To the Graduate Council:

I am submitting herewith a thesis written by William James Gerhardt entitled "Low Dose Chemical and Radiation Carcinogenesis in the Murine Lung." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Radiation Biology.

Robert L. Ullrich, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

A

LOW DOSE CHEMICAL AND RADIATION CARCINOGENESIS IN THE MURINE LUNG

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

William James Gerhardt

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This thesis is dedicated to my mother,
Debbie M. Gerhardt, and in memory of my father,
the late William H. Gerhardt.

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ABSTRACT

This study was designed to investigate the carcinogenic effects of combinations of chemical and/or physical agents, particularly at low doses. The animal model chosen for this investigation was lung tumor development in the Swiss Webster mouse.

The treatment combination of a known carcinogen and the non-carcinogenic chemical butylated hydroxytoluene (BHT) was evaluated in two series of experiments. One study evaluated the combination of urethan with BHT, while the other investigated fission neutron radiation plus BHT. An additional study looked at the effects of a combination of low doses of two carcinogenic agents, urethan and fission neutrons, in this animal system. Finally, a study of the BHT enhancement of lung tumorigenesis was done by investigating the related action of BHT to induce extensive cell proliferation in the murine lung.

A treatment sequence of a single high dose (1000 mg/kg) of urethan followed by eight weekly administrations of BHT (300 mg/kg) resulted in a significantly increased mean number of pulmonary tumors per mouse compared to urethan treatment only. A low dose of urethan (100 mg/kg) plus eight weekly BHT treatments, however, did not produce a significantly enhanced lung tumor multiplicity compared to low dose urethan treatment alone. No increase in tumor multiplicity was found in animals treated with fission neutrons ($\leq$50 rads) alone or fission neutrons followed by weekly BHT treatment. Mice given urethan (100 mg/kg) followed two weeks later by fission neutron irradiation developed a significantly increased pulmonary tumorigenesis over

untreated controls. This increase, however, was apparently due to urethan, as the tumor multiplicity of the combined treatment groups was similar to urethan treatment alone.

Following the first of a series of weekly BHT treatments significant cell proliferation, indirectly detected by the incorporation of radioactive thymidine into pulmonary DNA, occurred in both BALB/c and Swiss Webster mice. Significant cell proliferation, however, did not occur following further weekly BHT administration, but did again following BHT treatment when an interval of at least two weeks elapsed between injections of the chemical.

This thesis, therefore, showed that BHT significantly enhanced the lung tumorigenic effects of high doses of urethan, but not of low doses of the same chemical or of another carcinogen, namely fission neutrons. Second, a treatment combination of two carcinogens revealed that fission neutrons at low doses did not influence the tumorigenic effects of urethan. Finally, the lack of significant cell proliferation in the murine lung reported in this study following further weekly BHT administrations suggests that BHT enhancement of murine pulmonary tumor development is not entirely related to its induction of lung cell proliferation.

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LIST OF ABBREVIATIONS

2-AAF = 2-Acetylaminofluorene

BHT = Butylated hydroxytoluene

BP = Benzpyrene

DENA = Diethylnitrosamine

DMBA = Dimethylbenzanthracene

DMNA = Dimethylnitrosamine

2,7-FAA = N,N'-2,7-Fluorenylenebisacetamide

3-MCA = 3-Methylcholanthrene

MNU = N-Methyl-N-Nitrosourea

MMT = Methylcyclopentadienyl manganese tricarbonyl

TPA = 12-O-Tetradecanoylphorbol-13-Acetate

CHAPTER I

INTRODUCTION

Neoplasia is a large group of diseases characterized by uncontrolled growth and spread of abnormal cells. Currently, it is the second major cause of death behind heart diseases in the United States population. The National Cancer Institute estimates that 420,000 cancer-related deaths will occur, and a total of over 1,000,000 new cases of this disease will be diagnosed in 1981 [1].

Despite the prevalence of the disease, the etiology of most human neoplasms is unknown. An interplay of genetic and environmental influences is considered to be involved. Epidemiology has shown differences in cancer incidence in different geographic regions for certain organs. Migrating populations have been observed to acquire the predominant cancer pattern of the host countries and lose that of their home country [2]. This suggests a predominant influence of environmental factors.

A variety of environmental carcinogenic stimuli have been identified. Epidemiological studies have implicated a number of natural chemicals such as aflatoxin and synthetic chemicals such as diethylstilbestrol as inducing cancer in man [2]. Ultraviolet light has been linked to skin cancer [3], while ionizing radiation has been associated with the development of neoplasms in a number of sites [4]. Human exposure to these chemical and physical agents occurs as a consequence of occupation, personal habits such as diet and smoking, and water and air pollution. Most exposures are very low in dose and

appear to cause little, if any, detectable detrimental effects. However, it is possible that an interaction of agents or a cumulative effect of various agents may occur that influences the development of neoplasia or other pathology.

The hypothesis that a combination of agents can influence carcinogenesis has some speculative basis. First, very few cases of human cancer have been attributed to the influence of a single etiological factor. This has usually been the rare situation where exposure to high cumulative doses of a specific agent occurred. Second, some epidemiological data appears to support the involvement of more than one agent or factor. The majority of such data concerns lung cancer. Tobacco smoking and asbestos exposure together have been reported to significantly increase the risk of lung cancer compared to asbestos exposure alone [5]. A possible interaction between tobacco smoking and radiation exposure from airborne radon and radioactive substances present in uranium mines has been suggested by Lundin and co-workers [6]. Ten times greater respiratory cancer mortality was observed among miners who smoked compared to nonsmoking miners. According to the investigators, smoking alone could not explain the excess mortality. These reports indicate that possible interaction between chemical and physical carcinogens need to be explored further. Detailed investigation of the carcinogenic interaction between chemical and physical agents is not possible in humans and is best studied in experimental animal systems.

A useful model for experimental cancer study has been murine lung tumor development. The literature concerning the study of the

carcinogenic effects of individual chemical and physical agents in this animal is extensive (see Shimkin [7]). Studies investigating the carcinogenic influence of a combination of agents, however, are limited. A few reports have noted an enhancement of lung tumor development. Cole and Foley reported an enhanced incidence of lung tumors in LAF_1 mice given a combination of a low dose of the chemical carcinogen urethan and a low dose of x-rays [8]. Witschi and co-workers reported a treatment combination of urethan plus the noncarcinogenic chemical butylated hydroxtyoluene (BHT) to enhance the number of tumors per mouse [9]. These reports suggest that it is possible for a chemical or physical agent to interact with or enhance the carcinogenicity of another agent, and that the murine adenoma system is a suitable animal model for further investigation of the tumorigenic effects of a combination of agents.

The following section includes a review of human and experimental data concerning chemical and/or radiation carcinogenesis. The murine adenoma system is also reviewed, along with specific studies evaluating the tumorigenic effects of chemical and/or physical agents.

CHAPTER II

LITERATURE REVIEW

A. HUMAN DATA

Chemicals

Chemicals were the first environmental agents recognized as influencing human carcinogenesis. Percival Potts in 1775 wrote of the relationship between working as a chimney sweep and the development of cancer of the scrotum. Paris in 1812 described skin cancers among smelter workers exposed to arsenic. Bladder cancer among fuchsin dye workers was reported by Rehn in 1895 [10]. More recently, several chemical agents including arsenic, chloromethyl ethers, mustard gas, and nickel have been implicated in the development of lung cancer. Lynch and Smith [11] reported in 1935 the association of asbestos with lung tumorigenesis. Besides lung neoplasia, asbestos exposure appeared to increase the risk of development of other forms of malignancy such as respiratory mesothelioma. Overall, occupational exposure to chemical agents has been associated with a range of cancers, mainly of the skin, bladder, lungs, and nasal sinuses [2,12]. Today 25 chemicals are generally accepted as being carcinogenic in humans. Another 1500 chemical substances are listed as suspected carcinogens by the National Institute for Occupational Safety and Health [2].

Human exposure to chemical carcinogenic stimuli also occurs through social habits. Tobacco smoking has been associated with cancer of the lung and other sites. The significant increase in the

incidence of lung cancer since 1930 among males, and more recently among females in the United States population, has been attributed principally to smoking [1]. Although the mechanism through which tobacco influences cancer development is unknown, tobacco has been found to contain a number of chemicals such as polycyclic aromatic hydrocarbons which are known carcinogenic agents. Furthermore, other chemicals such as the N-methylcarbazoles have been identified which have promoting influences [13].

Ionizing Radiation

Substantial information suggests that radiation exposure can be an etiological factor in human cancer. Thus far, only ultraviolet and ionizing radiation are known to have carcinogenic activity [14]. Consideration here will be limited to ionizing radiation.

Information on the carcinogenic effects of ionizing radiation has been obtained from persons or populations receiving occupational, accidental, medical, and wartime exposure [4]. Historically, the first reports involved occupational exposures. Frieben in 1902 reported a skin carcinoma on the hand of a radiologist that was attributed to radiation exposure. Many similar reports subsequently appeared. Van Jagie in 1911 noted an association between leukemia and ionizing radiation among pioneer radiation workers. Seltser and Sartwell reported a higher incidence of leukemia among physicians and dentists who used x-rays compared to nonusers [15]. The higher incidence was largely confined to older radiologists who presumably received much of their exposure in the 1930-1950's and probably absorbed substantial cumulative radiation doses.

Exposures have also resulted from ingested or inhaled radioactive substances. Dial painters ingested radium through the practice of pointing the brush between their lips. The radionuclide was incorporated into the skeleton. A variety of bone tumors were reported among these workers, with the latency period varying from 10 to more than 25 years depending upon the radium content of the skeleton [14]. Several study groups of uranium miners in Europe and the United States had an elevated incidence of pulmonary cancer, attributed by many investigators to radiation exposure in the mines from radon and radon daughters [6,16,17]. Lundin found 62 cases of respiratory cancer deaths versus 10 expected deaths among 3414 white underground U. S. uranium miners [6]. Archer noted an increased incidence after 120 working level months of radon daughter exposure, and a continued increase with increasing exposure in this same study group [17]. Attributing radiation exposure to be the sole etiological factor has been disputed [18] and the involvement of other factors such as smoking has been considered.

