Pulmonary Carcinogenesis in Rats Given Implants of Cigarette Smoke Condensate in Beeswax Pellets [1,2]

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ABSTRACT—Lungs of inbred OM/NCR and outbred Sprague-Dawley rats were given implants, through a thoracotomy, of pellets of cigarette smoke condensate (CSC) suspended in a beeswax-tricaprylin vehicle. The pellets slowly released material into the surrounding parenchyma, which resulted in a dose-related increased incidence of lung cancer, predominantly invasive and metastasizing epidermoid carcinoma. A 42% prevalence of pulmonary carcinoma was present in the highest dosage group, which received 67 mg CSC, exposing approximately 1.65 cm² bronchiolar epithelium. Squamous metaplasia associated with the implanted site preceded the appearance of the carcinomas and was more severe, with the larger pellets having more concentrated CSC. No difference was observed in incidence of pulmonary carcinomas with the use of CSC containing high or low concentrations of nicotine. The potential values of this bioassay system were discussed.—J Natl Cancer Inst 61: 905-910, 1978.

Standardization of bioassay techniques for evaluating the carcinogenic effects of cigarette smoke or CSC on the respiratory system is necessary. These efforts are for the purpose of producing low-toxicity cigarettes for the prevention of diseases related to smoking (*1, 2*). Some of the promising bioassay systems under evaluation include intratracheal instillations of CSC in hamsters (*3, 4*), inhalation of cigarette smoke by rats (*5-9*), inhalation of cigarette smoke by dogs through tracheostomies (*10, 11*), and inhalation of cigarette smoke by monkeys (*12*).

Much work has been performed to develop a simple experimental model for lung cancer induction with CSC and its various fractions (*13-24*). One of the more promising procedures used by Stanton et al. (*22*) involves the mixing of the potential carcinogen with a vehicle composed of equal parts of beeswax and tricaprylin and then the injecting of the pellet directly into the lung. Carcinogens leaching from the pellet provide a chronic exposure to the lung tissue. The lung pellet procedure has been used for the induction of lung tumors in rats with 3-methylcholanthrene (*25*) and CSC (*22*).

The lung pellet bioassay technique offers some advantages over other bioassay procedures. Daily inhalation exposure of animals to smoke is very expensive and does not allow the fractionation of different carcinogens from the cigarette smoke. Bioassay of CSC by skin painting does not involve the respiratory tract, which is the tissue of concern for the human smoker. In addition, skin painting and intratracheal injections also require repeated exposures, which involve high labor costs.

This report describes studies done to standardize beeswax-tricaprylin lung pellet implantation as an effective bioassay technique to evaluate the pulmonary carcinogenicity of CSC. Inasmuch as nicotine and tars are the common variables in cigarettes, we studied CSC with HCN and LCN in three different-size pellets, each pellet size having three concentrations of CSC. The rats used were: inbred OM/NCR and outbred Sprague-Dawley.

MATERIALS AND METHODS

The lung pellet material was prepared from vials of CSC obtained from Dr. A. R. Patel, Meloy Laboratories, Inc., Springfield, Virginia. The CSC was prepared from cigarettes made with a research blend of tobaccos, either HCN or LCN, with 10.33 or 4.33%, respectively, nicotine in CSC. The cigarettes were smoked on an automatic machine, with the use of a 2-second puff of 35-ml volume, once a minute, to a butt length of not less than 20 mm. The smoke was trapped at −70° C, dissolved in acetone, and mixed with the beeswax-tricaprylin vehicle after evaporation of the acetone. The beeswax-tricaprylin vehicle was prepared from a single lot of white beeswax (Fischer Scientific Co., Fair Lawn, N.J.) and reagent grade tricaprylin (trioctonoin; Eastman Organic Chemicals, Rochester, N.Y.) that was heated together in equal weights in a water bath at 76° C until a solution was obtained. The different CSC were mixed with the vehicle at 76° C to make the three needed concentrations (1:50, 1:10, and 1:2) of each CSC.

OM/NCR rats were supplied by Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts, and Sprague-Dawley rats were purchased from Hilltop Laboratory Animals, Inc., Chatsworth, California. The Sprague-Dawley rats were housed in one room, and the OM/NCR rats were housed in two additional rooms; all were maintained at 21±1° C and 50±20% relative humidity on a 12-hour light-dark cycle. The rats were housed 2 to an 80-square-inch wire-bottom cage, with

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Abbreviations used: CSC=cigarette smoke condensate(s); df=degree(s) of freedom; H & E=hematoxylin and eosin; HCN=high concentration(s) of nicotine; LCN=low concentration(s) of nicotine; SNOP=Standardized Nomenclature of Pathology.

