CHAPTER D

In Vivo Bioassays for Carcinogenicity

Dietrich Hoffmann, Ph.D.
American Health Foundation
1 Dana Road
Valhalla, New York 10595-1599

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Chapter Contents

Introduction

In the 1989 Report on the Health Consequences of Smoking, the Surgeon General of the U.S. Public Health Service, in evaluating the health effects of smoking, concluded that "smoking is responsible for more than one of every six deaths in the United States" (1). Cigarette smoking alone increases the risk for coronary heart disease from 23/1000 to 54/1000, together with hypercholesterolemia cigarette smoking raises the risk to 103/1000, and together with high blood pressure, to 92/1000. All three risk factors, smoking, hypercholesterolemia, and high blood pressure, synergistically increase the risk for coronary heart disease to 189/1000 (1).

Cigarette smoking is also a major risk factor for chronic obstructive pulmonary disease, and here primarily for emphysema and chronic bronchitis. Eighty to 90% of the morbidity from chronic obstructive lung disease in the United States has been attributed to cigarette smoking (2).

Smoking of cigarettes is causally associated with cancer of the lung, larynx, oral cavity, esophagus, pancreas, renal pelvis and urinary bladder and is also linked with an increased risk for cancer of the nasal cavity, liver and the uterine cervix and possibly, related to cancer of the stomach (1). In 1992, the National Cancer Institute published a population-based case-control study that provided evidence for the association of cigarette smoking with several types of leukemia and thereby confirmed earlier prospective and case control studies (3). The National Cancer Institute estimated that in 1991 of the 514,000 cancer deaths at the seven sites causally associated with cigarette smoking, 30.6% are due to smoking (4).

Chemical analyses for the major known carcinogens offer a meaningful indication of the carcinogenic potential of cigarette smoke, especially in conjunction with chemical analytical data for the smoke of cigarettes already bioassayed for carcinogenic activity. In vitro assays for genotoxicity such as the Ames test with various bacterial strains, the DNA repair assay with primary rat liver cells, and the sister chromatid exchange assay have remained inconclusive in regard to the quantitative aspects of the genotoxic potencies of cigarette smokes (5). At present, conclusive data on the carcinogenicity of the smoke of new cigarettes can only be ascertained with long-term bioassays with laboratory animals (5-7).

Three animal species are primarily utilized for bioassays of whole cigarette smoke in inhalation experiments. These are mice, rats, and Syrian golden hamsters. All of the inhalation studies have the inherent shortcoming that the animals are obligated to breathe through the nose and that their inhalation of tobacco smoke

is shallow. They do not inhale smoke through the mouth as human smokers do. Nevertheless, cigarette smoke inhalation studies with mice, rats and Syrian golden hamsters have led to the induction of significant numbers of benign and malignant tumors in the respiratory tract of these animals.

The data from cigarette smoke inhalation studies with mice have not been fully accepted since only lung adenoma and lung adenocarcinoma have been elicited and not squamous cell tumors in the lung. Furthermore, most strains of mice have a fairly high rate of spontaneous lung adenoma. The rates of both adenocarcinoma and squamous cell carcinoma of the lung have increased in cigarette smokers and the current ratio of lung adenocarcinoma to squamous cell carcinoma in male smokers is 1:2-3 (8).

As will be discussed under "Inhalation Bioassays", the critique on the data from smoke inhalation studies with mice is no longer fully justified. A large-scale inhalation study with rats using highly advanced methodology presents encouraging data (9). However, until additional long-term inhalation bioassays have been completed with this exposure system, the database is too limited to recommend this rat bioassay for routine studies.

The largest database from cigarette smoke inhalation studies stems from assays with Syrian golden hamsters. As will be discussed, these long-term inhalation studies have only in a few cases led to lung tumors; however, they have induced highly significant incidences of benign and malignant tumors in the upper respiratory tract of hamsters. The tumors occurred primarily in the larynx.