Epidemiological studies of subjects who received medical exposures for therapeutic or diagnostic reasons have also suggested a radiation carcinogenic effect. Women subjected to repeated fluoroscopic examination of the lung in the treatment of pulmonary tuberculosis were reported to have an excess of breast cancer [14]. A Court Brown and Doll study of ankylosing spondylitis patients treated therapeutically with x-rays indicated an excess incidence of leukemia and tumors of the pharynx, stomach, pancreas, lung, and bone in heavily irradiated sites [19]. The relative risk of developing lung

cancer was approximately twice that in the general population. The average dose to the lungs of these patients had been estimated as about 400 rads [20]. More recent analysis, however, estimates the dose as approximately 200 rads [21].

The Japanese atomic bomb survivors probably have been the most extensively studied group. Elevated incidence of leukemias and solid tumors of the breast, lung, thyroid, and gastrointestinal tract have been observed during the greater than 30-year follow-up of this group. The risk of lung cancer in the segment of the Hiroshima population which received significant radiation exposure has been shown to be twice that in those who received negligible exposures. In contrast, the significantly irradiated population in Nagasaki did not show an excess risk over those exposed to negligible doses of radiation [4].

Interaction of Chemical and/or Physical Agents

Although there is considerable information that chemical and physical agents by themselves can be carcinogenic, data suggesting an interaction of chemical and/or physical agents in human carcinogenesis have been reported in only a few instances. As mentioned earlier, Selikoff and co-workers reported asbestos exposure and tobacco smoking to increase markedly the incidence of lung cancer above that associated with asbestos exposure alone [5]. Excessive consumption of alcohol has been reported by Wynder to significantly increase the risk in smokers for cancer of the oral cavity, larynx, and esophagus [13]. However, this enhancement is not considered attributable to a direct effect of alcohol, but rather to the nutritional deficiencies associated with alcoholism.

The possible carcinogenic interaction of chemicals and ionizing radiation has not been well documented. As discussed earlier, Lundin noted that lung cancer incidence among smoking uranium miners was 10 times greater than among nonsmoking miners [6], an increase that could not be attributed to smoking alone. Archer agreed with the findings of Lundin and concluded that some interaction between the effects of radiation and cigarette smoking appeared certain [17]. Smoking has been found to affect the latent period for lung cancer in the miners [22].

Carcinogenic interaction of chemicals and radiation has been suggested by another report involving therapeutic exposure to these agents. Canellos and co-workers reported a 9.7% incidence of secondary cancer among 452 Hodgkin's disease patients who received intensive cyclic chemotherapy and over 3500 rads of total nodal x-irradiation [23]. An incidence of 2.4% was observed in patients receiving either treatment alone. The secondary cancers were leukemias and tumors of the skin, lung, and chest wall. The authors considered the observed enhancement to possibly reflect the involvement of several factors: first, an impairment of cellular immunity that is characteristic of advanced Hodgkin's disease; second, the immunosuppressive effects of chemotherapy and radiation; and third, direct carcinogenic effects of the combined treatments upon cells.

Though these reports suggest that an interaction of certain chemical and/or physical agents influencing carcinogenesis in man is possible, many such associations cannot be firmly established. One

problem has often been a lack of quantitative data concerning exposure to the specific agents under investigation. Also, additional processes or factors, known or currently unknown, may be involved and thereby complicate an understanding of the final result observed, as noted in the Canellos study. To delineate and measure quantitatively the influence of a number of factors that may affect carcinogenesis is difficult in humans, and is better done in experimental studies.

B. EXPERIMENTAL DATA

Chemicals and Ionizing Radiation

The first report investigating the interaction of a chemical carcinogen and ionizing radiation was by Cramer [24]. Mice given twice-weekly tarring followed by radium exposures developed fewer skin tumors, and those that developed appeared at a later time than after tarring alone. The experimental conditions of this study were poorly defined, however. Also, the doses of radium were not known. Mottram [25] estimated the doses to be between 4000 and 8000 rads, which caused a great deal of tissue destruction. This was suggested as the basis for the observed reduced skin tumor incidence. In contrast, Mottram in 1937 reported that a twice-weekly treatment of 0.5% benzpyrene (BP) in benzene for 60 days followed by 160, 480, or 1440 rads of gamma radiation resulted in an enhanced skin tumor incidence. The degree of enhanced tumor incidence was related to the radiation dose. His data, therefore, indicated that radiation could interact with a chemical carcinogen to increase the tumor response if the radiation dose was not high enough to cause tissue destruction.

More recent reports have further investigated the interaction of a chemical carcinogen and ionizing radiation in mouse skin and other animal models. Cloudman reported additive skin tumor induction effects in mice given carcinogenic levels of beta radiation followed by 3-methylcholanthrene (3-MCA) [26]. The effects of reversing the sequence of treaments was not evaluated. Similar additive effects in the incidence of mammary adenocarcinomas were observed by Shellabarger in Sprague-Dawley rats 100 days after being given 3-MCA and x-ray or fission neutron radiation even when the treatment sequence was reversed [27]. Vogel and Zaldivar reported a synergistic increase in the incidence of liver neoplasms in rats given fission neutron radiation and N,N'-2,7-fluorenylenebisacetamide (2,7-FAA). Again, reversal of the sequence of treatments did not affect the final results. In contrast, an enhanced incidence of gastric neoplasia was noted in this study for the sequence of chemical carcinogen followed by neutron exposure, but not the reverse [28].

Of particular interest are several studies which suggest that subcarcinogenic doses of radiation can have latent carcinogenic effects. Shubik and co-workers reported that low doses of beta radiation, nontumorigenic by themselves, could produce tumors when followed by twice-weekly painting with croton oil [29]. A similar finding was made by MacGregor for skin tumor development in rats given beta radiation and subsequent cigarette tar applications [30]. The author attributed the effect to the tumor-enhancing activity of the cigarette tar. These reports are important in suggesting that

noncarcinogenic chemical agents are capable of influencing the tumorigenic effect of ionizing radiation.

Furthermore, the latent carcinogenic effects of a low dose of radiation may persist for long periods. This is suggested in a study by Hoshino and Tanooka [31]. Female ICR mice were treated with a sequence of beta-rays followed by 4-nitroquinoline 1-oxide, with the interval between exposure to the two agents varying from 11 to 408 days. Neither treatment alone produced skin tumors. However, the combined treatment resulted in an average incidence of malignant skin neoplasms of 12.4%, which was essentially the same for all time intervals between the treatments.

Chemical Agent Combinations

Mouse skin studies. The carcinogenic effects of a combination of chemicals have likewise been investigated. The most extensive studies have been on mouse skin tumor development. Single applications of a chemical carcinogen, such as BP or dimethylbenzathracene (DMBA), followed by repeated paintings of croton oil, were reported to enhance skin tumorigenesis over that seen from chemical carcinogen treatment alone [32]. Enhancement was still observed with low doses of carcinogen plus croton oil [33]. These results suggested to Berenblum that the process of carcinogenesis consists of separate independent processes, which he described as precarcinogenic, epicarcinogenic, and metacarcinogenic. Similarly, Friedewald and Rous in their investigation of rabbit skin carcinogenesis suggested a two stage process occurs which they defined as initiation and promotion [34]. This latter terminology for skin tumorigenesis became widely accepted.

Initiation has been suggested by Boutwell to involve permanent and heritable, but unexpressed changes in the cell genome by an agent [35]. The DNA is assumed to be the critical target. Slaga has described the initiation phase as requiring only a single application of either a direct or indirect carcinogen at a subthreshold dose [36]. Promotion presumably involves the gradual transition of the initiated cells to a growing tumor. A "promoter" has been defined by Slaga as an agent applied repeatedly after treatment with a tumor-initiating agent that results in tumors. This is in contrast to a "cocarcinogen" defined as an noncarcinogenic agent given concurrently with a carcinogen, which augments tumor development [36]. The process of promotion has been considered by Berenblum to be slowly acting, somewhat reversible, and less specific in action than initiation [37]. More recently, Boutwell has suggested that promotion can be divided into two steps: first, the "conversion" of an initiated cell into a dormant or latent tumor cell and, second, the "propagation" of the latent tumor cell to become an grossly visible tumor [35]. This division was based on experiments in which it was shown that after several weeks of treatment of initiated skin with croton oil, treatments with turpentine could be substituted and tumors would still develop. Turpentine induces epidermal hyperplasia, but has not been found to promote tumor development. The use of turpentine without prior promoter treatment was ineffective in influencing skin tumorigenesis.

Limitations of the two stage theory must be considered when applying it to other experimental systems. As discussed by Fishbein

[2], "initiator" and "promoter" are operational terms defined upon the temporal sequence of treatment. Initiator, then promoter presumably results in tumor enhancement. Reversal of the sequence would not. However, many agents considered promoters also have weak tumorigenic activity. Such an agent might be defined as a weak carcinogen or initiator. Thus the categorization of an agent as an initiator or promoter may be overly simplistic when considering mechanisms of action.

Observations in other animal systems. Besides mouse skin, the interaction of two chemical agents upon tumor development at other sites has been evaluated. Importantly, a number of recent reports have suggested that the two stage initiation-promotion theory might be applicable to carcinogenesis at other sites. Peraino and co-workers reported an enhanced incidence of liver tumors in rats fed a sequence of 2-acetylaminofluorene (2-AAF) and phenobarbital [38]. Simultaneous feeding of both agents, however, reduced the carcinogenic effects of 2-AAF. Phenobarbital alone had no tumorigenic effects. A similar enhancement of liver carcinogenesis was reported by Weisburger in rats given diethylnitrosamine (DENA) then phenobarbital [39]. DDT has likewise been reported to enhance the hepatic effects of a chemical carcinogen [40]. In a less definitive system Armuth and Berenblum reported the treatment sequence of DMNA given at birth, then repeated administrations of phorbol two weeks later enhanced the incidence of both lung and liver tumors [41]. However, waiting until the mice were 10 days older before DMNA was administered negated the enhancement.

Hicks and Chowaniec investigated the interaction of the carcinogen N-methyl-N-nitrosourea (MNU) and the artificial sweeters sodium saccharin or sodium cyclamate upon bladder tumorigenesis in Wistar rats [42]. A low dose of MNU instilled into the bladder failed to produce any tumors over a two year period. However, animals given the same subcarcinogenic dose of MNU and subsequent maintenance on a diet containing either saccharin or cyclamate over the same time period had a bladder tumor incidence of about 50%. Interestingly, saccharin has recently been reported to enhance the murine lung tumorigenic effects of urethan [43].