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40 cages to an automatically flushing rack. They were fed ad libitum Wayne Lab-Blox Laboratory rat diet. No significant incidence of pneumonia or other infectious disease was observed clinically or at necropsy. Mammary tumors were surgically removed to extend the life-span of the rats.

The lung pellets were implanted through a left intercostal thoracotomy while the rat was under ether anesthesia. The pellet material was warmed to 76° C and mixed with a vibrating machine before the pellet was injected into the left lung through a short 20-gauge needle attached to a 0.25-ml glass syringe. A total of 1,768 3- to 5-month-old female OM/NCR rats 1) were given implants of 0.05, 0.10, or 0.20 ml of the three CSC concentrations containing one of the two different nicotine levels, 2) served as vehicle controls, or 3) were untreated controls. The rats were given implants over a 12-week period with the use of a randomized block design with all treatments represented in each of the five blocks. No effects related to treatment in different blocks were observed. The 6.7% of the rats that were given implants and that died during the first 30 days were replaced with additional rats. An additional 250 Sprague-Dawley rats were given implants of 0.10-ml lung pellets of CSC with HCN or LCN, were treated with vehicle alone, or served as untreated controls. A total of 2,018 rats was used. the large number was necessary because of the great number of variables studied.

Groups of 10 OM/NCR rats killed 10, 30, 60, or 90 weeks after implantation with each concentration, pellet size, and CSC were necropsied. The surviving OM/NCR and Sprague-Dawley rats were killed 120 weeks after implantation. Histopathologic examination was made of routinely processed H & E-stained paraffin sections of left lung through the implant site, of the right diaphragmatic lobe, and of all gross lesions. Histopathologic diagnoses were SNOP coded with the SNOP system slightly modified for adaptation to rats, and the diagnoses were entered into a CYBER computer.

Statistical analysis included the use of the Kaplan-Meier representation (*26*) of the cumulative survivorship function to compute the percent survival at necropsy. By this method, the cumulative survivorship could be adjusted for scheduled killings. By a similar technique the prevalence of a lesion, defined as the probability that an animal will have the lesion present at a given age, could be estimated. Methods developed for nonlethal disease (*27*) were used for analyses of treatment effects on the prevalence of various lesions, with all of the analyses age adjusted. We tested treatment effects by arranging incidence data in a series of 2×K contingency tables (K=number of treatment levels), one for each combination of factors that might influence the results. A summary χ^2 for each effect was computed by means of the Mantel-Haenszel procedure (*28*). Estimates of relative risk (the risk of developing a lesion at a given factor level relative to the risk for controls), which were suggested by Mantel and Haenszel, were also computed. The scoring procedure suggested by Mantel (*29*) for obtaining 1 df χ^2 agreed perfectly with the Mantel-Haenszel procedure.

RESULTS

Pulmonary carcinomas developed around CSC lu pellets in OM/NCR rats, with an increased incide in rats with more concentrated CSC (table 1). significant effect of CSC concentration was obser (table 2), but the increasing pellet size (table 3) did affect the incidence of lung cancer. Percent morta was higher in the groups of rats receiving the hig concentration of CSC (text-fig. 1). There was difference in the incidence of pulmonary carcino between the CSC with HCN or LCN (χ^2=0.004; 3 with 34 pulmonary carcinomas developing from with HCN and CSC with LCN. The first pulmo

TABLE 1.—*Prevalence of pulmonary carcinomas in rats given pellet implants*

Lung pellet			Pulmonary carcinoma					Squamous metaplasia prevalence
CSC, mg	Size, ml	CSC concn	No. of rats*	No. HCN with CSC	No. LCN with CSC	Total No.	Prevalence†	
None			120	0	0	0	—	0.017
0	0.05	0	188	0	0	0	—	0
0	0.10	0	188	0	0	0	—	0.032
0	0.20	0	192	0	0	0	—	0.083
1	0.05	1:50	120	1	0	1	0.018	—
2	0.10	1:50	120	0	0	0	—	0.141
4	0.20	1:50	120	2	2	4	0.124	0.378
5	0.05	1:10	120	1	0	1	0.044	0.157
10	0.10	1:10	120	2	3	5	0.098	0.398
17	0.05	1:2	120	5	3	8	0.217	0.412
20	0.20	1:10	120	7	5	12	0.200	0.595
33	0.10	1:2	120	5	9	14	0.259	0.642
67	0.20	1:2	120	11	12	23	0.417	0.736
Total			1,768	34	34	68		

* Includes rats periodically killed and those that died before tumors developed from both CSC with HCN and CSC with LCN.
† Probability that rat will have a lesion 840 days post exposure.