Since the early 1960's, remarkable progress has been achieved in respiratory carcinogenesis. We have become well aware of the existence of carcinogens with organ-specificity for the respiratory tract of laboratory animals, and bioassays of aerosols and volatilized chemicals have also provided considerable evidence for their potential to induce tumors in the respiratory tract of mice, rats and hamsters (10).

I. Inhalation Bioassays

Three decades ago, the Leuchtenbergers (11) reported the first extensive inhalation experiments in which mice were exposed daily to air-diluted cigarette smoke in specially designed chambers. This smoke exposure led to early histological, cytological, and cytochemical changes in the major bronchi of the mice. The smoke exposure also caused various degrees of bronchitis associated with atypical proliferation of the bronchial epithelium. The investigators observed extracellular deposition of a brown pigment in the lungs of all the mice that underwent long-term exposure to cigarette smoke aerosols. After about 12-15 months the

smoke-exposed mice began to develop lung adenoma and lung adenocarcinoma in significantly higher numbers than did the control mice. In inhalation studies with the gas phase of cigarette smoke, lung adenomas have also been observed, though to a significantly lesser extent than with the whole smoke (12). The findings of the Leuchtenbergers (11, 12) were confirmed by Otto (13) who exposed inbred albino mice to cigarette smoke daily. After at least 12 months of smoke exposure, 23 of 60 mice developed lung adenomas, while only 3 of 60 control mice were found with such tumors. One mouse in the exposed group developed a squamous cell carcinoma of the lung after 16 months (13).

Several criticisms have been voiced in regard to the induction of lung adenoma and lung adenocarcinoma in mice by exposure to cigarette smoke. Concerns include the fact that such exposures caused tumors in the peripheral lung, and not in the bronchi, and that some of the tested strains of mice had a relatively high rate of spontaneous lung adenomas. It has been observed that not only the carcinoma in the bronchi, but also the incidence of lung adenocarcinoma, has significantly increased in cigarette smokers and that such tumors are now even seen in nonsmokers who have been exposed to environmental tobacco smoke, to carcinogenic chemicals, or to radiation (1).

In the past, it was not understood how the topical application of tobacco "tar" to the skin of mice could lead to the development of lung adenoma and adenocarcinoma. Today, we are aware that tobacco smoke contains also organ-specific carcinogens such as the tobacco-specific N-nitrosamines, which can induce adenoma and adenocarcinoma in the lung upon topical application to the skin of mice (14).

A major breakthrough in inhalation assays came with the development of new smoke-inhalation devices that facilitate the exposure to diluted tobacco smoke aerosols (15-17). When 80 rats were exposed seven times daily for intermittent periods (8.4 x 30 seconds) to 10% cigarette smoke aerosol for up to 2.5 years, most animals developed hyperplastic and metaplastic changes in the nasal turbinals, larynges and tracheas. Seven of the 80 smoke-exposed F344 female rats developed tumors in the respiratory tract, including 1 adenocarcinoma and 1 squamous cell carcinoma in the lung, compared to 1 alveologenic carcinoma only in the 93 control rats (9).

In another study, rats were exposed to diluted cigarette smoke six hours a day, 5 days a week, up to 40 weeks. Subsequently, DNA from nasal, lung and liver tissues was extracted and analyzed by the ^{32}P-postlabeling procedure. In the nasal mucosa at least four new DNA-adducts were seen; the amount of these adducts increased with the duration of smoke exposure. In the lung, one new DNA-adduct was detected; it also accumulated as smoke exposure progressed. It appears that the DNA adducts were aromatic and/or

hydrophobic in nature (18). In a similar assay, rats were exposed for 22 days to diluted cigarette smoke. In the nose-only intermittent exposure and nose-only continuous exposure 14 ± 0.9 and 9.9 ± 0.7 DNA adducts per 10^9 bases were determined in the lung (19). These studies demonstrate that minute amounts of genotoxic smoke components reach the lungs of rats in inhalation assays.

