a]Data of Slaga et al. (*7*).
[b]Experimental protocol: beginning 1 week after initiation, mice were treated twice weekly with 5 μg TPA.

tumor promoters, a wide variety of other compounds are known to have skin tumor-promoting activity (Table 4). After the phorbol esters and dihydroteleociden 'β, anthralin is the most potent tumor promoter of those listed in Table 4. Van Duuren and Goldschmidt (*11*) reported a fairly extensive structure-activity study with anthralin and derivatives. Likewise, Boutwell and Bosch (*12*) reported a structure-activity study of a number of phenolic compounds that are weak promoters in comparison to the phorbol esters and anthralin. Although several of the other compounds shown in Table 4 have moderate to weak activity as tumor promoters, no extensive structure-activity studies have been reported. We have recently found that benzo(e)pyrene and benzoyl peroxide as well as other free radical-generating compounds such as chloroperbenzoic acid and lauroyl peroxide are relatively good tumor promoters (*13,14*).

Table 4. Skin tumor promoters.[a]

Compound	Potency
Croton oil	Strong
Certain phorbol esters found in croton oil	Strong
Some synthetic phorbol esters	Strong
Certain euphorbia latices	Strong
Anthralin	Moderate
Certain fatty acids and fatty acid methyl esters	Weak
Certain long chain alkanes	Weak
Phenolic compounds	Weak
Surface active agents (sodium lauryl sulfate, Tween 60)	Weak
Citrus oils	Weak
Extracts of unburned tobacco	Moderate
Tobacco smoke condensate	Moderate
Iosoacetic acid	Weak
1-Fluoro-2,4-dinitrobenzene	Moderate
Benzo(e)pyrene	Moderate
Benzoyl peroxide	Moderate
Dihydroteleocidin β	Strong[b]

[a]See Slaga and Fischer (3) for individual citations.
[b]Dihydroteleocidin β has a promoting potency similar to that of TPA (Slaga and Sugimura, unpublished results).

The ability of TPA to promote tumor yield with respect to dose after DMBA initiation is shown in Table 5. For tumor promotion (as for tumor initiation, Table 3) a very good dose-response relationship is seen when either the number of papillomas per mouse at 15 weeks or the percentage of mice with squamous cell carcinomas at 50 weeks is considered. Similar results have been reported from studies using Charles River CD-1 mice (*15*) and ICR/Ha Swiss mice (*16*).

Unlike the initiation phase, the promotion stage requires a certain frequency of application to induce tumors and is reversible (*8*). In general, as the frequency of TPA application decreases the promoting activity also decreases (*8*). Even high doses of TPA once every 2 or 3 weeks are ineffective in tumor promotion (*8*).

Complete Carcinogenesis

Complete carcinogenesis in mouse skin refers to the production of tumors (mainly carcinomas) after repeated application of a carcinogen for terms of up to 1 yr. Compounds possessing both tumor initiating activity and tumor promoting activity will produce tumors in this regimen. An examination of six mouse stocks and strains using B(a)P and DMBA as the carcinogens (*9*) suggests the following ranking for sensitivity to complete carcinogenesis: SENCAR >CD-1 > C57BL/6 ≥ BALB/c ≥ ICR/Ha Swiss > C3H.

Complete carcinogenesis in mouse skin was used in the early 1900's to identify carcinogens in organic extracts of organic particulate samples and to try to link these materials to the etiology of human cancer. Passey (*17*) found that ether extracts of coal chimney soot produced both "warts" (papillomas) and "cancers" (carcinomas) when applied repetitively to the depilitated backs of mice. Campbell (*18*) confirmed these results with coal soot and also reported the carcinogenic activity on mouse skin of

Table 5. Dose-response studies on the ability of TPA to promote tumors after DMBA initiation in SENCAR mice.[a,b]

TPA dose, μg	Time to first papilloma, weeks	Mean papillomas per mouse at 15 weeks	Mice with papillomas at 15 weeks, %	Mice with carcinomas at 50 weeks, %
10	8	3.0	100	32
5	6	7.2	100	46
2	7	6.5	100	45
1	8	3.6	80	25
0.1	11	0.4	5	8

[a]Data of Slaga et al. (*7*).
[b]Experimental protocol: At 1 week after initiation with 10 nmoles DMBA, mice were promoted with various doses of TPA.

road dust extracts. Remarkably, Campbell also observed both dermal and lung tumors in mice exposed only to road dust particulates (*19*).