TABLE 2 —*Relative risks for CSC concentration in rats given pellet implants*

Lesion type	Relative risks at concentrations:[a]				χ^{2b}
	0	1:50	1:10	1:2	
Pulmonary carcinomas	1.0	3.57	8.33	16.67	70.082[c]
Malignant, other than pulmonary carcinomas	1.0	1.09	1.19	0.83	2.959[d]
All malignant tumors	1.0	1.01	1.43	2.70	29.253[c]
All malignant and benign tumors	1.0	1.05	0.99	0.97	0.474[d]
Squamous metaplasia	1.0	4.00	9.09	12.50	227.766[c]

[a] CSC/vehicle.
[b] 3 df.
[c] Highly significant.
[d] Not significant.

carcinoma was seen 385 days post exposure, with an average of 649±128 days post exposure, without relationship to pellet size, pellet concentration, or nicotine content.

Pulmonary carcinomas in the OM/NCR rats consisted of 63 epidermoid carcinomas, 4 undifferentiated carcinomas, and 1 adenocarcinoma. The epidermoid carcinomas replaced large areas of the left lung (fig. 1) and were composed of irregularly arranged sheets and lobules of keratinizing stratified squamous epithelium (fig. 2). The stratified epithelium surrounded the pellet cavity and was generally associated with large areas of necrosis in the center, with polymorphonuclear cellular infiltration associated with necrotic debris. The epidermoid carcinomas extended into the surrounding parenchyma or invaded pleura in all rats, invaded blood vessels in 56% of the rats, and metastasized to the mediastinal lymph nodes (fig. 4), right lung, kidney, rib, and/or vertebrae in 51% of the rats. The anaplastic carcinomas (1 from a rat given a 1-mg HN CSC pellet, 2 from rats given 4-mg LN CSC pellets, and 1 from a rat given a 17-mg LN CSC pellet) were composed of an irregular proliferation of spindle-to-round-shaped cells that invaded blood vessels or metastasized in 3 of the 4 rats with this neoplasm. The adenocarcinoma (from a rat given a 5-mg HN CSC pellet) was composed of an irregular proliferation of glandular epithelium surrounding the pellet site and metastasized to the mediastinum, right lung, and kidney.

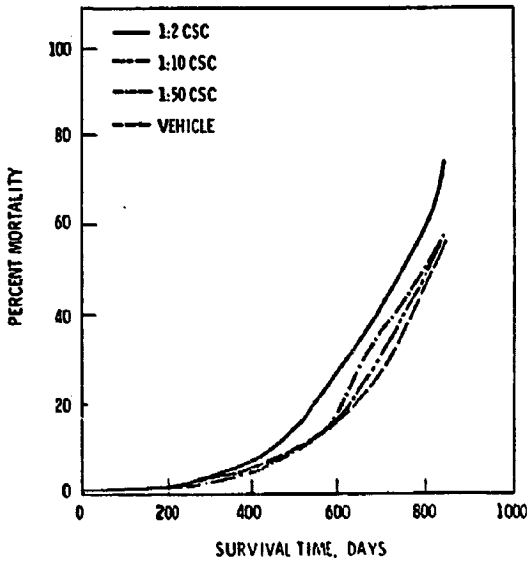
TABLE 3 —*Relative risks for pellet size in rats given implants*

Lesion type	Relative risks at pellet size:				χ^{2a}
	0 ml	0.05 ml	0.1 ml	0.2 ml	
Pulmonary carcinoma	1.0	1.0	1.0	1.0	1.582[b]
Malignant, other than pulmonary carcinomas	1.0	0.78	0.76	0.54	4.469[b]
All malignant tumors	1.0	2.43	1.36	1.01	3.617[b]
All malignant and benign tumors	1.0	0.96	0.90	0.84	1.693[b]
Squamous metaplasia	1.0	1.0	1.25	2.86	21.360[c]

[a] 3 df.
[b] Not significant.
[c] Highly significant.

