A REVIEW OF LONG-TERM INHALATION STUDIES WITH CIGARETTE SMOKE IN LABORATORY ANIMALS.

Christopher R.E. Coggins, Ph.D., DABT

Research & Development, R.J. Reynolds Tobacco Co., Winston-Salem, NC 27106.

ABSTRACT

A recent review (IARC, 1985) has stated *there is sufficient evidence that inhalation of cigarette smoke as well as application of tobacco smoke condensate cause cancer in experimental animals*. The data presented in this earlier review on inhalation studies are very limited (less than 20 papers). The present document constitutes an exhaustive review of the literature on long-term inhalation studies with cigarette smoke. Based on the evidence accumulated during this review, the conclusion is reached that the IARC evaluation of "sufficient evidence" is incorrect.

INTRODUCTION

A number of inhalation studies have been performed with cigarette smoke and experimental animals. This article will review the work that has been published over the last 20 years on inhalation studies with cigarette smoke. The most commonly used animals are rodents: rats, mice, hamsters (Chinese, Syrian-Golden and European), guinea pigs, and rabbits. There is relatively little published work on inhalation studies in larger animals such as dogs (see below for a discussion on tracheostomy) and primates. The review will consider the individual studies used with each of the main species, with the induction of pulmonary neoplasia as the sole concern. Within the different species various strains have been used (particularly for mice).

Reviews of the earlier literature are available (UICC, 1976; Wehner, 1983; Pepelko, 1984). The present document does not include short-term exposures, restricting the review to those studies with durations of at least several months. The decision on whether to include a

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study or not in such a review has already been discussed (Pepelko, 1984), varying primarily with the species of experimental animals being used.

There are several key areas that were examined in each of the studies: the cigarettes used, smoke chemistry data, the types of inhalation exposures (including the availability of deposition data), and the similarity (if any) of the histopathological changes produced with those reported in human smokers.

Cigarettes; smoke chemistry

The cigarettes used by many of the groups in this review were unfiltered and had very high yields of "tar" and nicotine. They are, thus, substantially different from cigarettes commercially available today. A typical cigarette used is the University of Kentucky 1R1 reference cigarette, made in 1969, which has a "tar" yield of 34.3 mg and a nicotine yield of 2.16 mg. The latest reference cigarette from the University of Kentucky, the 1R5F, has a sophisticated filter which results in a "tar" yield of 1.6 mg and a nicotine yield of 0.16 mg.

Few of the studies made measurements on the chemical composition of the smoke generated, relying instead on nominal dilutions of published yields of reference cigarettes.

Inhalation Technology

Most of the papers with rodents used nose-only exposure systems. Some of the smoking machines used single cigarettes in an attempt to mimic human smoking (Guerin et al., 1979; Griffith & Standafer, 1985). Others used large numbers of cigarettes on a rotating carousel, whereby a constant stream of smoke is generated and distributed (Baumgartner & Coggins, 1980). Some of the larger devices allowed the re-breathing of exhaled smoke by animals "downstream" (Henry et al., 1985). The manner in which the mainstream smoke was generated also varied.

In only a few of the inhalation studies were deposition data presented. It is well known that animals when exposed to cigarette smoke are capable of varying degrees of breath-holding

(Coggins et al., 1982; Coggins, 1985), a problem that is particularly pronounced in non-continuous or "bolus" exposures. Minimally, measures of blood carboxyhemoglobin (COHb) should be included to demonstrate that smoke was in fact inhaled, ideally accompanied by measures of the deposition of constituents of the smoke particulate phase such as plasma nicotine and cotinine. Some of the more complete studies used radiolabelled markers thereby providing definitive data on the amounts of deposition of "tar" in the lungs of the experimental animals.

In much of the non-rodent work, unphysiological approaches such as tracheostomy were often used (see below). The concerns listed above (e.g breath-holding and lack of deposition data) are also valid for these studies.

Histopathology

(TBD)

There follows a separate section on each of the major species of experimental animals as follows:

DOG

The work on dogs is divided into 2 areas: those animals exposed by inhalation, and those using a tracheostomy. The latter technique is extremely invasive and unphysiologic; most of the papers involving tracheostomies report large numbers of deaths, with lung infections also being very common. Dosimetry data are rare in these papers, and in many cases only small numbers of animals were used.

There follows a review of the papers which used tracheostomized dogs, including those papers where exposures were performed but no histopathology reported as follows: It is assumed in such studies that necropsy and histopathology were in fact performed and that there were no neoplastic changes induced as a result of the exposure. Such changes, if present, would undoubtedly have been reported.

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Based on work performed in the 1960's, Hammond et al. (1970) exposed beagle dogs to cigarette smoke via tracheostomy. The aim of this work was to determine whether dogs smoking cigarettes equipped with "efficient" filters would develop pulmonary emphysema and fibrosis to a greater degree, if at all, than dogs smoking the same number of non-filter cigarettes (n.b. pulmonary neoplasia was not a planned end-point). The unfiltered cigarettes used produced 35 mg of "tar" and 1.85 mg of nicotine; the filter cigarettes had yields approximately half this. No analysis was made of the smoke chemistry. Animals were exposed to smoke for up to 875 days; the mean number of cigarettes per day was 3.5 or 7. The authors calculated that the equivalent number of cigarettes per day for a 68 kg man were 21 or 42. ~~The~~ A calculation was also made of grams of "tar" per dog over the 875 days, around 100 or 200 grams per dog.

No measurement of blood COHb was made. Many of the dogs died during the course of the exposures, the principal causes of death being pulmonary oedema, bronchial pneumonia, pulmonary fibrosis and emphysema and cor pulmonale.

The pulmonary neoplasms were described in a second paper by Auerbach et al.(1970). Non-invasive bronchiolo-alveolar tumors were found in smoke-exposed and sham control animals. In the overall analysis, 25% of the non-smoking groups had tumors, defined by the authors as "lesions in which there were foci of neoplastic cells". Many of the bronchiolo-alveolar tumors were found by microscopic analysis, rather than by gross examination. Two animals showed invasive squamous cell carcinomas in the bronchus. Both animals had been exposed for approximately 880 days and had smoked 6,200 cigarettes.