Kotin and co-workers examined the carcinogenic effects of organic extracts of air particulate samples from the Los Angeles area. These extracts produced both malignant and benign tumors when applied repeatedly to the backs of C57BL/6 mice (*20*). Wynder and Hoffmann expanded these studies by administering air particulate extracts from the Detroit area to Swiss ICR mice (*21*). Both groups concluded that B(a)P alone could not account for all the carcinogenic activity observed. Kotin and his group (*22*) and Wynder and Hoffmann (*23–25*) also studied the carcinogenic effects of organic extracts of particulate emissions from gasoline engines; both concluded that these extracts produce tumors on mice. Kotin et al. studied extracts from particulates isolated from a diesel engine. In contrast to the positive results reported for air particulate and gasoline engine emissions, these investigators found C57BL/6 mice to be refractory to the diesel extracts (*26*). However, tumors were produced in strain A mice treated repetitively with the diesel mixtures (*26*). Von Haam and Mallette (*27*) and Võsamäe (*28*) were able to induce tumors in mice by applying extracts of industrial carbon black and oil shale soot, respectively.

Most of the studies described above used limited numbers of mice of various strains, did not explore potential sex differences, and used limited numbers of doses. These studies were not designed to produce comparative dose-response data.

We report here results from a study designed to produce extensive dose-response information, using the same mouse strain (SENCAR), on the tumorigenic and carcinogenic activities of organic extracts from a variety of particulate emission sources: diesel and gasoline vehicles, coke oven, and roofing tar. Earlier publications of results from these experiments reported papilloma formation after 6 mo in a tumor initiation protocol (*6,29–32*). This paper reports the production of carcinomas after 1 yr in both tumor initiation and complete carcinogenesis protocols.

Materials and Methods

Sample Generation and Isolation

The details of sample generation and isolation were reported previously (*33*). Vehicle emission samples were obtained from a 1973 preproduction Nissan-Datsun 220C (Nissan), a 1976 prototype Volkswagen TurboRabbit (VW Rabbit), and a 1977 Ford Mustang II-302 V-8 with catalyst and EGR (Mustang). Each vehicle was mounted on a chassis dynamometer and driven in a repeated highway fuel economy test (HWFET) cycle of 10.24 mi, an average speed of 48 mph, and a running time of 12.75 min. The Nissan and VW Rabbit were fueled with the same batch of No. 2 diesel fuel. The Mustang was fueled with unleaded gasoline. Particulate samples were collected with a dilution tunnel in which the hot exhaust was diluted, cooled, and filtered through Pallflex Teflon-coated fiber glass filters.

Topside coke oven samples were collected from the top of a coke oven battery at Republic Steel, Gadsden, AL, by use of a massive air volume sampler. Because of the topside ambient location and local wind conditions, an unknown portion of this emission sample contains particles from the local urban environment. The coke oven main sample was collected from a separator located between the gas collector and the primary coolers within the coke oven battery.

The roofing tar emission sample was collected from a conventional tar pot with external propane burner. Pitch-based tar was heated to 182–193°C and emissions were collected with a 1.8-m stack extension and Teflon socks in a baghouse.

Only one vehicle or source was used for each sample; therefore, each sample may not be representative of the particular technology. All samples

were extracted by a Soxhlet apparatus with dichloromethane, which was then removed by evaporation under dry nitrogen gas.

Tumor Experiments

Seven- to nine-week-old SENCAR mice (*8*) bred at Oak Ridge National Laboratory were used. There were 80 animals (40 of each sex) per treatment group. Animals were housed in plastic cages (10 per cage) under yellow light with hardwood chip bedding, fed Purina chow and water *ad libitum*, and maintained at 22–23°C with 10 changes of air per hour. All mice were shaved with surgical clippers 2 days before the initial treatment, and only those mice in the resting phase of the hair cycle were used.

Under the tumor initiation protocol, all samples at all doses were applied as a single topical treatment in 0.2 mL spectral quality acetone, except for the 10-mg dose, which was administered in doses of 2 mg for 5 days. Beginning 1 week after treatment, 2.0 μg TPA in 0.2 mL acetone was administered topically twice weekly. Under the complete carcinogenesis protocol, samples were administered in 0.2 mL acetone weekly (or twice weekly for the highest dose level) for 50 to 52 weeks. Under the tumor promotion protocol, mice were first initiated with 50.5 μg B(a)P in 0.2 mL acetone and then treated weekly (or twice weekly for the highest dose level) for 34 weeks with the sample.