A recent report by Goerttler and co-workers evaluated the carcinogenic effects of DMBA and the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) upon the murine forestomach [44]. C57BL/6 mice received a single intragastric dose of DMBA followed one week later by twice weekly intragastric administration of TPA for 35 weeks. An enhanced forestomach tumor incidence of 90% was found among this experimental group 37 weeks after DMBA treatment. DMBA itself caused a tumor incidence of 20%, while TPA elicited no forestomach tumors. Thus TPA appears to have promoted the forestomach carcinogenic effects of DMBA.

Other organ systems where application of a two stage theory of carcinogenesis has been suggested are the colon, mammary gland, stomach, and esophagus of the rat and mammary gland of the mouse [36]. Further research is needed, however, to more conclusively establish the possible mechanisms of carcinogenesis in these organ systems.

The reports cited in this section suggest that a chemical, which may be noncarcinogenic, can modify the known carcinogenicity of another chemical agent. A large number of chemicals have been investigated for possible modifying influences. One group of agents which has received particular attention are the phenolic antioxidants.

Modification of chemical carcinogenesis by phenolic antioxidants. Butylated hydroxytoluene (BHT) and other phenolic antioxidants, commonly used as food additives, have been shown to modify the carcinogenicity of a chemical agent in a number of studies. The type of modification has been shown to be critically dependent upon the sequence of treatment of the antioxidant and the carcinogen. For instance, when BHT was given prior to or concurrently with a chemical carcinogen, inhibition of tumorigenesis has been observed. When given after the carcinogen, tumor enhancement has been reported [45]. Several investigations appear to support this. Weisburger reported a concurrent treatment of BHT and azoxymethane had an inhibitory effect upon intestinal carcinogenesis in F344 rats. When BHT was given after the carcinogen, a slightly enhanced number of gastrointestinal tumors per rat was noted [46]. Peraino observed in female Sprague-Dawley rats given a brief diet of 0.02% 2-AAF, then 0.5% dietary BHT an enhanced hepatic tumor development [47]. Simultaneous feeding of BHT with 2-AAF resulted in a reduced hepatic tumor incidence [48]. Likewise, Clapp found that dietary BHT given prior to treatment with DENA inhibited forestomach cancer development [49].

The basis for the contrasting effects of BHT upon chemical carcinogenesis is not understood. BHT is an inducer of microsomal

metabolic enzymes. Wattenberg has suggested that the inhibitory effects of BHT may be due to this effect [45]. The microsomal mono-oxygenase enzymes are induced to increased activity by the presence of a variety of foreign compounds in the body. They represent a major pathway of metabolic detoxification and elimination of such compounds. When a chemical carcinogen is given subsequent to or concurrently with BHT, enhanced detoxification may occur over metabolic activation of the carcinogen. As a result, much of the carcinogen may be modified before it could interact with critical subcellular sites.

Though BHT enhancement has been reported in tumor development of a number of sites, it has been most extensively studied in murine lung tumor development. The reported role of BHT as a tumor promoter will be discussed later.

C. MOUSE LUNG TUMOR DEVELOPMENT AS AN ANIMAL MODEL

Anatomy of the Lower Murine Respiratory System

An excellent review of the anatomy of the murine respiratory system was written by Reznik-Schuller and Reznik [50]. The lower portion of the murine respiratory system consists of the trachea, bronchi, and lungs. The respiratory airway descends in the trachea to divide into two main bronchi. Subdivisions of the main bronchi subdivide repeatedly into passageways of gradually decreasing diameter. The terminal portions of the bronchi region are the bronchioles. These lead to alveolar ducts which end blindly in the alveoli, where the exchange of gases occurs.

A transition of types of epithelial cell populations occurs from one region to the next in the lower respiratory system. Pseudostratified ciliated epithelium with goblet and basal cells is found in the trachea. This becomes a simple columnar ciliated epithelium with Clara cells in the upper bronchi region. Lower bronchi regions and bronchioles exhibit a simple cuboidal epithelium consisting mainly of Clara cells. An identical epithelial cell population transition occurs in the human respiratory system but in different regions. The simple columnar ciliated and simple cuboidal epithelial populations are restricted to peripheral regions of the respiratory pathway, the bronchioles and terminal bronchioles, respectively. The alveolar regions of human and murine lungs are similar, possessing an epithelium composed mainly of Type I and Type II cells.

Type I alveolar cells have long thin cytoplasmic extensions which line the alveolar space. The ultrastructure is very simple. The cytoplasm is poor in organelles, with only a few mitochondria and a sparse, rough endoplasmic reticulum in the perikaryon. They do not divide and thus are considered end-stage cells. In contrast, Type II alveolar cells are very metabolically active. These cells are considered the source of pulmonary surfactant, a surface-active alveolar lining material composed predominantly of phospholipids. Surfactant functions to prevent the alveoli from collapsing. A second function is as stem cells for cell renewal and repair of injuries in the alveolar region. Type II cells proliferate and differentiate into new Type I cells to replace damaged or destroyed cells. Ultrastructurally, the cytoplasm is complex and abundant in organelles.

The most conspicuous features are osmiophilic lamellated inclusion bodies which are considered a diagnostic feature of these cells.

Murine Lung Tumors

Murine lung tumors, whether spontaneous or induced by a carcinogenic agent, usually originate from the peripheral regions of the respiratory system [51]. The majority of tumors of this region may be categorized histologically as adenomas and adenocarcinomas. Distinction between benign and malignant lung lesions has not been made by most investigators, and thus "adenoma" has been used often as a catch-all term for murine lung tumors. Distinction between benign and malignant lesions is difficult, but the uniform gross and microscopic appearance of the tumors makes them acceptable as a biological endpoint in carcinogenesis studies.

The murine lung tumor is viewed grossly as a pearly-white, discrete, round nodule. In cleared lungs it appears as an uniformly dense, spherical shape, found on both the surface and deep within the tissue when observed with a dissection microscope [Figure 1]. Under the light microscope, the tumor is usually found to consist of closely packed columns of cells. It is devoid of a capsule and compresses the surrounding pulmonary tissue [Figure 2].

Ultrastructurally, murine lung tumor cells resemble normal Type II alveolar cells [52-54]. Both have a columnar or cuboidal shape with a large round nucleus, often indented. Microvilli are present along the free cell surface. The cytoplasm is rich in organelles, the most prominent being the lamellar bodies. Based upon

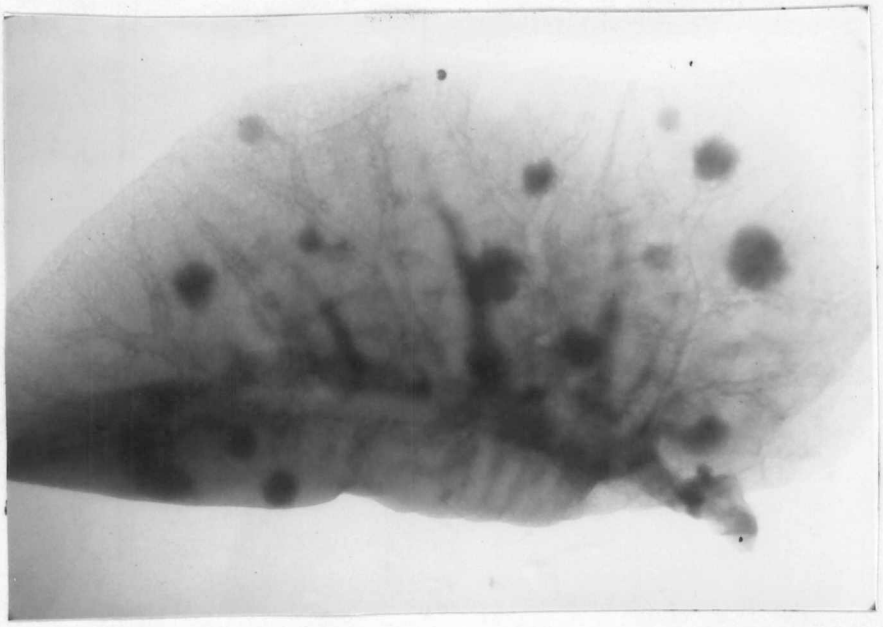

FIGURE 1. MURINE LUNG TUMORS IN CLEARED LUNGS

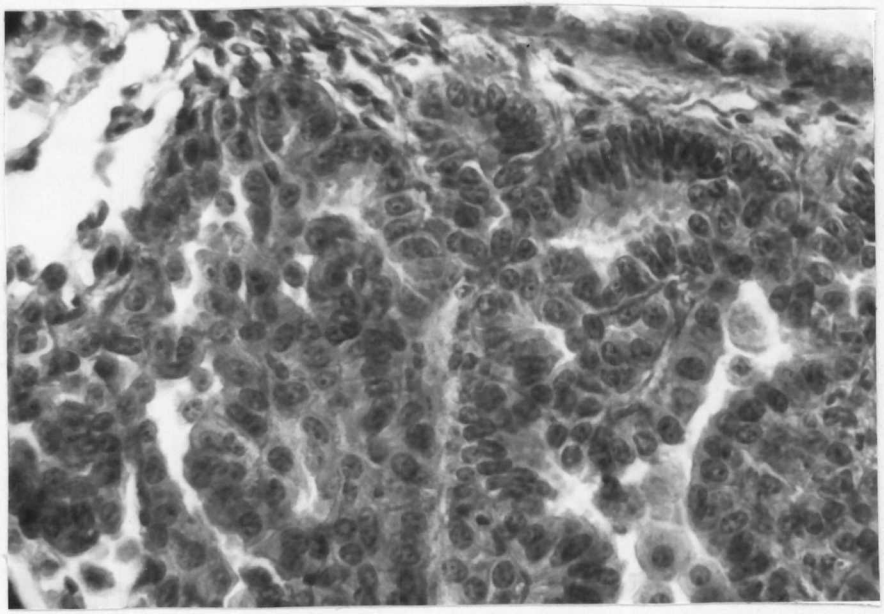

FIGURE 2. LIGHT MICROSCOPIC VIEW OF A MURINE LUNG TUMOR

these similarities, adenomas are generally considered to originate from Type II cells. However, this has not been conclusively proven [52]. It has been recently suggested by Kauffman that under certain circumstances some adenomas may originate from Clara cells [55].