Dontenwill and associates developed the "Hamburg II" smoke inhalation device in which small animals can be exposed to air-diluted smoke (Figure 1). Eighteen groups, each consisting of 80 female and 80 male random-bred Syrian golden hamsters, comprised this cigarette smoke inhalation lifetime assay. Animals in group 1 were exposed once daily for about 10 minutes, seven times each week to air-diluted smoke (7:1); those in group 2 had twice daily exposures to diluted smoke, hamsters in group 3 had 3 exposures to diluted smoke; and those in group 4 were exposed twice daily to the gas phase of diluted smoke, while group 5 consisted of sham-treated controls. In group 1, 38 animals developed papilloma and one animal had a carcinoma of the larynx (total 24%), hamsters in group 2 developed 69 papilloma and 17 carcinoma of the larynx (total 54%), corresponding tumor yields in group 3 were 77 papilloma and 11 carcinoma of the larynx (total 55%). Laryngeal tumors were not observed in group 4 (gas phase only) nor in group 5 (controls). Three hamsters in group 2 developed papilloma of the pharynx; tumors of the lung were not seen in any of the hamsters in this study (20).

In another assay, male Syrian golden hamsters from 2 inbred lines were exposed five times a week for up to 100 weeks to air-diluted smoke. In one inbred strain, 7 of 84 hamsters developed papilloma in the larynx, 9 had microinvasive cancer; in the second inbred strain, 11 of 87 animals had papilloma and 2 microinvasive cancers occurred in the larynx; none of the control hamsters developed laryngeal tumors (21). In a dose-response lifetime study with hamsters of a strain that is susceptible to the induction of laryngeal tumors, 70% of the animals responded to twice daily exposures to 22% cigarette smoke with papilloma and 47% with carcinoma of the larynx. The corresponding incidences in the hamsters exposed to 11% cigarette smoke twice daily were 27% and 7%, those in the control group 6% and 0%, respectively. In the high-dose group, 3 of 62 hamsters developed also tracheal papilloma (22). The Syrian golden hamsters are less susceptible than other laboratory animals to the toxicity of nicotine and of carbon monoxide and are therefore preferred for inhalation studies with tobacco smoke.

II. Bioassays with Cigarette Smoke Particulate Matter

Inhalation assays with Syrian golden hamsters have demonstrated that only whole smoke induces benign and malignant tumors of the respiratory tract in a dose-dependent fashion.

However, inhalation of smoke which is free of particulate matter ("tar") does not lead to tumors. This indicates that the dose of carcinogens in the gas phase by itself is not sufficient to induce tumors and that the majority of the carcinogens reside in the particulate matter of tobacco smoke. This consideration has led to in-depth fractionation studies and bioassays with tobacco smoke condensate in mice, rats and rabbits (6, 23, 24). The neutral subfractions B and BI that contain a concentrate of the polynuclear aromatic hydrocarbons (PAH), harbor the major tumor initiators (Figure 2). The PAH subfraction is also the only portion of the tar that, upon repeated intratracheal instillation, elicits tumors in the respiratory tract of rats (25).

Assays of the PAH concentrate explain only a small fraction of the total carcinogenicity of the tar. Results from bioassays of the PAH-subfraction in combination with the weakly acidic, non-carcinogenic fraction explain 70-90% of the carcinogenicity of the whole tar (23, 24). The weakly acidic fraction contains the major tumor promoters, volatile phenols, and the major cocarcinogens, catechols. In addition to tumor initiators, tumor promoters and cocarcinogens, tobacco smoke also contains carcinogens with organ-specificity. These act independently of the mode of exposure or site of application, by inducing benign and malignant tumors in specific organs. Table 1 presents a list of the known tumorigenic agents in tobacco smoke, their concentrations in the smoke of one cigarette, and the evaluation of evidence of their carcinogenicity by the International Agency for Research on Cancer (26 27). Table 2 is a listing of the likely causative agents for tobacco smoke-related cancers on the basis of organ-specificity of carcinogens and their various biological activities and concentrations in cigarette smoke.

The agents in tobacco smoke most likely to cause induction of cancer of the respiratory tract are PAH, the tobacco-specific N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the volatile aldehydes, acetylaldehyde and formaldehyde and , to a minor extent, polonium-210 (which stems from agricultural and environmental sources).