Skin tumor formation was recorded weekly, and papillomas >2 mm in diameter and carcinomas were included in the cumulative total if they persisted for 1 week or longer. The number of mice with tumors, the number of mice surviving, and the total number of tumors were determined and recorded weekly. At 6 months, the number of papillomas per surviving animal was recorded for statistical purposes. The tumors were histologically verified; also, nondermal tumors were histopathologically identified.

Statistical Analysis

Tumor incidence analyses were carried out on the papilloma data obtained at 6 mo and on the cumulative number of animals with carcinomas at 1 yr. The data were fitted to a probit model, taking into account the numbers of spontaneous tumors occurring in the control groups. The probit formula used is

$$P = \beta_0 + (1 - \beta_0)\,\phi\,(\beta_1 + \beta_2 \ln x)$$

where P is the probit proportion, x is the dose applied, and is the standard normal cumulative distribution function (*34*). The model parameters $\hat{\beta}_0$, $\hat{\beta}_1$, and $\hat{\beta}_2$, were estimated from the raw data by maximum likelihood methods (*35*). The dose that would produce a 50% tumor incidence in excess of the control rate was then estimated as a function of the model parameters. The 95% confidence limits were estimated using the asymptotic variance-covariance matrix estimated during the model-fitting process. Chi-square goodness-of-fit and likelihood ratio tests were also computed to examine the appropriateness of the model and the strength of the dose effect.

The papilloma scores at 6 months were also subjected to tumor multiplicity analysis by a nonlinear Poisson model (*36*):

$$\lambda_i = \beta_0 + e^{\beta_1 + \beta_2 \ln (x_i)}$$

where $\hat{\lambda}_i$ is the number of papillomas per mouse, x_i is the dose, and $\hat{\beta}_0$, $\hat{\beta}_1$, and $\hat{\beta}_2$ are the model parameters. Using maximum likelihood methods, the model parameters were estimated from the raw data and used to calculate the number of papillomas per mouse for a dose of 1 mg. Asymptotic 95% confidence intervals for these activities were obtained. Tests for the Poisson assumption, adequacy of the model, and strength of the dose response were also calculated (*36*).

Results

Tumor Initiation

The tumor initiation experiments were designed to compare the relative tumorigenic activities of the diverse complex mixtures (Nissan, VW Rabbit, Mustang, topside coke oven, coke oven main, and roofing tar extracts) and B(a)P. Animals were scored at 6 months for papillomas and at 1 yr for carcinomas. The carcinoma data represent the cumulative number of animals with carcinomas at 1 yr in each treatment group regardless of survival.

The B(a)P, topside coke oven, coke oven main, Nissan, and roofing tar samples produced an 89% or greater tumor incidence at the highest dose level applied (Tables 6, 7). Tumor multiplicity ranged from five to six papillomas per mouse in the roofing tar and Nissan groups to greater than seven in the B(a)P, topside coke oven and coke oven main groups. These groups also produced significant numbers of squamous cell carcinomas: 13 to 65% of the mice in each group bore carcinomas at the highest dose evaluated. In general, samples which produced a papilloma response of greater than five papillomas per mouse at 6 months produced a carcinoma response of 0.15 to 0.65 carcinomas per mouse, with 13 to

Table 6. Tumors observed following administration of B(a)P, topside coke oven extract, and coke oven main extract to SENCAR mice in the tumor initiation protocol.