Most investigators agree the murine lung tumor development does not simulate human lung carcinogenesis [7]. Tumors arising in the alveolar region are rare in humans. The majority of human lung neoplasms originate from higher regions of the respiratory tract in the tracheobronchial areas. Also, there are types such as oat cell carcinoma which rarely occur in mice [50]. The difference in type and origin of the majority of lung tumors in humans and mice in part may reflect the differences in the regional epithelial cell populations described earlier. These limitations, however, do not alter the usefulness of this system as an animal model for the study of carcinogenesis.

Mouse Lung Tumor Development as a Biological Assay Model

The value and characteristics of the model. Shimkin and Stoner consider the lung adenoma system to have two-fold value in cancer research [7]. First, it is a fast *in vivo* bioassay method for testing the carcinogenicity of chemicals. Second, the system is a reproducible, stable, rapid biological model for a variety of quantitative carcinogenesis studies.

The system requires the use of mouse strains such as A/J and Swiss Webster, which are susceptible to spontaneous development of lung tumors. In strain A mice, spontaneous lung nodules begin to appear as early as three to four months of age, with the tumor

incidence rising steadily to almost 100% by 24 months. The usual experimental procedure involves treatment of the mouse with a large dose of carcinogen and sacrificing the animals at the time point of interest after treatment, usually three or more months. The incidence of tumor-bearing mice and tumor multiplicity (mean number of tumors per mouse) are the two quantitative parameters measured at necropsy.

The murine lung adenoma system has been used in studies of the carcinogenicity of individual agents and of combinations of agents.

Chemical studies. Induction of lung tumors by chemical carcinogens was first demonstrated by Murphy and Strum using repeated applications of tar to mouse skin [56]. Reports of similar carcinogenic effects by other chemicals such as 1,2,5,6-dibenzanthracene and 20-methylcholanthrene appeared later in the literature [57]. In 1935 Andervont proposed the procedure of pulmonary tumor induction as a test for carcinogenic properties of chemicals. A variety of chemicals including alkylating agents [58], food additivies, and chemotherapeutic agents [59] have been evaluated by this method.

Urethan (ethyl carbamate) has been one of the most commonly used chemical carcinogens in studies of murine lung tumor development. It was first noted to be a carcinogen when C3H mice given urethan as an anestheia unexpectedly developed lung tumors. Subsequent study by Nettleship and co-workers confirmed its lung carcinogenicity [60]. It was later found to be carcinogenic in other mouse tissues [61] and other animal species [62]. It is not known if urethan is carcinogenic in humans.

The specific mechanism of the carcinogenic action of urethan is unknown. Like many chemical carcinogens, it is metabolized in the body to an active form which apparently binds to nucleic acids in the cells of the mouse lung and other organs [63]. The chemical is almost completely eliminated from the mouse body within 24 hours after administration.

Ionizing radiation studies. Limited studies have been conducted on the effects of ionizing radiation in murine lung carcinogenesis. The earliest report in the literature is by Furth and Furth who observed an increased incidence of lung tumors above controls in mice exposed to single or repeated large doses of x-rays [64]. However, mouse strain and experimental conditions were not specified. Later reports are more informative, but often conflicting. Lorenz showed a significant increase in lung tumor incidence in strain A mice chronically exposed to 8.8 rads daily of gamma radiation to a total dose of 2500 rads [65]. A similar result was observed in LAF_1 mice chronically exposed to 0.11 rads per day [66]. However, other reports have noted an inhibitory effect. Nowell and Cole observed that LAF_1 mice exposed to 800 rads of x-rays had a decreased lung tumor incidence below controls [67]. Upton made a similar observation in LAF_1 mice exposed to gamma radiation from an atomic weapon detonation. The lung tumor incidence appeared to decrease with increasing dose over the range of 0 to 697 rads [68]. Duhig observed that strain A mice given a fractionated schedule of exposure to total doses of either 500, 1000, or 1500 rads showed a reduced lung tumor incidence below controls at all three doses [69].

The results of these early reports are difficult to interpret. Properly defined control groups for comparison with experimental groups were not done in many of the studies. Also, the life shortening effects of radiation often were not taken into account, and appropriate age-adjustment corrections were done in only some of these investigations.

Even more limited is data concerning the carcinogenic influence of neutrons or other forms of densely ionizing radiation upon this animal system. Several reports investigated the influence of external exposures of densely ionizing radiation in the RF mouse strain. Upton observed a slightly increased incidence of pulmonary tumors in female mice given various doses of thermal neutrons [70]. Darden, however, failed to observed such an effect in females given various doses up to 400 rads of 14 Mev neutrons [71], as did Upton exposing mice to 1 and 5 Mev neutrons [72]. Clapp and co-workers reported a decreasing incidence of pulmonary tumors in female RF mice exposed to increasing doses of 60 Mev proton radiation [73]. Nowell and Cole reported high doses of fission neutrons decreased lung tumor incidence in LAF_1 mice below controls [67]. These early investigations provided qualitative, yet conflicting observations. None of these studies employed the lung tumor assay system used in later studies and in this investigation. Also, quantitative data on dose-response relationships are limited.

More recent studies have been better defined and quantitatively evaluated dose-response effects at low doses. Ullrich and co-workers observed an increased age-adjusted incidence of lung tumors in RFM mice over a 24 to 94 rad dose range with both chronic and acute

fission neutron exposure [74]. A more complex situation was observed in BALB/c mice [75]. After acute neutron exposure (5 R/min), lung adenoma incidence was lower than control levels over the 5 to 20 rad range, returned to control levels at 25 rads and remained at that level over the rest of the dose range. Chronic neutron exposure (1 rad/day) produced no reduced adenoma incidence at very low doses, and approximated that of controls except for a reduced incidence at a high total dose. Interestingly, in contrast to lung adenomas, the incidence of lung adenocarcinomas was greatly increased over the 5 to 20 rad range after acute neutron exposure, but not chronic exposure. Though lifespan information on the lung carcinogenic effects of fission neutrons is limited to these two mouse strains, a comparison of the two studies clearly suggests that the effects of low doses of fission neutrons varies among mouse strains. Also, within a mouse strain, the dose-response relationship may be quite complex, and vary as a function of both total dose and dose rate [75]. The BALB/c data suggests that dose effects may vary with the type of lung tumor as well.

Dose-response information on fission neutron effects upon lung tumorigenesis at a specific time point after treatment has apparently only been done with the RFM strain. Mice were given low dose neutron exposure locally to the thorax, and sacrificed nine months after treatment. The dose-response relationship over a dose range of 0 to 150 rads was reported by Ullrich and co-workers to show an increase in lung tumor incidence from 0 to 50 rads, a plateau between 50 to 100 rads, and a decline at higher doses [76]. The shape of this

dose-response relationship appears to be qualitatively similar to that reported by Yuhas and Walker [77] for the lung carcinogenic effects of x-rays in that over a 750 to 3000 rad dose range, they observed an increase in the tumor incidence and mean number of tumors per RFM mouse in the lower portion of the dose range, a peak at 1500 rads, and a gradual decrease to control levels at higher doses.

Investigations of a combination of agents.

1. Chemical carcinogens and ionizing radiation. Studies on the effects of a combination of a chemical carcinogen and ionizing radiation upon murine lung tumor development are few and have generally involved high treatment doses of both agents. Heston and co-workers observed a reduced lung tumor incidence in strain A mice given 900 rads of x-rays and nitrogen mustard compared to nitrogen mustard alone [78]. Similar results were reported by Foley and Cole in a series of papers which evaluated the combination of x-rays and urethan. A whole body dose of 880 rads given three hours before or up to one week after urethan was found to inhibit urethan carcinogenesis in the lungs of LAF_1 mice sacrificed 24 weeks after treatment [79]. This inhibition was again seen when the mice were given urethan and the same x-ray dose delivered only to the thorax [80]. This suggested to the authors that the inhibitory effect of the radiation was directly on lung, not a systemic effect. Further evaluation of this combination of agents at lower radiation doses revealed an enhancement. Three hundred rads of x-rays plus a carcinogenic dose of urethan increased the number of lung tumors per mouse [81]. Enhanced lung tumor incidence was observed when the same x-ray dose plus a low

subcarcinogenic dose of urethan were given [8]. Also, when the x-ray dose was given on a fractionated schedule of exposure in the same treatment combination, a greater number of lung tumors per mouse was observed compared to a single dose of 300 rads plus urethan. Cole and Foley concluded from their results that relatively low levels of ionizing radiation may be hazardous for lung tumorigenesis, particularly in conjunction with other carcinogenic or cocarcinogenic influences in the environment.

Little data exists on the interaction of densely ionizing radiation and a chemical carcinogen upon murine lung tumor development. Brightwell and Heppleston reported a significant inhibition of the capacity of urethan to induce pulmonary tumors in strain A mice exposed to radiation from inhaled plutonium dioxide [82]. Each mouse inhaled an approximate lung burden of 25 nanocuries of plutonium dioxide at the beginning of the experiment, and the lung was estimated to receive a total accumulated dose after 26 weeks of about 1000 rads. The inhibition of urethan tumorigenicity was concluded to be due to the high total dose of radiation, and to be similar to the effects of high doses of x-rays reported by Foley and Cole [79,80]. The influence of densely ionizing radiation at lower doses upon the lung carcinogenic effects of urethan, however, has been not evaluated and deserves investigation.

2. Urethan and butylated hydroxytoluene. The treatment combination of urethan and butylated hydroxytoluene has been investigated by Witschi and co-workers. As mentioned earlier, significant enhancement of lung tumorigenesis in Swiss Webster and A/J

mice from treatment with these agents was reported [9]. The experimental procedure consisted of the administration of a single carcinogenic dose of urethan followed one week later by a series of weekly BHT injections. Significant enhancement of adenoma formation was observed in Swiss Webster mice as early as 11 to 13 weeks after the start of treatment, and with as few as four BHT administrations [83]. It cannot be said whether the enhancing action of BHT involves either the acceleration of individual tumor growth or increasing the number of tumors actually formed. In two studies by Witschi and co-workers mice given urethan plus weekly BHT treatments developed a significantly increased lung tumor multiplicity at 14 weeks after the start of treatment compared to animals given urethan plus corn oil. Groups given urethan plus BHT, but sacrificed 24 weeks after urethan, also showed an increased lung tumor development compared to controls. This suggested that BHT did increase the actual number of lung tumors formed [83,84]. The increase in lung tumor multiplicity was greater in the first 14 weeks after the start of treatment in mice treated with urethan plus BHT than in controls given the carcinogen plus corn oil. In contrast, the increase in the following 10 weeks was greater in the controls. This suggested to the investigators that BHT may also accelerate lung tumor growth [83].