The main criticism that can be made of this early work, from which the design of many of the subsequent experiments with dogs was based, ~~is~~ are the extremely invasive nature of the tracheostomy, the substantial incidence of infections and deaths, the massive doses of "tar" deposited in the lungs of these animals, and the small number of animals in the treatment groups.

Ahmed et al. (1976) compared the relative effects of long-term smoking and nicotine administration using beagle litter mates prepared with a permanent tracheostomy. Animals were placed into three groups, namely a control of seven animals, nine animals smoking seven cigarettes a day and eight animals receiving comparable doses of nicotine parentally. No details are given of the "tar" yields of the cigarette. The cigarettes were without filter and produced 1.3 mg of nicotine per cigarette. Details on the overall exposure are lacking. The animals were apparently exposed for up to 22 months. No histopathology was reported.

Following this earlier work, Ahmed et al. (1980) examined myocardial effects of long-term cigarette smoking in tracheostomized beagle dogs. Animals were exposed to smoke from seven cigarettes of low nicotine content (0.2 mg per cigarette) and a group of animals were exposed to nicotine intramuscularly. As in the earlier work from this group, the data on dosimetry are very poor, although COHb concentrations of up to 5% were noted. Animals were exposed for 18 months. There were no reports of necropsy findings or of subsequent histopathology.

Brazell (1984) examined plasma nicotine and cotinine in 87 beagle dogs chronically exposed to cigarette smoke from cigarettes with three different levels of nicotine. Smoke was administered to the animals through tracheostomy tubes under standard puffing parameters. Approximately 3 or 12 cigarettes were smoked in each of the exposure sessions. The dogs were exposed for an average of two years prior to sample collection. Although blood samples were taken and analyzed for plasma nicotine and cotinine, no analyses were made of blood COHb. The data obtained indicated that both groups of animals retained about half of the nicotine offered by inhalation. Unfortunately, no measurement was actually made of nicotine in the smoke presented, but the concentrations in the plasma were related to the number of cigarettes smoked. Plasma

nicotine concentrations of up to 160 mg per ml were noted. Surprisingly, the concentrations of cotinine were much smaller, approximately 15 mg per ml. This difference between plasma nicotine and cotinine is probably due to the short time period over which the animals were exposed. The data indicate that cotinine is metabolized and eliminated at a rate which prevents accumulation of these exposure levels. Very large variations were noted for plasma nicotine and cotinine within an experimental group. There were no results relating to necropsy or histopathology.

Brazell et al. (1982) used tracheostomized beagle dogs exposed to smoke from the 1Al cigarette and from the 1A2 cigarette, which has approximately half of the "tar" yield of the 1Al. Animals were exposed for up to 292 days. From this work, chromatographic profiles were obtained from urine samples voided by the dogs. Although there was some qualitative differences found in the subjects, they were not consistent within a group. Blood COHb was not measured. There were no details of necropsy or of any subsequent histopathology.

Cox et al. (1984) used tracheostomized beagles exposed to cigarettes "calibrated to contain" 1.5 mg nicotine and 35 mg of "tar". Animals were exposed to smoke 12 cigarettes per day for two years, a total of 8,760 cigarettes. At the conclusion of the study, no neoplastic changes were noted. As with much of the work on the dog, dosimetry data are not available.

DeSanctis et al. (1987) exposed seven beagle dogs via tracheostomy to smoke from ten cigarettes a day, five days a week for ten months. Mucus was collected during the exposure in an attempt to determine any bronchoconstrictor effect of cigarette smoke. An additional end point of the study was to examine the variation of any responsiveness to methacholine to the quantity of mucus in the airway. The conclusion of this study was

that chronic cigarette smoke exposure reduces bronchial reactivity, as indicated by the response to a bronchial constrictor aerosol. There is no report on necropsy or subsequent histopathology.

Frasca et al. (1982) produced pulmonary fibrosis and emphysema in beagle dogs by the direct inhalation of cigarette smoke over two to four months at two to seven cigarettes per day, using tracheostomy. The cigarettes used had a "tar" delivery of 27 mg and 3.2 mg of nicotine. There was no attempt to assess dosimetry by nicotine or by blood COHb. Examination of the lungs by scanning and transmission electron microscopy showed a range of responses from the presence of numerous "smokers macrophages" to extensive alterations, including destruction and enlargement of alveolar ducts and varying degrees of enlargement of the alveolar spaces. There was no evidence of bronchitis and/or bronchiolitis or of a physical obstruction to the terminal airways in the early development of fibrosis and emphysema. No neoplastic changes were noted.

Hakim et al. (1985) exposed beagles to 50 cigarettes per week for 40 weeks using tracheostomy. Details of exposure are virtually absent; there was no report on necropsy or subsequent histopathology.

Humphrey et al. (1981) used intratracheal installations of crocidolite asbestos for periods of up to three years. Seven of the nine dogs used also smoked nine cigarettes per day, five days per week for six years. Unfiltered high "tar" cigarettes were used and this smoke was administered through a tracheostomy. ~~The total number of animals used in this experiment was very small.~~ Both dogs that were exposed to asbestos alone did develop a malignant tumor. Three of the seven dogs exposed to both asbestos and cigarette smoke did not develop a malignancy. The authors conclude that because of the

redundant

effectiveness of the asbestos in producing tumors, it was difficult to evaluate the role of cigarette smoking alone.

King et al. (1989) studied mucus hypersecretion and viscous elasticity changes in nine tracheostomized beagle dogs exposed to cigarette smoke. Each dog smoked 10 cigarettes per day over two and one half hours, five days per week for 10 months. This study showed that chronic mucus hypersecretion can be developed in dogs with 10 months of exposure to whole cigarette smoke, although this level of hypersecretion was highly variable. In this study, a measure of blood COHb was made; concentrations in smoking dogs were up to 14%, indicating that animals did indeed inhale the smoke presented to them. Although histopathology was performed, there was no mention of any neoplastic changes.

Roy et al. (1976) exposed dogs by tracheostomy to smoke from tobacco cigarettes and cigarettes fabricated from marijuana. These animals were exposed daily over a period of 30 months. The pathological findings showed that the incidence of alveolar dilatation in the tobacco and marijuana groups was larger than in controls, but that the tracheostomy procedure itself produced a high incidence of bronchiolitis. The pathological study of the conducting airways showed only two changes: a slight hyperplasia which occurred in all groups, and squamous metaplasia in the right bronchial tree. No neoplastic changes were noted. The dosimetry in this paper is very poor, although animals were exposed for well over three years.