Sample	Dose, μg/mouse	Sex	Mice surviving	Mice with papillomas, %[a]	Mean papillomas per mouse[a]	Mice with carcinomas, %[b]	Mean carcinomas per mouse[b]
B(a)P	0	M	37	8	0.08	5	0.05
	0	F	39	5	0.05	0	0
	2.52	M	40	45	0.50	5	0.07
	2.52	F	39	51	0.44	5	0.05
	12.6	M	40	73	1.8	20	0.20
	12.6	F	37	57	1.1	23	0.23
	50.5	M	39	100	5.8	25	0.25
	50.5	F	40	75	2.8	20	0.20
	101	M	38	95	10.2	30	0.33
	101	F	38	97	7.9	25	0.25
Topside coke oven	100	M	40	13	0.13	0	0
	100	F	40	10	0.20	8	0.08
	500	M	40	73	1.6	5	0.05
	500	F	40	70	1.8	15	0.15
	1000	M	37	95	2.6	15	0.15
	1000	F	39	72	2.0	3	0.03
	2000	M	39	95	4.0	13	0.13
	2000	F	38	90	3.5	10	0.10
	10000	M	39	100	7.1	13	0.15
	10000	F	40	100	7.7	20	0.23
Coke oven main	100	M	38	50	0.63	10	0.10
	100	F	39	31	0.38	25	0.25
	500	M	39	90	3.7	54	0.59
	500	F	39	92	2.2	54	0.54
	1000	M	39	87	3.3	53	0.53
	1000	F	39	90	3.1	48	0.48
	2000	M	40	78	3.1	48	0.48
	2000	F	40	100	5.3	45	0.45
	10000	M	38	100	8.9	55	0.55
	10000	F	37	100	8.1	65	0.65

[a]Scored 6 mo after initiation.
[b]Cumulative score 1 yr after initiation.

65% of the animals bearing at least one tumor at 1 yr.

The VW Rabbit sample (Table 7) produced dose-related increases in papillomas in both male and female mice, with the maximum activity for each sex at 10 mg. At this dose there were 0.34 to 0.47 papillomas per mouse, with 24 to 42% of the animals bearing tumors. Few carcinomas were scored at 1 yr.

The Mustang sample (Table 8) was tested at doses of from 0.1 to 3 mg/mouse due to sample limitations. The response was maximal in the females at 3 mg/mouse and activity reached a plateau at 2 to 3 mg/mouse in the males. Of the female mice, 20% developed carcinomas at the highest dose tested. The responses at the higher doses were significantly greater than those of the TPA controls (Table 6).

The lack of a monotonic dose response across the complete dose range tested of the VW Rabbit and Mustang samples may indicate a toxic response to these samples. Damage to the skin epidermal cells with result in a lower tumorigenic response for these complex mixtures. This is particularly clear with the Mustang sample, where a 3-fold increase in dose (from 1 to 3 mg/mouse) resulted in no increase in tumor response.

Complete Carcinogenesis

Four agents were examined for their ability to act as complete carcinogens in the SENCAR mouse skin system: B(a)P, coke oven main extract, roofing tar extract, and Nissan extract. Weekly applications of 50.5 μg B(a)P produced a carcinoma incidence of greater than 93%, with almost one carcinoma per mouse (Table 9). Higher doses did not increase the tumor multiplicity. No carcinomas were observed in the control animals (Table 9).

The coke oven main sample also produced a strong complete carcinogen response in both male and

Table 7. Tumors observed following administration of roofing tar extract, Nissan extract, and VW Rabbit extract to SENCAR mice in the tumor initiation protocol.

Sample	Dose, μg/mouse	Sex	Mice surviving	Mice with papillomas, %[a]	Mean papillomas per mouse[a]	Mice with carcinomas, %[b]	Mean carcinomas per mouse[b]
Roofing tar	100	M	40	10	0.13	5	0.05
	100	F	39	15	0.21	10	0.10
	500	M	40	28	0.35	10	0.10
	500	F	39	13	0.15	18	0.18
	1000	M	39	38	0.41	5	0.05
	1000	F	40	45	0.80	15	0.15
	2000	M	39	36	0.62	13	0.13
	2000	F	38	37	0.45	15	0.15
	10000	M	39	100	6.4	23	0.25
	10000	F	40	95	5.7	48	0.48
Nissan	100	M	37	0	0	0	0
	100	F	39	3	0.03	5	0.05
	500	M	38	26	0.34	13	0.13
	500	F	39	23	0.39	10	0.10
	1000	M	40	33	0.38	20	0.20
	1000	F	38	39	0.53	13	0.13
	2000	M	35	66	1.1	13	0.13
	2000	F	40	58	1.6	15	0.15
	10000	M	38	89	5.5	36	0.36
	10000	F	38	97	5.7	31	0.31
VW Rabbit	100	M	40	18	0.18	0	0
	100	F	37	14	0.14	0	0
	500	M	37	14	0.14	0	0
	500	F	40	5	0.05	0	0
	1000	M	38	21	0.21	3	0.03
	1000	F	39	18	0.26	3	0.03
	2000	M	38	21	0.24	5	0.05
	2000	F	35	14	0.17	6	0.06
	10000	M	38	24	0.34	5	0.05
	10000	F	38	42	0.47	10	0.10

[a]Scored 6 mo after initiation.
[b]Cumulative score 1 yr after initiation.