The specific mechanism of the BHT enhancement of urethan carcinogenicity in the murine lung is unknown. High doses of BHT are acutely toxic to murine lungs, causing necrosis of Type I alveolar cells [85]. Within two days after BHT, a cell proliferation response occurs. This response can be indirectly measured by the incorporation

of radioactive thymidine into pulmonary DNA [86]. A large portion of the proliferating cells are Type II alveolar cells which divide and differentiate into new Type I cells to replace the damaged cells. This proliferative response is not a direct effect of BHT, but a nonspecific adaptive reparative tissue reaction to many forms of toxic lung injury [87]. Repeated BHT-induced proliferation of lung cells initiated in the carcinogenic process by urethan was suggested by Witschi and co-workers as a explanation for the tumor enhancing influence of BHT [9].

Induced cell proliferation as the mechanism of tumor enhancement might apply to several earlier studies. DiPaolo observed that exposure to altered levels of oxygen after urethan enhanced lung tumorigenesis [88,89]. Strain A mice given a carcinogenic dose of urethan, then a single 48-hour exposure to 70% oxygen had a significantly greater number of lung tumors per mouse three months later [88]. Support for the possible involvement of induced lung cell proliferation in the DiPaolo study is suggested by Adamson and Bowden who reported that the exposure of mice for a few days to an atmosphere of 100% oxygen induced an extensive Type II cell proliferation [90].

Induced cell proliferation as an explanation for the tumor-promoting activity of BHT remains to be firmly established. If true, then it follows that other toxic stimuli to the lung such as ozone or elevated oxygen levels, proven to indirectly induce murine lung cell proliferation, might be likewise able to enhance urethan carcinogenicity.

3. Interaction of butylated hydroxytoluene with other agents.

Very little data exists on the interaction of BHT with chemical carcinogens other than urethan in murine lung tumorigenesis. Clapp observed in BALB/c mice given a brief DENA treatment plus dietary BHT for life an increased number of lung tumors per mouse at 18 months of age [91]. Recently, Witschi and co-workers evaluated the combination of either DMNA or 3-MCA and weekly BHT treatments. No enhancement was reported in Swiss Webster mice [92], but was in strain A [93].

Only one report could be located on the effects of a combination of ionizing radiation and BHT upon murine lung adenoma development. Clapp evaluated the tumorigenic effects of a whole body x-ray exposure of 250 rads and lifetime dietary BHT treatment in BALB/c mice. BHT treatment began three weeks prior to x-irradiation. At 18 months of age, an increased lung tumor incidence was observed with the radiation alone, while BHT + x-rays showed no such increase [49]. The influence of administering BHT subsequent to ionizing radiation, however, was not evaluated. Further investigation of the lung carcinogenic effects of a treatment combination of ionizing radiation and BHT is needed.

CHAPTER III

PURPOSE

The purposes of the work reported in this thesis were threefold:

(1) to examine the influence of BHT upon the lung carcinogenicity of urethan and fission neutrons;

(2) to examine the effects of a treatment combination of low doses of urethan and fission neutron radiation upon murine lung tumor development;

(3) to evaluate BHT-induced cell proliferation in the murine lung as a possible explanation for the tumor-promoting activity of this compound.

CHAPTER IV

MATERIALS AND METHODS

A. GENERAL METHODOLOGY

Mice

Two mouse strains were used in the experiments of this thesis. Specific-pathogen-free male BALB/c mice were obtained from the barrier facilities in the Biology Division, Oak Ridge National Laboratory. BALB/c mice are susceptible to the acute toxic lung effects of BHT [94], and were used for intercomparison with Swiss Webster mice.

Swiss Webster mice were purchased from Cumberland View Farms and Charles River Company. Upon arrival at the Biology Division, the animals underwent two weeks of quarantine to check for pathogens and overall health. Subsequently, they were transferred to conventional laboratory animal facilities. Swiss Webster mice are second to strain A in susceptibility to spontaneous development of lung tumors [7]. Tumor incidence in 12- to 18-month-old animals reaches 40 to 50% [57]. This strain was chosen because many of the experimental studies concerning BHT toxic effects and interaction with carcinogens in the murine lung have involved this mouse strain.

All mice were placed in groups of 10 in plastic cages with metal tops and a bedding of hardwood shavings. The type of cage bedding used was important since cedarwood shavings have been shown to block the toxic effects of BHT in murine lungs [95]. This inhibition may be related to the induction of drug metabolizing enzymes by cedar terpenes contained in the shavings. Water and laboratory chow were

provided ad libitum. Bedding, water, and food were changed twice weekly.

Chemical Treatments

Urethan (Sigma Chemical Company, St. Louis, Missouri) was dissolved in sterile 0.9% sodium chloride solution (Cutter Laboratories, Berkeley, California). Single intraperitoneal injections at a dosage of either 100 or 1000 mg/kg body weight (0.1 or 1.0 mg/ml) were administered via a 1 cc disposable syringe (Becton-Dickinson Company, Rutherford, New Jersey) with a 27 gauge needle. Equivalent volumes of sterile saline solution were given to appropriate controls.

Butylated hydroxytoluene (Sigma Chemical Company) was dissolved in corn oil (Mazola). All BHT administrations were given as intraperitoneal injections at a dosage of 300 mg/kg body weight (0.3 mg BHT/ml) via a 1 cc syringe with a 21 gauge needle. Injections of the corn oil vehicle alone at 10 cc/kg body weight were given to controls.

Radiation

Fission neutron radiation exposures were conducted at the Health Physics Research Reactor. The exposure conditions were identical to those described by Ullrich [76]. Briefly, a shield of 50 centimeters of borated paraffin backed by 1.25 centimeters of lead was used. Radiation exposure to the thoracic region was given via rectangular openings measuring 1.59 X 1.91 centimeters. The distance from the mouse thorax to the reactor core was 2 meters. At this distance the dose to the rest of the body behind the shield was 12% of the dose

through the collimator. Also, the ratio of neutron to gamma ray dose in air through the collimator with no shield is 6.7:1. The gamma ray component of the low dose exposures done in this study was small, and omitted from consideration.

Since extensive dosimetry was done previously, only routine monitoring of the neutron fluence was necessary. Therefore, neutron dosimetry for this study consisted of sulfur pellets placed beside the reactor core. Based on previous studies, the neutron air dose determined from the pellets was then multiplied by a conversion factor of 0.586 to give the value of the free space tissue kerma through the collimator at 2 meters distance from the core. The neutron dose absorbed by the thorax was calculated by multiplication of the free space tissue kerma by a factor of 0.96 [96].

The procedure for neutron irradiation was as follows. Mice were placed in polycarbonate tubes. The tubes were set in an upright position behind the shield, with the thoracic region of the mouse facing the reactor core through the rectangular openings. Single exposures of 10 and 50 rads of neutron radiation at a dose rate of approximately 1 and 5 rads/minute, respectively, were done. Fifteen mice were exposed per reactor run. The actual estimated neutron dose to the thorax (mean ± standard error) was 9.6 ± 0.2 rads for the low dose exposures and 48.9 ± 0.4 rads for the high dose treatment.

Determination of ^{14}C-thymidine incorporation

The procedure of indirectly detecting cell proliferation in tbe murine lung by measuring the incorporation of radioactive thymidine into pulmonary DNA was reported previously [86]. Briefly, the mouse

was intraperitoneally injected with a ^{14}C-thymidine (Becton-Dickinson Immunodiagnostics, Orangeburg, New York) solution of 0.5 microcurie in a volume of 0.2 ml sterile saline. Ninety minutes later the mouse was sacrificed by cervical dislocation. The lungs were removed, weighed, and then homogenized in a volume of 5 ml of ice-cold water. Two and one-half milliliters of cold 0.6 N perchloric acid ($HClO_4$) was then added. After centrifugation at 2100 rpm for 15 minutes at 4°C, the supernatant was discarded and the pellets washed three times with cold 0.2 N HCl04. The pellets were then treated for 20 minutes at 75-80°C with 4 ml of 0.5 N $HClO_4$ to extract the pulmonary DNA. This extract was centrifuged at 2000 rpm at room temperature for 10 minutes and the supernatant placed into clean tubes. Two milliliters of extract were mixed with 10 ml of Aquasol (New England Nuclear Company) for liquid scintillation counting. Adjusting for background, the activity of the extracted pulmonary DNA was determined as disintegrations/minute (dpm).

The DNA content of the extract was determined using the diphenylamine method described by Burton [97]. One-half milliliter of the extract was added to 3.5 ml of freshly prepared diphenylamine reagent. This reagent consists of 1.5 g diphenylamine, 100 ml of glacial acetic acid, 1.5 ml of concentrated 10 M sulfuric acid, and 0.5 ml 2% acetaldehyde. The tubes were allowed to sit overnight (16-20 hr) at room temperature, and the absorbance (OD) was measured on a spectrophotometer at a wavelength of 600 nm. Different known DNA concentrations were also measured to establish a standard curve of OD versus DNA concentration. Comparison of the lung sample OD values to

this standard enabled determination of the DNA content of the samples. The incorporation of ^{14}C-thymidine into pulmonary DNA was measured as dpm/mg DNA.

Lung Clearing and Tumor Counting

Mice were sacrificed by cervical dislocation six months after the start of treatment. The thorax was opened to expose the trachea and thoracic viscera. The lungs were inflated with Tellyesniczky's solution given via a 3 cc syringe with a 25 gauge needle inserted into the trachea. The lungs were removed and subsequently processed through a staining and clearing technique described previously [98].