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Tulenko et al. (1988) exposed beagle dogs for two years by tracheostomy to cigarette smoke, using the 1A1 reference cigarette. No histopathology was reported.

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Zwicker et al. (1978) compared the clinical and pathological effects of high and low nicotine cigarette smoke on twelve male beagles. There were control and tracheostomized groups consisting of three groups each. Animals were exposed to smoke seven days per week for five months. Clinical disease, primarily tracheobronchitis, was noted in most of the exposed animals. The dogs were treated with antibiotics, but infections often recurred after the apparent recovery. Histopathological changes were found in all smoke-exposed dogs with slightly more severe lesions in the dogs exposed to twelve cigarettes per day.

In this study a measure was made of blood COHb. Values of up to 32% were noted in the high exposure group, indicating that animals had indeed inhaled large quantities of smoke. Histopathological alterations related to smoke exposure involved the nasal turbinates and included focal basal cell hyperplasia and focal squamous metaplasia along with rhinitis. Laryngitis was noted in all but one of the dogs; in the trachea extensive basal cell hyperplasia was noticed. There was no apparent group differences. Squamous metaplasia was also noted in the trachea and there was tracheitis. Examination of the bronchial tree showed basal cell hyperplasia in all smoke-exposed dogs, accompanied in some by focal squamous metaplasia. This latter lesion was most often found at the tracheal bifurcation. All dogs had bronchitis and bronchiolitis. Alveolar emphysema was an infrequent finding. Smoke granulomas were generally found in the walls of the small veins and bronchioles.

Electron microscopic examination showed degenerative changes in thickened alveolar septa, reactive hyperplasia of the alveolar type 2 cells, increased amounts of collagen and macrophages containing lipid. The tracheobronchial lymph nodes of smoke-exposed dogs all had similar histologic alterations consisting of hyperplasia of the lymphoid centers with numerous secondary germinal centers and sinuses that were packed with pigment-filled histiocytes.

Presumably, the turbinate alterations resulted from the exhalation of smoke not deposited in the trachea, lungs or larynx. Despite a very large exposure of smoke over a reasonable period of exposure, there were no neoplastic changes induced. This is one of the few dog studies that had extremely competent histopathology throughout the respiratory tract.

There are a small number of papers where the dogs were exposed by inhalation rather than by the totally unphysiologic tracheostomy technique reported above. The inhalation technique usually consisted of a mask over the nose.

Park et al. (1979), using beagle dogs, developed an animal model for the evaluation of the effect of chronic cigarette smoking on pulmonary defence and function and on lung structure. In this work a smoking apparatus was used that allowed the dog to actively inhale properly diluted smoke directly from the cigarette through a mouth piece. Using the 1R1 cigarette, animals were exposed up to four exposure sessions per day using a 1 to 4 dilution of the mainstream smoke. Measurements of blood COHb showed concentrations over 5%. The durations of the exposures were six months ~~and one year~~.

At the end of these periods, the results obtained included impairment of tracheal mucociliary transport and the bacterial suppressive activity of the alveolar macrophages, with little change in pulmonary function. Morphologically significant lesions were noted in the central airways and bronchial walls, consisting of tracheal epithelial basal cell hyperplasia, proliferation of goblet cells in the central airways and peribronchiolar infiltration by inflammatory cells. Morphometry of bronchiolar size and alveolar surface area, however, failed to show significant differences between the groups.

In this study it was shown clearly that animals inhaled large quantities of smoke in a physiologically correct manner. Although histopathological examinations were made of

the lungs, no neoplastic lesions were reported. An additional section to this piece of work was the conclusion of a reversibility period; there was only a small indication that morphological alterations produced by cigarette smoking for one year did persist for a one month period after cessation of smoking.

Cross et al. (1982) exposed a group of beagle dogs to smoke from 10 or 20 cigarettes per day, seven days a week, for up to 65 months. Other groups ~~of animals~~ were also exposed to radon, radon daughters and uranium ore dust, as well as cigarette smoke. The cigarette used was the University of Kentucky 1R1 research cigarette. Unlike many of the other studies reported here, a deposition study was performed using carbon-14 labeled dotriacontane. The dotriacontane data confirmed that about 30% of the inhaled smoke was, in fact, deposited in the lung, in agreement with the COHb concentrations (around 5%).

Dogs exposed to 10 cigarettes/day had no significant respiratory lesions. However, three of the dogs exposed to smoke from 20 cigarettes per day had severe respiratory tract changes, including focal areas of pleural thickening, alveolar fibrosis and sub-pleural inflammation. The authors quote however, *the quantity of smoke from that number of cigarettes was very high when compared on an organ or body weight basis and, if inhaled may be unparalleled in all but the most avid of human cigarette smokers.*

should you use standard quoting "—"?

In the animals exposed to radon daughters and uranium ore dust, the histopathological changes were much more prevalent and severe than those in the cigarette smoke groups. Some of these dogs had adenomatous lesions which progressed to squamous metaplasia of the alveolar epithelium, epidermoid carcinomas (associated with large cavities noted within the lung parenchyma) and bronchiolo-alveolar carcinoma.

The overall incidence of lung tumors was 37% in the animals exposed to radon, radon daughters and uranium dust, but only 5% (one animal) in the group with added cigarette smoke. Neoplastic changes were also prominent in the nasal mucosa, where

again the radon daughters plus cigarette smoke group showed a lower incidence of nasal carcinomas than did the radon daughters alone. There were no tumors (pulmonary or nasal) in the group of animals exposed to smoke alone.

The results of the experiment indicated that cigarette smoke had a mitigating or "beneficial" effect on radon daughter induced respiratory tract cancer. This difference was statistically significant, and the authors suggest two reasons for this. First, that smoking could cause an increase in mucus production that would result in a smaller radiation dose to bronchial and bronchiolar epithelial cells, and second, the amount of cigarette smoke inhaled could have a net stimulatory effect on mucociliary clearance. The former theory was subseqently disproved by Gies et al. (1987), who concluded that increased mucus production is the more likely explanation for the smaller numbers of respiratory carcinomas in the smoke-exposed group.