Table 8. Tumors observed following administration of Mustang extract to SENCAR mice in the tumor initiation protocol.

Dose, μg/mouse	Sex	Mice surviving	Mice with papillomas, %[a]	Mean papillomas per mouse[a]	Mice with carcinomas, %[b]	Mean carcinomas per mouse[b]
100	M	39	5	0.05	5	0.05
100	F	39	13	0.23	13	0.13
500	M	39	13	0.15	0	0
500	F	38	18	0.24	10	0.10
1000	M	40	18	0.18	5	0.05
1000	F	40	10	0.13	10	0.10
2000	M	37	22	0.24	15	0.15
2000	F	39	21	0.23	13	0.13
3000	M	34	18	0.24	5	0.05
3000	F	40	23	0.28	20	0.20

[a]Scored 6 mo after initiation.
[b]Cumulative score 1 yr after initiation.

female mice (Table 10). Male mice seemed to be more sensitive: 98% of the males bore approximately one carcinoma, while only 75% of the females responded. The roofing tar sample produced a significant response only at the highest dose applied (4 mg/mouse/week), with 25 to 28% of the mice bearing tumors.

The Nissan sample was essentially inactive as a

Table 9. Tumors observed following administration of B(a)P to SENCAR mice in the complete carcinogenesis protocol.

Dose, μg/ mouse/week	Sex	Mice with carcinomas, %[a]	Mean carcinomas per mouse[a]
0	M	0	0
0	F	0	0
12.6	M	10	0.10
12.6	F	8	0.08
25.2	M	63	0.63
25.2	F	43	0.43
50.5	M	93	0.93
50.5	F	98	0.98
101	M	80	0.83
101	F	90	0.98
202	M	80	0.80
202	F	93	0.98

[a]Cumulative score after 1 yr.

complete carcinogen at the doses applied (Table 10).

Tumor Promotion

The coke oven main and roofing tar samples were applied weekly to mice previously initiated with a single dose of 50.5 μg B(a)P. The coke oven main sample was 1/1000 as active as TPA (Table 11). The roofing tar was also active as a tumor promoter and produced a dose-related effect up to the highest dose applied. Mice treated with only a single dose of B(a)P produced no tumors.

Quantitative Analysis

The data were subjected to computer modeling and statistical procedures specifically designed for

Table 10. Tumors observed following administration of coke oven main extract, roofing tar extract, and Nissan extract to SENCAR mice in the complete carcinogenesis protocol.

Dose, μg/ mouse/week	Sex	Mice with carcinomas, %[a]			Mean carcinomas per mouse[a]		
		Coke oven main	Roofing tar	Nissan	Coke oven main	Roofing tar	Nissan
100	M	5	0	0	0.05	0	0
100	F	5	0	0	0.05	0	0
500	M	36	0	0	0.36	0	0
500	F	30	0	0	0.30	0	0
1000	M	48	3	0	0.55	0.03	0
1000	F	60	0	0	0.60	0	0
2000	M	82	3	0	1.00	0.03	0
2000	F	78	8	0	0.78	0.08	0
4000	M	98	25	3	0.98	0.28	0.03
4000	F	75	28	5	0.85	0.28	0.05

[a]Cumulative score after 1 yr.

Table 11. Tumors observed following administration of coke oven main extract and roofing tar extract to SENCAR mice in the tumor promotion protocol.

Dose, μg/mouse/week	Sex	Mice with papillomas, %[a]		Mean papillomas per mouse[a]	
		Coke oven main	Roofing tar	Coke oven main	Roofing tar
0[b]	M	0	0	0	0
0[b]	F	0	0	0	0
100[c]	M	3	0	0.02	0
100[c]	F	10	0	0.10	0
500	M	26	5	0.44	0.05
500	F	38	0	0.83	0
1000	M	53	20	1.2	0.27
1000	F	68	16	1.2	0.36
2000	M	84	23	2.5	0.32
2000	F	85	13	3.1	0.15
4000[d]	M	100	55	8.2	1.2
4000[d]	F	100	30	8.8	0.6
TPA, 4 μg[e]	M	86	100	3.1	5.2
TPA, 4 μg[e]	F	97	100	5.9	7.2