III. Bioassay on Mouse Skin

Inhalation studies with Syrian golden hamsters have clearly demonstrated that the major carcinogenic activity of whole cigarette smoke resides in its particulate matter (tar), as discussed earlier. This has led to extensive bioassays of cigarette tar in both the connective tissue of rats and the skin of mice (6). Since the induction of sarcoma in the connective tissue of rats can be influenced by the physical form of the tar. by the presence of insoluble particles (Oppenheimer-Nothdurft effect; 28, 29) the mouse skin bioassay is now the preferred method for estimating the tumor potency of smoke condensates especially

when comparing tars of experimental cigarettes that vary from the control cigarette only in a few aspects.

The tars obtained from a smoking machine (Chapter B), such as a Borgwaldt-30 cigarette smoker (30) or other devices (6), are stored in the dark at refrigerator temperature until needed for biological testing, but should not be older than 3 weeks when applied. Before use, the suspensions in acetone are thoroughly mixed in a mechanical shaker for at least 3 hours, a sample is poured into a 60-ml glass-stoppered reagent bottle. Since tars are not always fully dissolved in the acetone (1:1), vigorous shaking of the bottle is essential before each use.

Anywhere from 30 to 100, usually 50 but preferably 100, Ha/ICR/Mil (Swiss albino) female mice are used for each tar to be tested. The random bred Ha/ICR/Mil (Swiss albino) mice are sturdy animals, and they are quite resistant to nicotine toxicity. Compared to two inbred strains of mice often used in skin carcinogenesis (CAF_1, C57BL), they are more susceptible to the carcinogenic activity of tobacco tars (31). Female mice are used for the bioassay since they do not fight as do the males, which results in skin scratches. Thus, females can be housed 5 to a cage, while males require one cage for each mouse. The maintenance of female mice is therefore significantly more cost effective (6). At the onset of bioassays the mice are 5 to 7 weeks of age and weigh 22 to 25 g. They receive feed and water ad libitum. Their cages are cleaned twice weekly.

Before each tar application, the dorsal hair of the mice is shaved with a Model A2 (size 40) Oster animal clipper. The tar is then applied in 0.1 ml of an acetone suspension containing 50 mg tar with a full No. 5 camel hair brush, or by pipette. The treatment is repeated three times weekly, allowing at least one day between applications for absorption of the tar before the next application. It is sometimes necessary, especially at the onset of the experiment, to skip a painting if the mice exhibit poor absorption or low tolerance of the tar.

Mice that survive the first month usually tolerate the toxic effects of the tar solutions (LD_5). All mice lost during the first month in an experiment are replaced by mice of the same age. Therefore, the initial number of animals to be scheduled for each assay must exceed the requirements for the control and experimental groups by about 10%. If the toxicity persists, even though the number of applications is cut down, the tar must be applied at a lower concentration with the necessary revision of the protocol. In recent years, however, such modifications have usually not been necessary, owing to the generally lower levels of nicotine in tobaccos. The bioassay is not terminated until 90% of the mice in the tar group with the longest survival rate have died or were moribund and had to be killed; this takes usually 18-20 months of tar application.

Average weights of the mice are recorded at the onset of the experiment and again at 2 weeks, 4 weeks, and thereafter at monthly intervals as an indicator of the general health of the mice. A reduction in weight could be associated with a reduced tumor yield, particularly if the weight loss takes place in the tumor promotion phase. It is essential that weight records be kept in experiments with tobacco carcinogenesis.

Constant observation of the animals is also essential. Any change in appearance, habit, or reaction is noted; any lesion on the back is described as to [1] type, i.e. ulcer, infection, or tumor; [2] date of appearance or change in appearance; and [3] exact location. When such a notation is first made, the animal, is marked on the head with a yellow dye (picric acid). Diagrammatic representations of the animal's back are used to facilitate the recording.