The procedure for the counting of lung tumors was as follows. Cleared lungs were separated into the individual lobes and observed for tumors under a dissection microscope. Questionable lung areas were blocked in paraffin and processed onto slides of sections stained with hematoxylin and eosin. Light microscopic observation of the slides was done to establish final diagnosis. The gross and microscopic characteristics of lung adenomas were described earlier and served as the criteria for identifying and distinguishing lung tumors from infection and artifacts. Lung tumor data were quantitated as the tumor incidence and tumor multiplicity or mean number of tumors per mouse.

Statistical Analysis

The tumor multiplicity values were analyzed first by an overall analysis of variance, using the method described by Snedecor and Cochran for samples of unequal sizes [99]. The null hypothesis was

that the means of a collection of treatment groups were not significantly different from each other. A variance ratio value greater than the critical value at a probability level of 0.05 was considered significant.

If the overall analysis of variance was significant, the treatment group means were further analyzed by Kramer's extension of Duncan's multiple range test for group means with unequal numbers of samples [100]. The numerical difference between any two means was compared against a critical value computed partially from a list of significant studentized range numbers at a probability level of 0.05. If the difference between any pair of means exceeded the critical value, the two means were considered significantly different. The analysis by this method was done with the assistance of George Cotsonis of the Computer Science Department, Biology Division, Oak Ridge National Laboratory.

Evaluation of ^{14}C-thymidine incorporation data consisted of testing for significant differences between BHT treatment group values against corn oil controls using a Student's t-test procedure described by Snedecor and Cochran [101] at a probability level of 0.05.

B. EXPERIMENTAL SERIES

Experiment 1

The objective of this experiment was to evaluate the influence of BHT upon lung tumor development in mice given urethan. The treatment groups and details of this experiment are presented in Table A.1 (see Appendix I). Briefly, groups of six-week-old male Swiss Webster mice

were initially given injections of urethan at a dosage of 100 mg/kg or 1000 mg/kg, or of saline. One week later half of the animals of both urethan-treated groups were started on a series of eight weekly BHT administrations at a dosage of 300 mg/kg. A control group receiving BHT only was also begun. All mice were sacrificed six months after the start of treatment.

Experiment 2

The objective of this experiment was to evaluate the influence of a treatment combination of fission neutron radiation and BHT upon lung tumor development in the Swiss Webster mouse. Treatment groups and details of this experiment are presented in Table A.2 (see Appendix I). Briefly, groups of six-week-old Swiss Webster mice were given fission neutron doses of either 10 or 50 rads locally to the thorax. Two weeks later half of the animals of both dose groups were begun on a series of weekly BHT administrations. The other half received weekly corn oil treatments. An additional set of control groups receiving either BHT or corn oil treatments only were also included. The animals were sacrificed six months after the start of treatment.

Experiment 3

The objective of this experiment was to study the effects of a treatment combination of fission neutrons and urethan upon murine lung adenoma development. The details of this experiment are presented in Table A.3 (see Appendix I). Briefly, groups of six-week-old Swiss Webster mice were given injections of a low dose (100 mg/kg) of urethan. Controls received saline only. Two weeks later the animals

were either given fission neutron doses of either 10 or 50 rads locally to the thorax or sham-irradiation. The mice were sacrificed six months after the start of treatment.

Experiment 4

The objective of the experiment was to further evaluate BHT-induced cell proliferation in the murine lung. Two mouse strains, Swiss Webster and BALB/c, were used in this experiment for intercomparison. Specific treatment groups and other details of this experiment are shown in Table A.4 (see Appendix I). Briefly, groups of seven- to eight-week-old male Swiss Webster and BALB/c mice were begun on a series of weekly BHT treatments at a dose of 300 mg/kg body weight. Control groups received corn oil injections. Two days after each BHT treatment, eight mice were taken from each group and received injections of ^{14}C-thymidine. The lungs were subsequently evaluated by the procedures described earlier to measure the incorporation of the radioisotope into pulmonary DNA.

CHAPTER V

RESULTS

A. EXPERIMENT ONE

The data on the lung tumorigenic effects of a treatment combination of urethan plus BHT are summarized in Table B.1 (see Appendix II). The high dose of urethan (1000 mg/kg) significantly increased the mean number of pulmonary tumors per mouse compared to untreated controls. Further, BHT administrations for eight weeks produced a significant increase in the lung carcinogenic effects of the high urethan dose. A low dose of urethan (100 mg/kg) caused a slight, but significant increase in pulmonary tumor development compared to untreated controls. Weekly BHT treatments, however, did not significantly enhance low dose urethan carcinogenesis above that of urethan treatment alone.

B. EXPERIMENT TWO

The effects of fission neutron radiation and a combination of fission neutrons plus BHT on lung tumorigenesis are presented in Table B.2 (see Appendix II). Ten and 50 rads of fission neutrons did not significantly increase the lung tumor multiplicity from that of unirradiated controls. Furthermore, weekly BHT treatments did not significantly increase the lung carcinogenic effects of either dose of fission neutrons.

C. EXPERIMENT THREE

The results of the urethan plus fission neutrons study are presented in Table B.3 (see Appendix II). As discussed earlier, the low dose of urethan (100 mg/kg) alone significantly increased the mean number of pulmonary tumors per mouse. Low doses of fission neutrons (10 and 50 rads), however, did not significantly influence lung tumor development in either saline controls or urethan-treated mice. This suggests that the significantly increased lung tumor multiplicity in mice given urethan plus fission neutrons was due entirely to the urethan treatment.

D. EXPERIMENT FOUR

The data on the influence of weekly BHT treatments on lung cell proliferation are summarized in Table B.4 (see Appendix II). Following the first BHT administration significant cell proliferation in the lung, as indicated by ^{14}C-thymidine incorporation into pulmonary DNA, occurred in both BALB/c and Swiss Webster mice. However, after the second weekly BHT treatment, ^{14}C-thymidine incorporation levels in Swiss Webster mice were equivalent to corn oil controls. A reduced but still significant increased incorporation occurred in the BALB/c strain. Following a third BHT treatment no significant thymidine incorporation occurred in either mouse strain.

Since further weekly BHT administrations did not elicit significant cell proliferation in the murine lung, the experimental procedure was altered by varying the length of time before the next

BHT or corn oil injection was given to determine if significant thymidine incorporation (indicating cell proliferation) might again be induced by BHT. Significant thymidine incorporation occurred in both mouse strains following BHT treatment given two weeks subsequent to a previous injection. Similar results were observed when intervals of three and four weeks elapsed between BHT administrations. Finally, other treatment groups were given a fourth BHT or corn oil injection four weeks subsequent to the last of three weekly administrations. A fifth injection was given one week later. Again no significant thymidine incorporation occurred in either strain following this successive weekly BHT administration.

CHAPTER VI

DISCUSSION

Urethan Plus Butylated Hydroxytoluene

Butylated hydroxytoluene significantly enhanced the lung tumor multiplicity in Swiss Webster mice pretreated with 1000 mg/kg urethan. A similar but not statistically significant increase occurred in mice given 100 mg/kg of urethan. Although the mean number of pulmonary tumors per mouse in either the low or high dose urethan plus BHT groups of this study are higher than those reported by Witschi and Lock [84] in similar treatment groups evaluated at 24 weeks after urethan, the results of this experiment are qualitatively similar to their data. This study, therefore, independently confirms Witschi's observations of BHT enhancement of the lung tumorigenic effects of high doses, but not low doses of urethan.

In view of his findings on the influence of BHT upon urethan lung carcinogenicity, Witschi has proposed that the two-stage theory of carcinogenesis might apply to murine lung adenoma development [84]. Further support for this is suggested by reports of phorbol [41] and saccharin [43] enhancement of lung tumorigenesis in mice. Although BHT does not fit all the criteria of a "promoter" defined from mouse skin studies, there are several lines of evidence which support this. First, BHT was observed to enhance lung tumorigenesis only when given subsequent to urethan [9]. Enhancement occurred even if BHT was not administered up to five months after urethan [92]. Enhancement was observed regardless of the route of administration of BHT, whether by

injection [84], gavage [102], or diet [103]. BHT enhanced the lung carcinogenicity of other chemicals, namely 3-MCA and DMN, in strain A mice [93].

On the other hand, several observations tend to limit the definition of BHT as a promoter. First, it did not enhance the effects of low doses (≤100 mg/kg) of urethan in this study as well as in an earlier study by Witschi [83]. Second, no enhancement occurred in mouse strains, such as the BALB/c, which are less susceptible to urethan tumorigenesis [83]. Third, no enhancement of carcinogenesis after treatment with other carcinogens (i.e., 3-MCA or DMNA) was observed in Swiss Webster mice [92]. Lastly, a significantly increased urethan tumorigenesis occurred in A mice given urethan two weeks subsequent to a single BHT administration. This would appear to conflict with an earlier report by the same investigator of enhanced urethan lung tumor development occurring only when BHT was given subsequent to urethan [9]. This earlier investigation, however, used a different mouse strain (Swiss Webster), and studied a time interval of only up to seven days between BHT and urethan treatments. The basis for the enhanced urethan tumorigenesis subsequent to BHT is unknown. Bojan and co-workers have also reported a significantly increased pulmonary tumor development in female CFLP mice when urethan was given six or seven days after BHT [104]. This enhancement was also dependent on the dose of BHT. Whether increased lung tumorigenesis might occur in other mouse strains when urethan is administered subsequent to BHT is unknown, and deserves further investigation.

Neutrons Plus Butylated Hydroxytoluene

BHT did not enhance lung tumor development in mice exposed to low doses of fission neutrons. An explanation for this finding is unknown. However, several points should be considered. First, the neutron doses may have been too low to be influenced by the repeated BHT treatments. This might be analogous to the lack of BHT enhancement of low doses of urethan observed in this study. Fifty rads of neutron radiation by itself failed to produce even a slight tumorigenic effect. In contrast, the same neutron dose greatly increased both the lung tumor incidence and tumor multiplicity in RFM mice [76]. Previous studies [74,76] have indicated lung tumor development in the RFM strain is sensitive to low doses of fission neutrons. Similar information on the Swiss Webster strain was previously unknown. This study suggests that lung tumorigenesis in this mouse strain is relatively insensitive to low doses ($\leq$50 rads) of fission neutrons.