[a]Scored at 34 weeks.
[b]Mice initiated with 50.5 μg B(a)P and subsequently treated weekly with acetone.
[c]Mice initiated with 50.5 μg B(a)P and subsequently treated weekly with coke oven main or roofing tar extract.
[d]Mice initiated with 50.5 μg B(a)P and subsequently treated twice weekly with 2 mg coke oven main or roofing tar extract.
[e]Mice initiated with 50.5 μg B(a)P and subsequently treated twice weekly with 2 μg TPA.

the analysis of tumor multiplicity and incidence data using an interactive computer terminal graphics system. Tumor incidence data were applied to a probit model with background correction. From the model, the dose that would elicit tumors in 50% of the surviving animals in excess of the control rate (TD_{50}) was estimated. An example of this probit analysis (papilloma data, Nissan sample) is shown in Figure 1. The value of TD_{50} and the associated 95% confidence intervals calculated from the fitted parameters are shown as well as the raw data. In Table 12, such estimates are presented only if the observed data adequately fit the calculated model. The TD_{50} values ranged from 0.0036 to 2.1 mg; B(a)P was the most active sample and roofing tar extract was the least active sample. The VW Rabbit and Mustang experiments did not result in any tumor incidences of 50% or more, and calculations were not made for these samples. Within the 95% confidence bands, there were no sex differences for any sample.

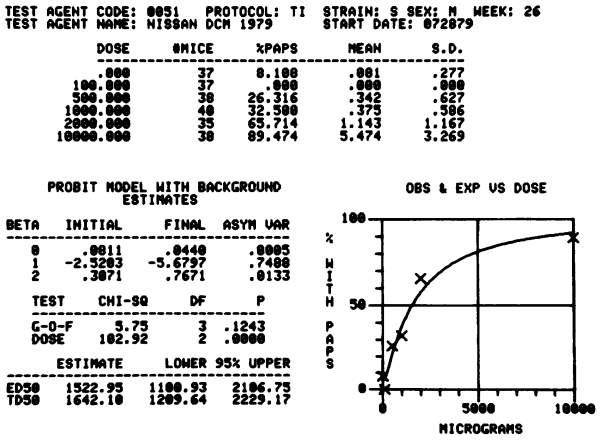

FIGURE 1. Computer-generated Probit analysis of tumor incidence. Computer graphic output of sample identification; raw data; initial and final parameter estimates; (---) TD_{50} estimates with 95% confidence intervals; (——) plot of expected response (solid line) and (x) observed responses.

Tumor multiplicity data were analyzed by a nonlinear Poisson model with a background correction term. The data were fitted to the model and the model parameters were estimated; from these values the number of papillomas per mouse at 1 mg and the associated 95 % confidence intervals were estimated. An example of the graphics display is shown in Figure 2. The calculated tumor multiplicity data ranged from 0.17 to 2.2 papillomas per mouse, a 10-fold range (Table 12). Data from the B(a)P experiments could not be used, since they were not obtained in the 1-mg dose range, but linear extrapolation from the 0.1-mg dose used yields an estimate of 79 to 100 papillomas per mouse. No sex differences were evident from any of these calculations.

The carcinoma incidence data (tumor initiation and complete carcinogenesis protocols) were applied to the probit model. Estimates for B(a)P and the coke oven main sample are compared in Table 13. With the coke oven main sample, the estimated values of TD_{50} are similar for both the tumor initiation (single application) and complete carcinogenesis (weekly application) protocols. However, B(a)P as a tumor initiator was much less effective in

Table 12. Nonlinear Poisson and probit model estimates based on papilloma incidence at 6 mo in SENCAR mice in the tumor initiation protocol.

		Nonlinear Poisson		Probit	
Sample	Sex	Papillomas/mouse at 1 mg	95% confidence intervals	Dose for 50% papilloma incidence (TD_{50}), mg	95% confidence intervals
B(a)P	M	NC[a]		0.0036	0.0021 - 0.0062
	F	NC[a]		0.0091	0.0057 - 0.015
Coke oven main	M	ND[b]		0.079	0.027 - 0.23
	F	ND[b]		0.19	0.14 - 0.28
Topside coke oven	M	2.2[c]	2.00–2.40	0.30	0.22 - 0.40
	F	2.0[c]	1.90–2.20	0.42	0.31 - 0.58
Nissan	M	0.49[c]	0.38–0.63	1.60	1.2 - 2.2
	F	0.68[c]	0.57–0.79	1.50	1.1 - 1.9
Roofing tar	M	0.38[c]	0.30–0.49	1.8	1.2 - 2.7
	F	0.44[c]	0.35–0.55	2.1	1.5 - 2.8
VW Rabbit	M	0.21	0.14–0.30	NC[d]	
	F	0.17	0.11–0.25	NC[d]	
Mustang	M	0.17	0.12–0.24	NC[d]	