Argument does not follow!

In conclusion, this report, using very long exposures and comparatively complete deposition data, showed no effect of inhaled cigarette smoke in terms of induction of pulmonary carcinomas in beagle dogs.

Wanner et al. (1973) studied tracheal mucus velocity in eight pure bred beagle dogs exposed to 100 cigarettes per week for 13 ½ months. There was a significant decrease in the tracheal mucus velocity in animals exposed to cigarette smoke compared with controls, but no differences in lung compliance and resistance or other physiological parameters. Unfiltered, high nicotine cigarettes were used in a fashion whereby every tenth breath involved cigarette smoking. Part of the problem in this type of apparatus is that the animals quickly learn that every tenth breath will contain cigarette smoke and maintain a very much lower tidal volume during that breath. No measurements of blood COHb were measured. There were no reports of necropsy or any subsequent histopathology.

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MOUSE

In contrast to dogs, large numbers of mice can be exposed simultaneously using sophisticated nose-only exposure devices (no recourse to invasive tracheostomy). An additional problem is encountered with this species however, in that there can be very significant background incidences of neoplastic disease (depending on the strain used). The small size of the experimental animal makes blood sampling to obtain deposition data very difficult unless the animals are killed.

Chalmer et. al. (1975) exposed C57Bl and Balb/c mice for seven to eight minutes per day for varying periods up to 30 weeks, before subcutaneous or intratracheal inoculation of viable tumor cells. In this work, 16 mg "tar" filter cigarettes were used in a Borgwald smoking machine (fixed 1:7 dilution). Blood COHb concentrations were approximately 20% in the C57Bl mice, but only around 5% in the Balb/c mice. As in the earlier experiment (Thomas et al., 1974), exposure to smoke for periods up to 15 weeks enhanced immune responsiveness. Long-term exposure to cigarette smoke ultimately resulted in a lowered capacity to mount humoral and cell mediated immune response. The growth rate of subcutaneously established melanoma was lower in animals that had undergone short-term exposure than in appropriate controls. No details on necropsy or histopathology were presented.

Harris et al. (1974) exposed C57Bl mice to smoke from four different cigarette types (all without filters, no smoke chemistry presented) for 114 weeks. Each batch of animals was exposed for 12 minutes per day, for up to 129 weeks. In one of the cigarette types, animals were exposed to smoke that had been passed through a Cambridge filter. No data on dosimetry are presented. Confounding factors in this experiment were the fact that animals were presented with different influenza viruses by aerosol. Several of the animals died from hepatitis, so some groups of animals were treated orally with

antibiotics. Control mice were apparently maintained under the same conditions as the experimental animals, but they were not given any antibiotic treatment. Smoke-exposure was performed consecutively for 2 of the groups: it is not clear if a separate control group was used for each of these smoke exposures. No antibiotics were used in the Cambridge filter group.

Histopathologic examination showed well circumscribed pulmonary adenomas protruding from the lung surface, apparently similar to those seen in the lungs of strain A mice. The occurence of adenocarcinomas was reported but no data are presented. The incidence of the adenocarcinomas in all but one of the various smoke-exposed groups was not significantly different from that in the single group of controls (see above). When the data from this single group were combined with a similar group exposed 2 years earlier, then the combined incidence was also greater than that in the single group of controls. This selective combination of data from groups exposed at different time points and subsequent comparison with a single group of controls is statistically a suspect procedure and the scientific value of such a procedure likely to be small.

There was no effect of Cambridge filtration of the smoke: tumor incidence in this group was similar to that in non-filtered groups. The incidence in the Cambridge-filtered group was also not significantly different from that in controls. The only group to show an effect of smoke had an incidence of adenomas of 5.8%; in controls the incidence was 1.3% and in the Cambridge-filtered group, 2.9%. The authors correctly point out that the small incidence of "spontaneous" lung adenomas in the controls means that the smoke exposures could only be accurately reported as eliciting a higher response, rather than being causative.

There was no synergistic effect of the influenza infection and smoking.

Henry and Kouri (1986) performed a chronic inhalation study in male mice exposed to smoke from the 2R1 reference cigarette. The report is a distillation from a much

larger report from the Council for Tobacco Research (1988). Mice were exposed five days per week for 110 weeks. Animals were inoculated at the beginning of the study with Sendai virus. ~~The 2R1 cigarette from the University of Kentucky was used.~~ This is a non-filtered reference cigarette with a delivery of approximately 44 mg of tar and 2.4 mg of nicotine.

A problem in terms of the inhalation technology is that the animals were exposed sequentially, rather than in a parallel manner. In this way, animal number 2 received the exhaled smoke from animal number 1. As there were over a thousand animals being exposed simultaneously, there could be significant differences between the animals: "downstream"~~: the smoke these~~ animals received could have been inhaled and exhaled many times previously. There is also a problem in the aging of the smoke ~~going from one~~ being distributed to over 1,000 animals. No chemical analysis of the smoke actually presented to the animals was made; assumptions were made as to the physical composition of this smoke.

In a parallel experiment using radiolabeled dotriacontane, smoke particle deposition was estimated at between 125 and 200 µg tar per day per mouse lung. Blood COHb concentrations were estimated in smoke-exposed groups to be around 17%. The authors calculate that up to 100 mg of tar could have been deposited per mouse lung over the course of the study, although no actual attempts were made to measure this.

Large numbers of animals died during the experiment, some of the deaths being related to the method by which the animals were restrained during these smoke exposures. In particular, large numbers of animals had neck lesions. This was also noticed in the sham-exposed animals. The sham animals died in a significant rate from around 80 weeks of exposure; approximately 60% of these animals died from neoplastic diseases and 40% of non-neoplastic diseases. The major non-neoplastic diseases were pneumonia, nephritis and conditions were no major disease was found. The major neoplastic diseases observed

were hematic tumors, sarcomas, fibrosarcomas, lung adenocarcinomas, liver carcinomas and mammary carcinomas.