A second reason for the lack of an observed effect of BHT after neutron irradiation might be the time interval used in this study. Six months may not have been sufficient time for the effects of the treatment combination upon lung tumorigenesis to become apparent. This might also apply to low doses of urethan plus BHT. However, based upon data from other mouse strains this argument does not appear to apply. Ullrich observed that the number of lung tumors per RFM mouse plateaus six months after either x-ray or fission neutron radiation [76]. Yuhas reported a similar observation in RFM mice given urethan [105]. Shimkin and Polissar noted in strain A mice the

number of urethan-induced pulmonary tumors at the lung surface plateaued six months after treatment [106]. Whether similar observations might occur in Swiss Webster lung tumorigenesis following either urethan or fission neutron treatment is unknown.

The individual experimental variables in the fission neutron radiation plus BHT study need better definition before the results of this study can be properly interpreted. Definition of the fission neutron dose-response relationship for the Swiss Webster mouse, enabling use of a radiation dose which increases tumor multiplicity, is needed. A time point dose-response study similar to the Ullrich RFM mouse study [76] mght be done. Alternatively, a lifespan study similar to those on the BALB/c [75] and RFM [74] mouse might be done to gain data on the development of lung tumors and possibly other types of tumors that could be useful for intercomparison with other mouse strains. The effects of fission neutrons plus BHT in other mouse strains might also be examined. As discussed earlier, Witschi and Kehrer reported no enhancement of lung tumorigenesis in Swiss Webster mice four months after a treatment of either DMNA or 3-MCA plus four subsequent BHT administrations [92]. This might be analogous to the failure of BHT to enhance neutron carcinogenesis in this study. A similar experiment, however, using strain A/J mice showed enhancement by BHT [93]. One option, therefore, might be to investigate the neutrons plus BHT combination using strain A mice. However, this study would be limited by a lack of data concerning neutron radiation influence upon lung tumorigenesis in this strain. Fission neutron dose-response data is available on the RFM mouse

[76], and suggests a second alternative of using this mouse strain. However, information on BHT effects in this strain is lacking.

Urethan and Fission Neutrons

The slight increase in lung tumor development in the experimental group given a treatment combination of low doses of urethan and fission neutrons was apparently due to the urethan treatment alone. As with the neutrons plus BHT experiment, an explanation for the lack of a neutron carcinogenic effect is unknown. No previous reports in the literature have evaluated the treatment combination of urethan and fission neutrons in the murine lung tumor system. Since the neutron doses in this study did not induce a significant lung tumorigenic effect, it might be considered to use higher doses of fission neutrons. However, the limited dose-response data available on fission neutron lung carcinogenesis in the mouse [76] suggests that any quantitative increases in tumorigenesis induced by densely ionizing radiation may be small compared to the increases in tumor multiplicity produced by urethan. Thus, significant neutron-induced changes may be difficult to detect. Using higher neutron doses is further complicated by an increasing probability of inhibitory effects upon lung tumor development. This would be analogous to the inhibition of urethan tumorigenesis by high doses of x-rays reported by Foley and Cole [79,80].

Modification of the experimental variables of the urethan plus fission neutrons study might be done as a way of better examining any interactions. One variable which may be important is the mouse strain used. This is because few mouse strains have been shown to be

sensitive to lung tumorigenesis at relatively low doses of neutrons. The LAF_1 mouse might be studied. As discussed earlier, enhanced lung tumorigenesis was reported in this strain from a treatment combination of urethan and x-rays [8]. Dose-response information on fission neutron lung carcinogenesis, however, in this mouse is lacking. A second alternative might be the RFM strain. As discussed earlier, studies have indicated that this strain is sensitive to lung tumor induction after neutron exposures [74,76]. Dose-response data on the lung carcinogenic effects of urethan [104] and fission neutrons [76] in RFM mice are available and would be useful for suggesting doses of both agents for a treatment combination study.

Another variable for possible investigation might be the influence of altering the sequence of treatments. The lung tumor enhancement reported by Foley and Cole occurred using a treatment sequence of ionizing radiation followed by urethan [8]. This suggests that a revised treatment sequence of fission neutrons, then urethan, might be investigated.

BHT-induced Cell Proliferation and Tumor Enhancement

Significant cell proliferation in the murine lung, as measured by the level of radioactive thymidine incorporation, was detected after the first BHT treatment, but not after further weekly injections. Importantly, this occurred in both BALB/c and Swiss Webster strains, suggesting this observation might apply to other mouse strains where acute BHT toxic effects and induced cell proliferation in the lungs have been reported [94]. Although a total of only three weekly BHT treatments was done in this study, and whether the lack of significant

cell proliferation would continue after further weekly administrations of BHT is not known, a recent study by Witschi [107] indicates that this lack of BHT-induced cell proliferation persists. In this study pulmonary labeling indices were used to measure cell proliferation following each of a series of eight weekly BHT administrations. A significant proliferation was detected after the first BHT treatment, but substantially reduced or no proliferation following each of seven more weekly treatments.

An explanation for the lack of induced cell proliferation in the mouse lung following further weekly BHT treatments is unknown. It might be suggested that the murine alveolar epithelium one week after an initial BHT insult is in a state resistant or insensitive to a second BHT treatment. The reparative and proliferative processes of the murine lung following a toxic BHT insult occur over a nine day period [85]. Proliferation of Type II cells, occurring mainly between days 2 and 5, is followed by differentiation to new Type I cells. Perhaps these new young Type I cells, while progressing towards full development, are insensitive for a period of time to BHT toxicity. This state might be analogous to the lungs of very young mice less than three weeks old which Malkinson observed to be insensitive to high doses of BHT [95]. If so, then exposure to a second BHT treatment one week after the first would presumably cause limited, if any, damage to the lung and hence no induced cell proliferation. However, with the passage of another week, these young Type I cells would presumably reach full differentiation and become sensitive to BHT toxicity. The rest of one week would then presumably enable a

third weekly BHT treatment to adversely affect alveolar epithelium, and thus induce cell proliferation. However, no proliferation was detected in this study following a third BHT administration.

An alternative explanation relates to the induction of drug metabolizing enzymes by BHT. BHT apparently requires conversion to an active form which mediates its acute toxic lung effects [108]. BHT is catabolized in the rodent liver to a number of forms excreted in the feces and urine [109]. It is possible that early successive weekly BHT treatments might induce a heightened activity of drug metabolizing enzymes, maintained by BHT treatments given later, where detoxification is favored over metabolic activation of BHT. This might be analogous to the reported effect of BHT and certain other agents of reducing the carcinogenicity of various chemicals when given prior to or concurrently with a chemical carcinogen [45].

Significant cell proliferation in the murine lung has been reported to occur following toxic lung damage caused by a number of inhaled or bloodborne agents [87]. Such studies have generally involved single dose exposures to the toxic agent. The effects of a series of treatments of a toxic agent, as done in this study, has not been evaluated. It is possible that the lack of cell proliferation following each of further weekly BHT treatments might occur with other toxic agents. This needs to be further studied. A simple comparative study might be done evaluating the effects of a series of weekly 100% oxygen exposures upon lung cell proliferation versus BHT-treated mice.

As mentioned earlier, repeated BHT-induced proliferation of lung cells in urethan-treated mice was suggested by Witschi as an

explanation for the tumor enhancing influence of BHT [9]. The results of the ^{14}C-thymidine incorporation experiment and of the Witschi pulmonary labelling index study [107] suggest that BHT enhancement of murine lung tumorigenesis is not entirely related to its induction of lung cell proliferation. Recent work by Witschi and co-workers appears to further support the dissociation of induced cell proliferation from tumor enhancing action of BHT. In one study [92] Witschi and Kehrer found that doses of BHT which were too low to acutely damage the lung and induce cell proliferation still enhanced lung adenoma development. Also, Swiss Webster mice given urethan and subsequent concurrent treatment of BHT plus SKF-525A or piperonyl butoxide showed an increased lung tumorigenesis. SKF-525A and piperonyl butoxide are inhibitors of drug metabolizing enzymes, and were reported to prevent the acute toxic lung effects of high BHT doses, presumably by blocking the metabolic conversion of BHT to its active toxic form. As a result, no cell proliferation occurred [108]. Furthermore, mice given urethan and then six weekly 72-hour exposures to 100% oxygen, a treatment reported by Adamson [90] to induce significant lung cell proliferation, showed no enhancement of lung adenoma development four months after treatment. This last observation appears to be in contrast with the reports by DiPaolo [88,89], discussed earlier, which noted exposure to altered oxygen levels after urethan enhanced lung tumor development. However, single 48-hour exposures to altered levels of oxygen immediately following urethan administration were done, and thus these reports cannot be directly compared with Witschi and Kehrer.

It has been further shown by Witschi and co-workers [93] that methylcyclopentadienyl manganese tricarbonyl (MMT), an agent capable of inducing murine lung cell proliferation, failed to enhance urethan tumorigenesis in strain A mice. MMT injures lung tissue, particularly Clara cells, and induces significant cell proliferation within two days after a single administration [110]. The cell kinetics of this proliferation, however, are presently unknown. The antioxidant properties of BHT in relation to tumor enhancement were also evaluated. Mice given urethan plus the antioxidant butylated hydroxyanisole (BHA) or Vitamin E did not show lung tumor enhancement. Based upon the accumulated data of the two studies [92,93], Witschi concluded that the effects of BHT on lung tumor development in susceptible mice are not related to either its properties as an antioxidant or its capability to produce cell proliferation in the lung.

Another possible explanation for BHT tumor enhancement might be an alteration of the metabolism of urethan. As discussed earlier, BHT induces an increased activity of hepatic drug metabolizing enzymes, and thus might affect urethan metabolism. However, available data does not support this. Mice given 13 weekly BHT administrations prior to urethan did not show lung tumor enhancement four months after urethan [84]. Also, the administration of BHT at various time intervals before or after urethan was only found to influence lung tumorigenesis when given seven days after urethan [9]. Since urethan is rapidly eliminated from the mouse within 24 hours, it seems

unlikely that BHT, given one week after urethan, would affect its metabolism.