[a]Not calculated since data were obtained at a lower dose range.
[b]Not determined.
[c]The distribution of tumors at some dose levels was not Poisson, as the variances exceeded the means.
[d]Not calculated since tumor incidence did not exceed 50%.

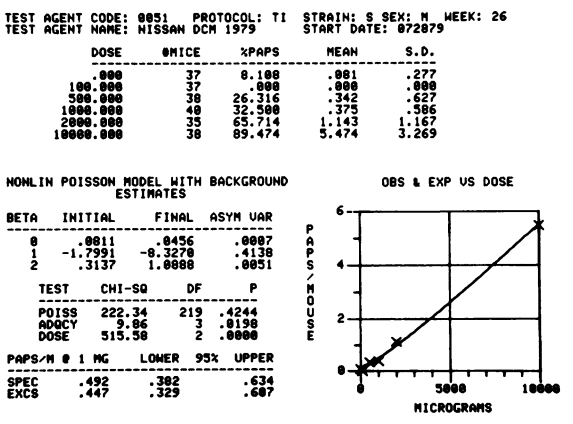

FIGURE 2. Computer-generated nonlinear Poisson analysis of tumor multiplicity data. Computer graphic output of sample identification; raw data; initial and final parameter estimates; estimated papillomas/mouse at 1 mg with 95% confidence intervals; (——) plot of expected response and (x) observed responses.

producing carcinomas than was the B(a)P as a complete carcinogen. When the comparison was made on the total amount applied to the mice, the tumor initiation protocol was more sensitive than the complete carcinogenesis protocol for both agents.

Discussion

The SENCAR mouse, specifically bred for increased sensitivity to two-stage (initiation-promotion) carcinogenesis, has demonstrated its responsivity to carcinogens (*10,38,39*). Of mouse strains and stocks examined, the SENCAR mouse was most sensitive to the initiating and complete carcinogenic effects of B(a)P and DMBA (*9,10*).

These studies of the effects of complex mixtures and B(a)P on SENCAR mouse skin are the most extensive to date, and the results confirm the applicability of this mouse strain to the analysis of complex mixtures.

The B(a)P, coke oven main, and roofing tar samples were positive in both sexes as tumor initiators, producing both papillomas and carcinomas, and were also positive as tumor promoters and complete carcinogens. In general, those agents which produced a strong tumor-initiation papilloma response also produced carcinomas in the same animals when scored at 1 yr. The two diesel engine samples were positive as tumor initiators, as was the unleaded gasoline engine sample. Additional work with emission extracts from other diesel vehicles and engines has demonstrated their activity as mouse skin tumor initiators (*6,31,32*).

Of the strong tumor initiators (B(a)P, coke oven main, roofing tar, Nissan), only Nissan was not also a complete carcinogen at the doses tested. Kotin et al. (*26*) did obtain tumors from diesel particulate extract on strain A mice; this indicates that higher doses might induce tumors in SENCAR mice. However, on a weight basis, coke oven main and roofing tar are much more active than Nissan as complete carcinogens.

Of the strong tumor initiators (B(a)P, coke oven main, roofing tar, Nissan), only Nissan seemed not to possess tumor-promoting activity. The presumed lack of tumor-promoting activity in the Nissan sample is probably as function of the composition of the Nissan mixture. The skin tumorigenesis results results indicate that the coke oven main sample was a stronger tumor promoter than the roofing tar sample. Chemical fractionation and mutagenesis studies of the diesel, roofing tar, and coke oven main samples show that both the chemical composition of the fractions and their capacities to induce genetic alteration are significantly different (J. Lewtas, personal communication).

Chemicals which seem to only be tumor initiators on mouse skin may also possess complete carcinogenic activity when administered by other routes to mice and rats. Urethane (*40*) and triethylenemelamine (*41*) are both probably pure mouse skin tumor initiators: repeated applications of these agents on mouse skin do not yield tumors. However, urethane administered intraperitoneally, subcutaneously, or orally

Table 13. Comparison of probit model estimates based on cumulative carcinoma incidence at 1 yr in SENCAR mice in the tumor initiation and complete carcinogenesis protocols.