The implantation of CSC pellets tended to stimulate the proliferation of bronchiolar epithelium along the inner lining of the cavity formed by the pellet. Squamous metaplasia was graded as follows: very slight, the cavity having only foci of stratified epithelium; slight, a larger portion of the cavity lined with stratified epithelium that may have keratinized; moderate, stratified epithelium nearly completely lining the cavity and having small extensions into the surrounding fibrous capsule; marked, prominent extensions of the stratified epithelium into surrounding fibrous capsule; extreme, pseudoepitheliomatous hyperplasia and metaplasia, difficult to distinguish from carcinoma in situ. The pellet cavities with more severe grades of squamous metaplasia also tended to have more prominent fibrous capsules and mixtures of polymorphonuclear cells and necrotic debris mixed with keratin. Squamous metaplasia surrounding the pellet site in the OM/NCR rats increased in severity with the larger pellets that contained more concentrated CSC (table 1). There was a highly significant pellet size effect (χ^2=21.360; 3 df) and concentration effect (χ^2=227.766; 3 df) on development of this lesion (tables 2, 3). The average severity of squamous metaplasia decreased with time as the incidence of pulmonary carcinoma increased, which suggests that the pulmonary carcinomas replaced the more severe forms of squamous metaplasia. However, a clear morphologic distinction between squamous metaplasia and carcinoma, with few transition stages, was observed.

Pulmonary carcinomas and squamous metaplasia developed in a similar fashion in Sprague-Dawley rats. Epidermoid carcinoma of the lung, which invaded



TEXT FIGURE 1 —Mortality of OM/NCR rats given implants of pellets in their lungs

blood vessels and metastasized intrathoracically, occurred around the pellets of 2 rats from the group of CSC with HCN and 2 rats from the group of CSC with LCN (50 rats in each group). In addition, an undifferentiated carcinoma associated with a lung pellet of LCN and an adenocarcinoma associated with a pellet of HCN were seen. The grade of squamous metaplasia in the lung surrounding the pellet sites averaged 2.1 for the CSC with HCN and 1.5 for the CSC with LCN.

The vehicle pellet alone elicited a foreign-body-type granulomatous reaction including macrophages, multinucleated giant cells, and a thin fibrous capsule. The fibrous capsule was thinner than that associated with the pellets containing CSC, and very little squamous metaplasia or associated polymorphonuclear cellular infiltration was exhibited. The left lung of 1 vehicle control contained a sarcoma composed of large, polyhedral to slightly spindle-shaped cells, with an occasional multinucleated cell and numerous mitotic figures. The left thoracic walls of 2 additional rats given implants of the vehicle contained sarcomas that were possibly related to the vehicle or surgical treatment. Occasionally, lung abscesses and acute bronchopneumonia were associated with the pellets, but these were present in both vehicle and CSC-treated rats and were probably related to the thoracotomy. No other lesions related to either the vehicle or CSC were found.

DISCUSSION

This work confirms earlier studies which reported that pulmonary carcinomas can readily be induced in the lungs of rats given implants of CSC in a vehicle which allows slow release of a carcinogen (*14*, *22*). In addition, we have shown a clear dose relationship in OM/NCR rats, with the more concentrated CSC inducing a greater incidence of carcinomas, and no difference in the incidence of pulmonary carcinomas between HCN or LCN. The induced carcinomas, usually epidermoid in type, invaded blood vessels and frequently metastasized.

Inasmuch as CSC consists of various chemicals at varying concentrations (*30*) and the release of chemicals from a binding matrix is a function of the diffusibility and solubility of each component of the mixture in the matrix (*31*), it is expected that the various chemical components of CSC would have dissimilar diffusion rates and would provide varying chronic exposures to lung tissue. In effect, the beeswax-tricaprylin matrix would act as a selective filter, which provides a time-dependent fractionation of the carcinogens (*32*).

Dosimetry studies (*33*) demonstrated that the in vitro release profiles from the vehicle for nicotine, 3-methylcholanthrene, and phenol are related to the chemical nature of the material. The order of polarity and rate of release for the three compounds was nicotine> phenol>3-methylcholanthrene. We also demonstrated the large variation in release properties among these compounds by comparing the percent of starting component lost from the pellets with their release rates. The 3-methylcholanthrene had a nearly constant release rate, whereas the more polar phenol and nicotine were rapidly leached during the initial phase but slowed as the equilibrium solubility was approached. The release rate of these materials was concentration- and geometry-dependent; however, the chemical nature of the release compounds dominated the concentration, and geometric factors governed the overall release profile. The more rapid release of nicotine indicates that the beeswax pellet may be inadequate to demonstrate the carcinogenic potential of nicotine alone.