The only lung cancers observed were diagnosed as alveolar adenoma carcinoma. No squamous cell carcinomas or poorly differentiated carcinomas were found. A total of 19 of 978 smoke-exposed mice and 7 of 651 sham-exposed mice were observed with alveolar adenocarcinomas. The difference between these groups was not statistically significant. The data were analyzed in the CTR book in a number of different ways; under no circumstances could a statistical significance between sham and smoke-exposed groups be noted. At no time in this study was the incidence in the smoke-exposed mice higher than that in the sham-exposed animals. An analysis was made using actuary tables. Using this technique, the results showed that there was no difference in the incidence or latency of alveolar adenocarcinomas between the smoke-exposed and sham-exposed mice.

Although no differences were observed between sham and smoke-exposed mice in an experiment using good dosimetry with chronic exposures, the authors conclude that the 2R1 cigarette smoke has week carcinogenic activity. The statistics are quite clear and showing that there is no such difference between smoke-exposed and sham animals.

A full analysis of the histopathology noted in the study is missing. There is no accurate description of the alveolar adenoma carcinoma. Additional problems with this study include the unknown effects of the inoculation of the Sendai virus, and also the physical restraint causing large numbers of mortalities in the study.

Holt et al. (1976) exposed Balb/c and C57Bl mice to smoke from high-"tar" (c. 16 mg) or low-"tar" (c. 6 mg) cigarettes for 7-8 minutes per day, 5 days per week, for up to 36 weeks. No figures are presented for the CO yields of the 2 cigarette types. Measurements were made of blood COHb: concentrations approached 30% in the C57Bl mice exposed to smoke from the high "tar" cigarette, but were only around 9% in the low

"tar" cigarette. Concentrations in the Balb/c mice were around 10% for both cigarette types. These COHb concentrations were accompanied in some cases by leukopenia.

Mice exposed to smoke from the high "tar" cigarettes exhibited alterations in humoral immune responsiveness. However, cell-mediated immune responsiveness to bacteial and tumor-specific antigens were depressed similarly in animals exposed to low or high "tar" cigarettes.

Standard necropsy and histopathology techniques were used. Histological findings included marked signs of persistent bronchitis, which was apparently more noticeable in the high "tar" groups than in the low "tar" groups or in controls. The alveolar parenchyma of some animals apparently showed interstitial pneumonia. However, no data are presented on the comparative incidences of this change. No information on disease status was presented, although the authors hypothesize that the pneumonia may have resulted from infection.

There was no report of any neoplastic change.

Keast and Ayre (1980) exposed C57Bl and Balb/c mice to high "tar" (16 mg per cigarette) or low "tar" (5 mg per cigarette) filtered cigarettes for eight minutes per day, for up to 30 weeks. No measurements of the smoke composition were made, nor were any estimates made of dosimetry by plasma nicotine or blood COHb. Phagocytic and degradative properties of the liver and spleen were assessed using opsonized radioactive sheep erythrocytes, injected intravenously into naive or immune animals.

The results demonstrated that both the high and low "tar" exposure of susceptible and resistant mice often modify systemic clearance mechanisms and decrease the ability of the liver to trap circulating antigen with concomitant variations in the rate of antigen breakdown. These results were thought to provide at least a partial explanation of the observed delays in antibiotic production by tobacco smoke-exposed mice, and to indicate that a genetic tobacco smoke susceptibility may exist, either through variations in the

ability to metabolize tobacco smoke toxins or direct immunological susceptibility to tobacco smoke toxicity (possibly emitted through vapor phase compounds).

There were no reports of necropsy or histopathology.

Using similar methods, Keast & Ayre (1981) exposed Balb/c mice for varying periods of time, up to 83 weeks. The exposure system was as described above, except that animals were exposed to the body weight equivalent of 20-30 cigarettes. The aim of the work was an extended study of the effect of smoke on the murine immunological system.

The results showed, as in the earlier work, some deterioration of the immune response with age. The immunological responsiveness of smoke-exposed mice was similar to that of age-matched controls.

There were no reports of necropsy or histopathology.

Keast and Taylor (1983) exposed C57Bl and Balb/c of mice to smoke from 16 mg "tar" filter cigarettes for up to 32 weeks, using the Borgwald exposure apparatus. There were no estimates of dosimetry by either plasma nicotine or blood COHb. The aim of the study was to determine the phagocytic and killing capacity of polymorphonuclear leukocytes (PMN) of the smoke exposed mice.

The results indicated that the PMN cells from mice chronically exposed to tobacco smoke exhibit significantly depressed bacterial phagocytic and killing abilities in the presence of opsonized bacteria. This effect was less pronounced in Balb/c mice than in C57Bl mice. The authors theorized that the major differences in phagocytic and killing abilities between smoke-exposed and control cells could be the result of the modification by tobacco smoke of the complement receptor site and/or associated metabolic requirements.

There was no report of necropsy or any subsequent histopathology.

Keast et al. (1981) exposed Balb/c mice once daily for seven to eight minutes, five days per week for 95 weeks, to smoke from 30 high "tar" cigarettes (around 16 mg of "tar" per cigarette) . The mainstream smoke was diluted in a Borgwaldt smoking machine in a ratio of 1 to 7. No measurements were made of the composition of the smoke presented to the animals, nor were any measurements made of dosimetry. Tobacco smoke exposure was found to produce a marked rise in mortality after approximately 80 weeks. The authors attributed the greater incidence of mortalities to an increased incidence in bronchial adenomas and lymphocytic lymphoma. A second major difference in mortality patterns between smoke exposed and controls was the incidence of large organizing ovarian hematomas. These were noted in seven control mice, but only two smoke-exposed mice.

A detailed breakdown of the type of lung lesions detected showed that almost all tobacco exposed and control mice displayed at least one type of abnormality. However, the smoke-exposed mice were invariably more severely compromised. Squamous metaplasia was not detected in any of the animals. Smoke-exposed mice were found to exhibit fewer liver lesions than controls and to be significantly protected from fatal ovarian and para-ovarian hematomas. Liver lesions, when present in smoke-exposed mice, were of a less severe character than those found in controls.

The conclusion made by the authors was that the murine model for the study of the effects of long-term cigarette smoke exposure has obvious major differences histopathologically from that of the human smoker: the preponderance of bronchial adenomas rather than the squamous cell carcinomas was "to be expected", they stated. The authors also concluded that smoke-exposed mice exhibited fewer liver lesions than controls, and were significantly protected from other changes.