		Tumor inititation[a]		Complete carcinogenesis[b]	
Sample	Sex	Dose for 50% carcinoma (TD_{50}), mg	95% confidence intervals	Dose for 50% carcinoma (TD_{50}), mg	95% confidence intervals
B(a)P	M	> 0.10	–	0.025	0.017–0.037
	F	> 0.10	–	0.029	0.023–0.036
Coke oven main	M	1.9	0.83–4.4	0.78	0.62 –0.99
	F	1.5	0.57–3.7	0.93	0.69 –1.2

[a]Estimates based on single dose administration.
[b]Estimates based on weekly dose administration.

to mice produces a variety of lesions, including lung, liver, and lymphoid tumors. Urethane administered orally to rats also produces multiple tumors (*40*). Triethylenemelamine produces lung tumors in mice after intraperitoneal injection and muscle tumors in rats after subcutaneous injection (*41*).

It is compelling to postulate that the B(a)P in these complex mixtures accounts for their tumorigenic activity, since mouse skin is exquisitely sensitive to this agent. The results presented here reveal that a single application of less than 5 μg B(a)P as a tumor initiator yields a 50% tumor incidence. However, the relationship between the B(a)P content of each mixture and the papilloma response for each mixture is not linear (Fig. 3). Probably none of the activity of the coke oven sample can be explained by B(a)P content, as the B(a)P-induced tumor response at the B(a)P level in the coke oven sample is quite small. Even the B(a)P level in the Nissan sample (11 μg/10 mg extract) can only account for 20 to 30% of the papilloma response elicited by the Nissan sample. This conclusion has also been stated by Kotin et al. (*20*) and Wynder and Hoffmann (*21*), based on the analysis of other complex mixtures. Other components of the mixtures may play an important role in their tumorigenic activities. For example, β-propiolactone, a mouse skin tumor initiator, has been identified in diesel exhaust particulate extracts (*42*).

Quantitative methods for the analysis of tumor data are many and employ tumor incidence, tumor multiplicity, and tumor latency data. Statistical methods have been employed using Poisson and other distribution assumptions, as well as both uni-and multivariate analytical approaches (*43–46*). We chose to apply a nonlinear Poisson model to the papilloma incidence data. This model assumes a Poisson distribution of tumors, that tumor multiplicity is related to dose, that the response may be nonlinear, and that there is a background response. Results from the nonlinear Poisson model suggest the following ranking: topside coke oven > Nissan ≥ roofing tar ≥ VW Rabbit = Mustang. The values calculated are only estimates and in some cases the Poisson assumption made to derive the estimates is only partially fulfilled.

A probit model was chosen to evaluate the tumor incidence data. The probit model examines animals with tumors (regardless of multiplicity) and animals without tumors. Results from the probit analysis suggest the following ranking: B(a)P > coke oven main ≥ topside coke oven > Nissan = roofing tar. These are not the only models that can be applied to these data, and although they appear effective in this case, more effort is being placed in improving statistical and modeling techniques.

In addition to the tumorigenesis studies described above, detailed gross and histopathological analyses of selected animals have been undertaken. Results from these detailed pathological studies on the formation of internal tumors and on the appearance of tumors with longer latency periods will be presented at a later date.

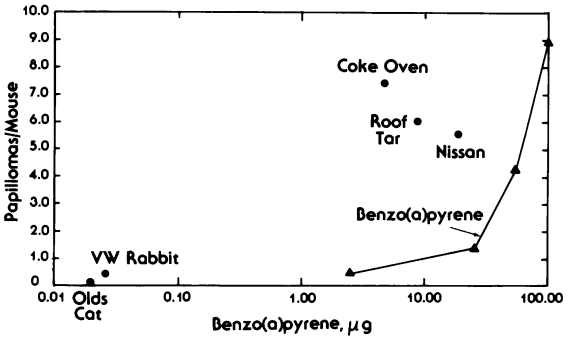

FIGURE 3. Skin tumor initiating activities of pure B(a)P and of six complex mixtures. The tumor and B(a)P concentration data are from the results obtained at the 10-mg dose of complex mixture. CAT is a diesel sample not described in this paper.

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