The most striking aspect of these studies with lung pellets was the high incidence of pulmonary carcinoma with low concentrations of CSC. In this study, a 42% prevalence of pulmonary carcinoma was produced with 67 mg CSC, the approximate equivalent of only three high-tar cigarettes; and pulmonary carcinoma was produced with concentrations as low as 1 mg CSC. The dose response for CSC, not adjusted for any specific carcinogen, seems compatible with a nonthreshold linear model similar to that proposed for radiation-induced lung tumors (*34*). Assuming the 0.2-ml pellet formed a sphere with 1.65 cm^2 bronchiolar epithelium exposed to CSC, we can calculate a prevalence of pulmonary carcinoma in the rat of 0.0153/mg CSC/cm^2 of bronchiolar epithelium. The relative risks could be calculated for different tobaccos, tobacco fractions, tobacco substitutes, filters, and their combinations. The lung pellet technique may also provide an animal model for evaluations of cocarcinogenic activity, immunologic modulations, or studies of the therapy of lung tumors produced with a tobacco product, the common cause of lung cancer in man.

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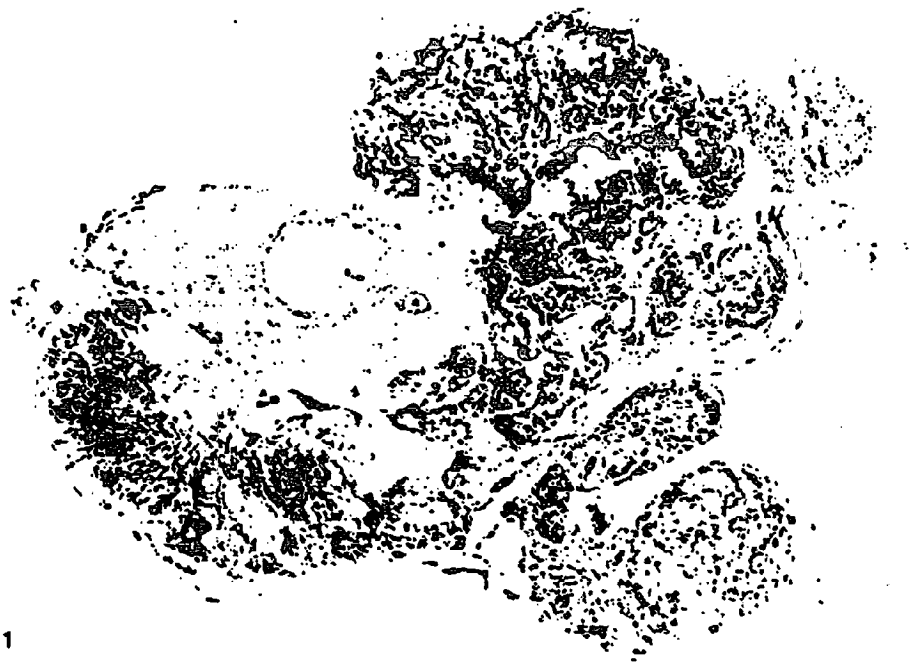

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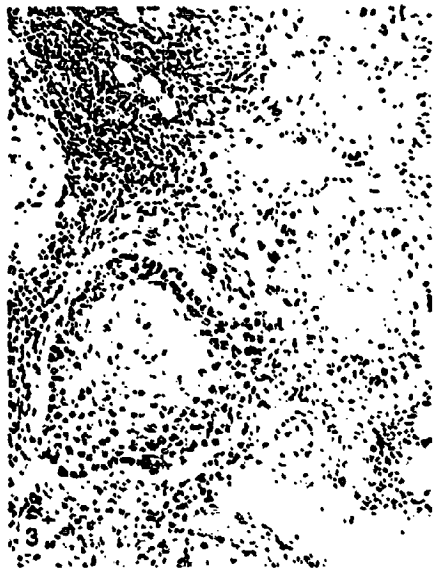

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FIGURE 1 —Epidermoid carcinoma surrounding pellet site in lung of rat #1173. Trichrome × 4
FIGURE 2 —Higher magnification of epidermoid carcinoma in lung of rat #1173 H & E × 125
FIGURE 3 —Metastatic epidermoid carcinoma in mediastinal lymph node of rat #1173 H & E × 125