Leuchtenberger and Leuchtenberger (1974) examined the differential response of two strains of mice to chronic inhalation of cigarette smoke. Details of the cigarettes and

of the smoke exposure are missing. Animals were apparently exposed for up to 26 months and in some cases animals were exposed to smoke that had been passed through a Cambridge filter. No details of the smoke chemistry are presented, nor of blood COHb or plasma nicotine.

The two strains of mice showed significantly different incidence of pulmonary tumors, both adenomas and adenocarcinomas. Following smoke exposure, one of the strains (C57Bl) did not develop any adenocarcinoma of the lung, nor were any adenomas noted. In the second strain of mouse Snell's there were incidence of adenomas and adenocarcinomas in both controls, whole smoke and gas vapor-phase groups. Although no details are given of the statistical methodologies used to compare control and smoke, using Fisher exact test there is no significant difference for either sex between the incidence of adenocarcinomas in the whole-smoke group or in sham-exposed animals. In male animals only, there appears to be a significant difference for the incidence of adenocarcinomas and animals exposed to vapor phase when compared with controls.

No bronchogenic carcinomas were observed in this study, although the authors claim that the adenocarcinoma is of the same histopathological type of lung cancer as the bronchogenic carcinoma. A unique problem with this study is that the Snell's mice carry a recessive gene for pituitary dwarfism and have not been used by any other workers in the area of pulmonary carcinogenesis. A further problem concerning the animal health is that no investigative serology was performed to determine disease status of these animals the were bred under conditions much poorer than those currently available.

Matulionis (1984) studied the effects of long-term cigarette smoke inhalation on the morphologic and functional integrity of lungs of old and young C57Bl mice. Smoke exposure occurred over a nine month period, beginning when the animals were either two or nine months old. At the termination of the experiment, microscopic and morphometric evaluations were made of pulmonary tissue showing peribronchiolar and perivascular

accumulations of lymphocytes and macrophages in lungs of both young and old smoke-exposed mice. Such lesions were never observed in sham-treated animals of either age.

In this extensive work, the THRI single-port reverse smoking machine was used, in conjunction with the 2A1 University of Kentucky reference cigarette. Smoke from a single cigarette was allowed to remain in the exposure chamber for 15 seconds after which time it was flushed with room air. Forty five seconds were allowed to elapse before the next smoke puff was delivered into the exposure chamber. Animals were exposed twice per day in this pulsatile fashion to 10 puffs of smoke from one cigarette. Although no measurement of the smoke composition was made, an estimate of the blood COHb was made: concentrations were around 10% in the young animals only. No estimates were made in the old mice due to the small number of animals; the number of animals available at the termination of the experiment varied was as low as 4 per group.

A number of animals in this experiment sustained fractures of the upper limbs while being loaded into or from the restraining system. A number of old animals also developed skin lesions over the nasal bones as a result of trauma sustained while being restrained during the smoke exposure.

Light and electron microscopic assessment of lung tissue from young and old sham-treated and control animals revealed normal morphology. Lymphocytic infiltrations were noted at the light microscopic level in young and old smoke-exposed animals. In close proximity, and often mingled with the aggregations of lymphocytes, were large vacuolated macrophages containing brown pigment. Histopathologic examinations were restricted to lungs. Serologic tests prior to and at the termination of the exposure were negative for both Sendai and *Mycoplasma pulmonis* (which is itself though to cause lymphocytic infiltration). In the old smoke-exposed animals there was a reduction of alveolar space surrounded by normal parenchyma and an increase in thickened septa and cellularity of lungs. These abnormalities resembled pulmonary fibrosis.

Pulmonary function tests substantiated the morphologic and morphometric data, suggesting that the abnormal conditions manifest as a result of an interaction between smoke inhalation and aging. Thus, physiologic data suggest that the anatomical abnormalities observed in the old smoke-exposed animals are restrictive in nature and conform most closely to pulmonary fibrosis.

In this well-controlled study, with small numbers of animals exposed for up to ten months of time, there was no evidence of any neoplastic change in the lungs of the smoke-exposed animals. A quantitation of the pulmonary macrophage response was made in a subsequent paper (Matulionis and Simmerman, 1985).

Matulionis et al. (1985) studied the combined effects of cigarette smoke inhalation and hydrocortisone acetate on lungs of C57Bl male mice, in a sub-chnonic (56 day) study. It was found that smoke inhalation or HCA administration had no effects on the animals. The data indicated that manifestation of pathologic conditions resembling pulmonary fibrosis and pulmonary alveolar proteinosis were a result of the interaction between drug and cigarette smoke. A measurement of the smoke concentration of total particular matter was made: around 10 mg per exposure session. The COHb concentrations approached 8%. A full set of necropsy and histopathology procedures were reported; no neoplastic changes were noted.

Matulionis and Yokel (1988) exposed C57Bl mice twice a day to smoke from the 2Al reference cigarette, for up to 8.5 months. The aim of this experiment was to study the effects of added kaolin to the cigarette smoke.

Significant attrition of old smoke-exposed and sham-treated mice occurred during this study, apparently due to the handling of the animals. The results of this study showed a greater increase in parenchymal tissue, and a decrease of air space, occurred in older than in young mice subjected to smoke from cigarettes containing high doses of kaolin.

Qualitative and quantitative alterations of lung macrophage ultrastructure, fibrocyte population size and abnormal macrophage aggregations were seen in old versus young mice inhaling higher amounts of kaolin. The consequence of inhaled kaolin was not understood.

A full set of necropsy and histopathology was reported for lung tissue; no neoplastic changes were reported. A relation between the abnormal function of the macrophages and manifestation of fibrotic-like lesions was suggested by these authors.

Nguyen and Keast (1986) exposed AKR mice (animals congenitally destined to 100% fatal incidence of virus-induced leukemia) for seven to nine minutes per day, five days a week to fresh tobacco smoke from 30 cigarettes with yields of around 15 mg of "tar". No measurements were made of smoke composition or of dosimetry.

The results showed that daily exposure to low levels of tobacco smoke produced significantly different mortality profiles associated with both the sex of the animals and the age at which tobacco smoke exposure commenced. The females were susceptible and died sooner than males, where a significant proportions of animals survived longer than age matched controls. This prolongation of life appears to be due to a failure of the leukemic state to be mobilized in the tobacco smoke-exposed males. Exposure of both the females and the males to tobacco smoke did not induce significant detectable immunological reactivity against the leukemic cells for several parameters tested, possibly due to a significant enhancement of suppressed activity in the serum of the chronically exposed animals, over and above that which also occurs in age matched control animals.