The application of the test material by painting or pipetting may lead to benign skin tumors which are recorded when they have attained a diameter of 1 mm. They enlarge by nodular growth (papilloma) or by lateral invasion (carcinoma); some may not enlarge, but regress. Those tumors that remain 1 mm or grow larger for 21 consecutive calendar days are counted and become the raw tumor yield data. Macroscopically, the lateral invasion of the tumor into adjacent skin is considered as transformation into a carcinoma. Continued growth of such lesions, however, is required before they can be recorded as macroscopically observed carcinomas.

Mice with carcinomas are killed by cervical dislocation and the tumor is excised and examined histologically. All suspicious lesions are likewise examined. The animals are autopsied for distant metastases and the occurrence of other tumors, especially pulmonary adenomas and lymphomas.

Testing the biological effects of tobacco smoke may be encumbered by the toxicity of some smoke condensates. For this reason, experiments with tobacco smoke condensate should no longer be carried out with less than 100 animals. All mice lost during the first month of the experiment are replaced. The most careful recording of experimental observations demands equally careful statistical evaluation of the final data. Therefore, some pertinent statistical considerations will be discussed.

Assuming that in a given experiment none of the animals in the control group has a tumor and 6 or more in the experimental group have tumors, one may utilize the table prepared by Vos based on chi-square analysis with "Yates correction". This shows that the difference between the groups is significant at $P < 0.05$ when the number in each group varies from 10 to 50 or more.

This situation merely identifies that one is dealing with a tumorigenic agent and does not allow a quantitative assay of tumor-producing agents of varying potency. If, for example, there are as many as 4 tumor-bearing animals in a given group treated with a weak carcinogen, the number of animals in each group becomes important. If there were 10 animals in each group, all of them would have to bear tumors to indicate a difference at the $P < 0.05$ level of significance. In a group of 50 animals treated with a stronger tumorigenic agent, only 12 need to show tumors. Information about differences in tumor yield necessary for significance at $P < 0.05$ between two groups for groups of 10 to 50 animals may be readily extracted from tables by the Food Protection Committee of the U.S. Academy of Science (30).

We calculated that finding a difference with 80% statistical power for the tumorigenic activity on mouse skin of a condensate of an experimental cigarette [with reduced ignition propensity] vs. the condensate of the control cigarette would require 90 mice in each group, plus 30 mice for the negative control group.

IV. Inhalation Bioassay with Syrian Golden Hamsters

Inhalation studies with whole smoke are strongly indicated to compare the tumorigenic activities of cigarette smoke condensates. As discussed earlier, the Syrian golden hamster (SGH) is presently the animal of choice for long-term inhalation assays with cigarette smoke (6, 20-22). One should be aware that inhalation bioassays with whole cigarette smoke will rarely lead to lung tumors in SGH or rats (9) but it will lead to papilloma and carcinoma in the larynges of the animals. Since the larynges of inbred strains of male SGH are apparently most susceptible to the carcinogenic effects of cigarette smoke (21, 22) one is inclined to prefer this animal model. However, it is not without difficulties to obtain such inbred strains of animals and these hamsters are not as resistant to the acute toxicity of the smoke as are random-bred SGH; thus, the latter are generally used for inhalation studies (7, 31).

Three inhalation devices have been developed for exposure of SGH and rats to cigarette smoke. These are the "Hamburg II" device (20, 34), the "Oak Ridge" smoke inhalation exposure device (9, 17) and the "Walton-reverse smoker" (21). All 3 machines are well developed and the "Oak Ridge" device has especially favorable features in respect to forced smoke inhalation by laboratory animals. However, the "Hamburg II" device with SGH is recommended for comparing the tumorigenicity of whole smoke from various cigarettes. The device has been widely used and most data on the tumorigenicity of whole cigarette smoking, including a dose-response study, were generated with it (15, 20, 34). In general this bioassay requires 24-26 months.