A question of interest which arises from all of these studies is whether the sensitivity of a tissue to neoplastic transformation can be altered by agents which induce cell proliferation. Although, according to Oehlert, there is a lack of correlation between the magnitude of cell turnover and the frequency of tumor development in many tissues [111], there is evidence for hepatic tumorigenesis which suggests that induced hyperplasia can alter tissue sensitivity to carcinogenic agents. Chernozemski and Warwick observed an enhanced incidence of hepatic tumors in male B6AF mice given urethan shortly after partial hepatectomy [112]. Partial hepatectomy induces a burst of DNA synthesis and hepatocyte proliferation occurring within several days after the operation. A similar enhancement of urethan tumorigenesis of the liver in Hall mice was observed by Pound and Lawson [113]. Induced hyperplasia has also been reported to alter hepatic sensitivity to radiation carcinogenesis. Wiley and co-workers observed partial hepatectomy shortly before exposure to gamma or fission neutron radiation to significantly enhance liver tumor incidence in LAF_1/Jax mice [114]. Enhancement was still observed even when partial hepatectomy occurred 8-13 weeks after irradiation.

Whether induced hyperplasia affects murine lung sensitivity to transformation is less clear. Urethan given during BHT-induced cell proliferation did not result in an enhanced pulmonary tumorigenesis in studies by Witschi and co-workers [9,93]. In contrast, Bojan and coworkers reported significant lung tumor enhancement when urethan was

given subsequent to either BHT or paraquat [104]. The influence of exposure to other carcinogens, especially ionizing radiation, during induced lung cell proliferation is unknown. A study of radiation carcinogenic effects might be done using a sequence of a single large dose of BHT to induce cell proliferation followed by exposure to various doses of fission neutrons or other radiation. This would be a modification of the procedure used by Meyer and Ullrich in a study on the effects of x-rays and fission neutrons on BHT-induced cell proliferation in the BALB/c lung [115]. Since the lung carcinogenic effects of fission neutrons and gamma rays in this strain have been characterized in previous reports [76,116], this mouse strain may be a suitable animal model for a BHT plus ionizing radiation study.

Conclusions

1. Butylated hydroxytoluene (BHT) significantly enhanced pulmonary tumorigenesis in male Swiss Webster mice given a carcinogenic dose of urethan (1000 mg/kg), but did not significantly enhance the lung tumorigenic effects of a low dose of urethan (100 mg/kg).

2. Butylated hydroxytoluene did not influence the lung carcinogenic effects of fission neutrons over the 0 to 50 rad dose range (i.e., 10 and 50 rads).

3. Doses of fission neutron radiation of 10 and 50 rads did not influence lung tumorigenesis in urethan-induced mice.

4. Significant cell proliferation in the lung occurred in both BALB/c and Swiss Webster mice following a single large dose administration of BHT (300 mg/kg).

5. Additional weekly administrations of BHT did not induce significant lung cell proliferation in either mouse strain.

6. Significant cell proliferation in the lungs of both mouse strains was again induced by BHT when administration of the chemical was delayed two weeks or longer after a previous treatment.

Recommendations

The following recommendations are made to suggest further topics of investigation and possible means of improving the informational background of the experiments reported in this thesis.

1. A quantitative dose-response study of the lung carcinogenic effects of fission neutron radiation in Swiss Webster mice. This might be done as a time point and/or lifespan investigation.

2. Modification of one or more of the experimental variables in the fission neutrons plus BHT, and urethan plus fission neutrons treatment combination studies. Suggestions include a longer time interval of study; substitution of another mouse strain; the use of higher doses of fission neutrons; and evaluating the reverse treatment sequence of fission neutrons, then urethan.

3. An investigation of the influence of a series of weekly treatments of other toxic pulmonary agents upon cell proliferation in the murine lung.

4. A study investigating whether the sensitivity of murine lung tissue to neoplastic transformation by ionizing radiation is altered when in a proliferative stage.

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APPENDICES

APPENDIX I

TABLE A.1

EXPERIMENT ONE: URETHAN + BHT

Treatment groups
1) None
2) Saline (10 ml/kg)
3) BHT (300 mg/kg), eight weekly injections
4) Urethan (100 mg/kg)
5) Urethan (100 mg/kg) + (one week later) BHT (300 mg/kg), eight weekly injections
6) Urethan, 1000 mg/kg
7) Urethan, 1000 mg/kg + (one week later) BHT (300 mg/kg), eight weekly injections

TABLE A.2

EXPERIMENT TWO: FISSION NEUTRONS + BHT

Treatment groups
1) None
2) BHT (300 mg/kg), eight weekly injections
3) Corn oil (10 ml/kg), eight weekly injections
4) Fission neutrons (10 rads) + (one week later) BHT (300 mg/kg), eight weekly injections
5) Fission neutrons (10 rads) + (one week later) corn oil (10 ml/kg), eight weekly injections
6) Fission neutrons (50 rads) + (one week later) BHT (300 mg/kg), eight weekly injections
7) Fission neutrons (50 rads) + (one week later) corn oil (10 mg/kg), eight weekly injections

TABLE A.3

EXPERIMENT THREE: URETHAN + FISSION NEUTRONS

Treatment groups
1) None
2) Saline
3) Urethan (100 mg/kg)
4) Urethan (100 mg/kg) + (two weeks later) Fission neutrons (10 rads)
5) Urethan (100 mg/kg) + (two weeks later) Fission neutrons (50 rads)
6) Saline + (two weeks later) Fission neutrons (10 rads)
7) Saline + (two weeks later) Fission neutrons (50 rads)

TABLE A.4

EXPERIMENT FOUR: BHT-INDUCED LUNG PROLIFERATION

Mouse Strain	Treatment
Swiss Webster	BHT (300 mg/kg), once weekly
Swiss Webster	Corn oil (10 ml/kg), once weekly
BALB/c	BHT (300 mg/kg), once weekly
BALB/c	Corn oil (10 ml/kg), once weekly

APPENDIX II

TABLE B.1

LUNG TUMOR INCIDENCE AND LUNG TUMOR MULTIPLICITY IN SWISS WEBSTER MICE TREATED WITH URETHAN AND/OR BHT

Treatment	Number of Animals	Lung Tumor Incidence (%)	Lung Tumor Multiplicity (Mean ± Standard Error)
None	26	15.4	0.15 ± 0.07
Saline	30	23.3	0.27 ± 0.10
BHT	29	24.1	0.38 ± 0.15
Urethan (100 mg/kg)	29	62.0	1.34 ± 0.32[a]
Urethan (100 mg/kg) + BHT	27	100.0	2.30 ± 0.39[c]
Urethan (1000 mg/kg)	29	100.0	18.55 ± 1.95[a]
Urethan (1000 mg/kg) + BHT	32	100.0	29.80 ± 3.37[b,c]

[a] Significantly different from saline control at a P = 0.05.

[b] Significantly different from urethan (1000 mg/kg).

[c] Significantly different from BHT control.

TABLE B.2

LUNG TUMOR INCIDENCE AND LUNG TUMOR MULTIPLICITY
IN SWISS WEBSTER MICE TREATED WITH
FISSION NEUTRONS AND/OR BHT

Treatment	Number of Animals	Lung Tumor Incidence (%)	Lung Tumor Multiplicity[a] (Mean ± Standard Error)
None	26	15.4	0.15 ± 0.07
BHT	29	24.1	0.38 ± 0.15
Corn Oil	26	30.8	0.38 ± 0.13
Neutrons (10 rads) + BHT	43	23.2	0.26 ± 0.08
Neutrons (10 rads) + Corn oil	25	16.0	0.20 ± 0.10
Neutrons (50 rads) + BHT	38	23.7	0.34 ± 0.12
Neutrons (50 rads) + Corn oil	20	15.0	0.15 ± 0.18

[a]No significant difference among the means was indicated at a P = 0.05.

TABLE B.3

LUNG TUMOR INCIDENCE AND LUNG TUMOR MULTIPLICITY
IN SWISS WEBSTER MICE TREATED WITH
URETHAN AND/OR FISSION NEUTRONS

Treatment	Number of Animals	Lung Tumor Incidence (%)	Lung Tumor Multiplicity (Mean ± Standard Error)
None	26	15.4	0.15 ± 0.07
Saline	30	23.3	0.27 ± 0.10
Urethan (100 mg/kg)	29	62.0	1.34 ± 0.32[a]
Urethan (100 mg/kg) + Neutrons (10 rads)	20	60.0	1.20 ± 0.38[a]
Urethan (100 mg/kg) + Neutrons (50 rads)	28	71.4	1.75 ± 0.30[a]
Saline + Neutrons (10 rads)	29	21.4	0.28 ± 0.10
Saline + Neutrons (50 rads)	25	16.0	0.24 ± 0.12

[a]Significantly different from saline controls at a P = 0.05.

TABLE B.4

^{14}C-THYMIDINE INCORPORATION IN THE LUNGS OF SWISS WEBSTER AND BALB/C MICE FOLLOWING WEEKLY BHT OR CORN OIL TREATMENT

Total Number of Weekly Adminstrations of BHT or Corn oil/Time Between Successive Administrations of BHT or Corn Oil	^{14}C-Thymidine Incorporation (dpm/mg DNA)[a,b]			
	Swiss Webster BHT	Swiss Webster Corn Oil	BALB/c BHT	BALB/c Corn Oil
1 Experiment 1	2100 ± 586[c]	488 ± 110	4059 ± 1136[c]	270 ± 54
Experiment 2	2066 ± 450[c]	—	4028 ± 918[c]	—
2	754 ± 110	731 ± 108	908 ± 184[c]	322 ± 27
3	498 ± 91	302 ± 82	393 ± 101	278 ± 27
4 (two weeks after the third administration)	2749 ± 784[c]	485 ± 101	3189 ± 958[c]	249 ± 25
4 (three weeks after the third administration)	1890 ± 710[c]	263 ± 48	3170 ± 741[c]	213 ± 31
4 (four weeks after the third administration)	1366 ± 733	356 ± 76	2098 ± 773[c]	225 ± 12
5 (one week after the fourth administration)	324 ± 70	535 ± 123	474 ± 84	693 ± 402

[a] All values represent the mean ± standard error of the mean.

[b] Eight mice per data point.

[c] Significantly different (≤ 0.05) from corn oil controls.

VITA

William James Gerhardt was born in Philadelphia, Pennsylvania on July 15, 1953. He attended elementary schools in that city and was graduated from Central High School in June 1971. The following September he entered the Pennsylvania State University, and in May 1975 he received a Bachelor of Science degree in Biology.

In September 1978 he accepted a teaching assistantship at the University of Tennessee, Knoxville and began study towards a Master of Science degree with a major in Radiation Biology. The degree was awarded in December 1981.

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