It is difficult from the text of this paper to determine exactly how long animals were exposed to smoke. A large proportion of the animals died from ill-defined causes. There are no details given of necropsy or histopathology.

Priest et al. (1989) conducted an experiment to determine the effect of chronic exposure to cigarette smoke on the incidence of plutonium induced lung tumors in mice. Animals were exposed to $^{239}PuO_2$ to give an initial alveolar burden of 100 Bequerels, and then treated in three ways. One third received no treatment and was held for a period of 18 months; the remainder were either sham-exposed or exposed to mainstream smoke for one year and then held in the animal house for a further six months. After this time, animals were killed and the number of tumors present in the lungs determined by histopathological examination.

The Geneva smoking machine was used to expose the animals. The cigarettes used were commercial United Kingdom middle "tar" filter cigarettes, using a target concentration for particulates of approximately 1.3 mg per liter, one hour per day, five days per week, for one year.

Deposition data were obtained using cigarettes labeled with ^{123}I-iodohexadecane, a hydrocarbon with a boiling point equivalent to the mid-point of those of cigarette "tars". The studies showed that the smoking procedure resulted in an average daily cigarette "tar" deposition in the lungs of the smoking mice of 90 μg. In addition to the above, the lungs of some mice from each experimental group were analyzed to determine their radium content at death. The lung contents of the control and sham-exposed mice were very similar, but the lungs of animals exposed to cigarette smoke contained approximately four times as much plutonium, indicating that the cigarette smoke inhibited the clearance of the radium.

A total of 48 tumors were identified histopathologically. Of these, eight were judged malignant and 40 benign. Of the benign tumors, 13 were solid tumors of alveolar origin, showing no tubular differentiation. Twenty two showed tubular differentiation and were judged to be of bronchiolar origin; four were mixed or uncertain origin. More than twice as many primary tumors were found in the lungs of the mice in the cage control group as those that were exposed to cigarette smoke. The number of tumors found in the

sham-exposed group was intermediate between those of the other groups. There was no difference between the number of tubular bronchiolar benign tumors found in each of the experimental groups, but the other tumor types were generally much less prevalent in the lungs of mice that were exposed to cigarette smoke. With the exception of a single tumor in one sham animal, all the malignant tumors were found in the cage control mice.

The authors had difficulty interpreting the above results. The data may indicate that the effects of α particles and cigarette smoke on the lung are antagonistic and that cigarette smoke had a protective, rather than synergistic effect. An alternative theory made by the authors is that the promoting effect of cigarette smoke, together with the greater radiation dose to the lungs of the mice which received cigarette smoke, may have had a lowering effect on the tumor incidence (the lowest number of tumors were found in the group that had been exposed to smoke). The authors conclude, that under conditions of the experimental design, the effects of cigarette smoke and of α irradiation of the lung can be antagonistic. This result contrasts with the common expectation of synergy.

Rasmussen et. al. (1981) exposed mice chronically to the smoke from the 1A1 reference cigarette and measured DNA replication and unscheduled DNA synthesis. No analysis was made of the chemical or physical composition of the smoke. Large numbers of animals were exposed at the same time causing problems with aging of smoke and with rebreathing of exhaled smoke from upstream animals. The overall exposure was 17 weeks.

Within one week of beginning smoke exposure, the DNA replicative activity was increased more than two-fold over sham-exposed controls and remained elevated as long as smoke exposure was continued. If the mice were removed from smoke exposure, DNA replicative activity returned to normal levels within one week. However, the unscheduled DNA synthesis response to DNA damage remained depressed for up to five months after ending the smoke exposure.

The authors hypothesized that the reason for the overall reduction in unscheduled DNA synthesis was due to an accumulation of a stable population of cells that have a substantially reduced repair capacity. The authors could not comment whether the accumulation of unrepaired or unrepairable DNA has any relevance to oncogenesis.

No necropsy appears to have been performed and there were no results of histopathological examinations presented.

Talbot et al. (1987) reported on preliminary studies of the interaction between cigarette smoke and $^{239}PuO_2$. This work used the Geneva smoking machine with estimates made of the composition of the smoke in terms of total particular matter and of carbon monoxide. Measurements were also made of breathing frequencies, resulting in sound dosimetry data. Unfortunately, no measurements of blood COHb were made.

There was a significant increase in the numbers of polymorphonuclear macrophages. In absolute terms this was small and not evidence of a market inflammatory response. As well as producing an increased number of pulmonary alveolar macrophages in the lungs, exposure to smoke also produced a considerable increase in the mean diameter of the macrophages. This was not a uniform change, but was an increase in the very large diameter cells.

Histological examination of the lungs exposed to smoke for three months showed only minimal changes relative to the sham-exposed or caged control mice. The authors stated that the most important finding of this experiment was the daily smoke exposure regime inhibited the clearance of plutonium from the mouse lung, while sham exposure had no effect.

Thomas et al. (1974a) exposed C57Bl mice in a Borgwaldt machine to smoke at an air dilution of one to seven, once daily for seven to eight minutes, for up to 294 days.

There are no details given of the cigarettes used or of the smoke composition, nor were any estimates made of dosimetry.

Mice were inoculated intratracheally with sheep red blood cells and examined for immunological parameters. The intratracheal inoculations induced plaque forming cells in the spleen, the regional lymph nodes of the respiratory tract, and the lungs.

The main results of the study are that cigarette smoke exposure appears to progressively impair the primary immune response, rather than exerting an immediate toxic effect. There is no information on necropsy or histopathology.

Thomas et al. (1974b) examined the sub-cutaneous growth of the transplantable Lewis lung tumor in C57Bl mice chronically exposed to smoke. There are no details of the cigarettes used or of any measures of dosimetry. Animals were exposed to smoke for up to 38 weeks before inoculation; the authors state that exposure continued throughout tumor growth, but it is not clear how long the total exposures were (at least 25 days).