The inhalation studies with SGH require twice daily exposure of the air-diluted smoke (7:1) of one cigarette each, seven times weekly, for the entire lifespan of the animals. Because of the high CO concentration in undiluted smoke (2.8 - 4.6 vol%) the MTD is 10 minute exposure twice daily of cigarette smoke diluted by air 1:7. Each time 10 SGH can be exposed concurrently to diluted cigarette smoke in one Hamburg II device. Since 80 male hamsters are needed for each test cigarette, the bioassay is very labor intensive and is recommended only as a last step in the cascade of assays. (Details for the inhalation assay with SGH are presented in Dontenwill et al.; 15, 20).

Inhalation assays using F344 rats and employing the "Oak Ridge" inhalation device for exposure to cigarette smoke (9, 17) appear promising. Although it can not yet be recommended for toxicity testing, it is hoped that the methodology will be confirmed by additional studies.

Critique

Ideally one establishes the carcinogenicity of an aerosol by inhalation assays which lead to the induction of benign and malignant tumors in the respiratory tract of laboratory animals. In the case of cigarette smoke this goal has been only partially reached. In the Syrian golden hamster, papilloma and carcinoma have been induced in the larynx with cigarette smoke in a dose-related fashion, however, with a few exceptions, squamous cell tumors have not been induced in the lung (15, 20-22). In a lifespan inhalation assay with rats a most advanced smoke inhalation device has led to only a few isolated lung carcinoma (9, 17). Epidemiologists claim correctly that several hundred prospective and case-control studies have demonstrated that cigarette smokers face an increased risk for lung cancer and, therefore, confirmation by inhalation bioassays is not needed. The inability to reproduce this biological process in an animal model is frustrating to the experimentalist.

Simulation of human smoking behavior in terms of deep inhalation of cigarette smoke into the lungs has not been successful in laboratory rodents. However, in comparisons of the relative tumorigenicity of the whole smoke of cigarettes with reduced ignition propensity to that of a control, the inhalation bioassay with hamsters should clearly reflect possible changes in the carcinogenic potential in the number of tumors observed in the larynges.

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Legends to Figures

Fig.1. Hamburg II Smoke-Inhalation Device for 10 Hamsters (20).

Fig.2. Fractionation of Cigarette Smoke Condensate (2).

Glossary of Terms

Adenocarcinoma	Malignant tumor of a glandular structure, such as in the peripheral lung.
Adenoma	Benign tumor of a glandular structure, such as in the peripheral lung.
Carcinoma	Malignant tumor of epithelial orgin.
DNA	Deoxyribonucleic acid. DNA is localized in the cell nucleus and is the molecular basis of heredity in many organisms.
Genotoxicity	Damage to the DNA structure.
In vitro	Experimentation with microorganisms, isolated cells, tissues, or isolated organs in biological media.
In vivo	Experimentation with live animals, such as mice, rats and hamsters.
Papilloma	Benign tumors (warts) due to a proliferation of epithelial tissue.

Cost Estimates for Carcinogenicity Bioassays

Bioassay	Animal	Groups	Number of Animals[2]	Cost[1]
Inhalation	SG hamster (random bred)	exptl. cigarette I	60	$185,000-$250,000[3]
		exptl. cigarette II	60	
		sham control	60	
Skin	mouse (Ha/ICR/Mil)	exptl. cigarette I	90	$39,000
		exptl. cigarette II	90	
		sham control	60	

Estimates pertain only to direct cost. The costs exclude the overhead as approved for individual institutes by the U.S. Department of Health and Human Services. Direct total costs include animal purchase, health screening of the animals, maintenance, treatment (smoking of hamsters or tar application to mouse skin), weighing (first 8 weeks weekly, subsequently monthly), recording, autopsy and histology.

Estimates do not include purchase of cigarettes (inhalation study requires about 280,000 cigarettes/group; mouse skin bioassay @ 1.5 kg/group requires about 75,000 cigarettes, assuming one cigarette yields 20 mg tar), or the smoking of cigarettes for the preparation of the tar for the mouse skin bioassays.

The number of animals per group is calculated for a difference between two groups with 80% statistical power either for the tumorigenic activity in the larynx of hamsters of whole cigarette smoke or for the tumorigenic activity on mouse skin of a tar.

This includes the overtime for twice daily exposure on Saturday and Sunday.