There were no observations of lung tumors in animals exposed to smoke but not to the Lewis tumor, nor of any other lung changes induced by the smoke exposure. Tumor cell growth during short term exposure was indistinguishable from controls. The authors hypothesized that the cigarette smoke exposure depressed the ability of the mice to elicit an immune response against the tumor cells.

When murine sarcoma virus induced tumor cells were inoculated into Balb/c mice only those animals chronically exposed (20-31 weeks) to smoke died with tumor cells in the lungs: this part of the study is poorly documented; small numbers of animals were used and no data are presented on animals that did not die. Bronchopneuomonia was noted as a probable confounder.

NON-HUMAN PRIMATE

Ando & Yanagita (1981) exposed rhesus monkeys with or without the simultaneous use of other reinforcers to mainstream cigarette smoke. Fourteen monkeys were trained to suck air and puff on cigarettes using sweetened liquid reinforcer. After smoking had been established, reinforcement was removed. Smoking without the reinforcement was then observed during 20-hour daily sessions. The maximum figures recorded for a single 20-hour session were up to 16,000 puffs in one monkey corresponding to approximately 47 cigarettes. No measurements were made of puff volume. The cigarettes used contained low amounts of nicotine or were nicotine-free. The latter cigarettes were made from tobacco leaves grown from tobacco stems which has been grafted onto the stems of tomato bushes. Nicotine was reported to be undetectable in the smoke of this cigarette. The other cigarette produced approximately 0.3 mg of nicotine. Both cigarette types produced 8 mg of tar for each cigarette.

Even after lengthy training periods, only 2 of the 14 monkeys exposed to the training process actually performed voluntary smoking for extended periods of time. The authors reaffirmed the difficulty of shaping voluntary patterns in rhesus monkeys. In the two animals that did develop voluntary smoking, the numbers of puffs from cigarettes used varied from session to session, unlike figures for human smokers. The monkeys in this study took approximately 100 puffs or more per cigarette compared to the 10 to 12 puffs taken by humans. Although no data presented on blood COHb, there are some details given on the plasma nicotine concentrations. In one of the animals, these concentrations were found to approach 40 ng/ml, showing that the nicotine was being significantly absorbed by the animals, be it by puffing, swallowing or by inhaling.

Comparisons of the nicotine and nicotine-free cigarettes did not show unequivocal results. Small decrease in smoking with the nicotine-free cigarettes would seem to suggest an important role performed by nicotine in maintaining smoking behavior. In this

study, no mention is made of necropsy or any histopathology of the smoke-exposed rhesus monkeys.

Raymond et al. (1982) exposed 21 adult male macaques 10 days a week for up to five years to cigarette smoke at a human equivalent of three or one packs per day. Six animals served as sham controls. Blood COHb concentrations measured immediately after smoking showed concentrations of up to 6% for the high exposure animals. In this work, the smoke exposure involved restraint of animals and custom-fitted chairs and aluminum inhalation masks. The masks were connected via a one way valve to a multi-port reversed puff smoking machine timed electronically to provide access to a three second exposure of diluted smoke every 40 seconds. The masks contained a bit which held the mouth open and a balloon which occluded the nostrils when inflated, thus ensuring oral administration of smoke. The University of Kentucky 2R1 cigarettes were used.

Chronic cigarette smoke inhalation failed to alter plasma lipid or protein levels. No significant differences were seen in total plasma cholesterol or lack of protein cholesterol concentrations measured at four intervals over a period of one year. No details are presented in this study of necropsy or of subsequent histopathology.

Rogers et al. (1988) exposed baboons to the smoke from more than 40 2R1 reference cigarettes (37 mg of tar and 2.6 mg of nicotine) per day for periods of up to 3.3 years. A total number of 30 animals was used, with sub-groups of animals placed on an atherogenic diet. An estimate was made of blood COHb concentration: values were approximately 1½% in the smoke-exposed animals and 0.5% in controls. The animals had mean puff volumes of 47 ml and had longer puffs than the FTC standard.

A number of different parameters were examined in this study, including hematologic variables and atherosclerotic lesions. The latter were obtained through

histologic sections of the abdominal aorta. There were no differences between smokers in controls in the extent of fatty streaks or in the prevalence of fibrous plaques. The necropsy results were restricted to cardiovascular system; however, it is clear that most examinations were made of all tissue. Cigarette smoking for up to three years did not increase the extent of diet-induced experimental atherosclerosis. The authors concluded that it was unlikely that this failure was due to the use of an ineffective method of smoke exposure.

One of the criticisms that had been made of cigarette smoke inhalation studies in primates is that because of the complicated anatomy of the nasal passages, only small amounts of materials may reach the lungs of the smoke-exposed animals. This has been shown to be not the case for baboons, through work performed by Rogers et al. (1981). In this work, very similar to that described by Rogers et al. (1988), baboons were exposed to 14C-labelled dotriacontane delivered in a manner providing extensive deposition of particulates. Lungs of these passively exposed animals were lavaged so that the efficiency of recovery of the lavage procedure could be determined. A second phase of this work was to expose nine baboons to actively-smoked labelled cigarettes, followed by the lavage of the lungs of these animals to recover 14C-labelled dotriacontane. The total amount of particular matter present in the lungs was estimated using the efficiency factor previously determined.

Smoking baboons retained an average of 9% of the total cigarette particular matter, in proportions similar to that retained by other animal smoke inhalation models. In this work, no data were given on the percentage lung deposition of the cigarette particulate matter content. However, the authors were confident that is should be possible to increase the fraction considerably to values of approximately 10%.

Sopori et al. (1985) exposed 11 adult macaque monkeys to either a low dose (human equivalent of one pack per day) or high dose (human equivalent three packs per day) of high tar, high nicotine reference cigarette smoke for four to eight years. Animals were exposed seven days a week, 30 and 90 minutes per day. The inhalation protocol was carried out twice per day using the University of Kentucky 2R1 reference cigarettes. No details of blood COHb or plasma nicotine were reported, nor was any mention made of necropsy or subsequent histopathology.

Parameters of immunological response were compared to those seen in six non-smoked controlled animals. The results suggested that cigarette smoking does not significantly affect the responses of spleen cells to mitogens. However, spleen cells subjected to the high dose of smoke demonstrated a reduction in their natural killer cell mediated activity.

RATS

Probably more inhalation experiments have been performed with this species than with any other.

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