Respiratory Infections and the Pathogenesis of Lung Cancer*

P. Nettesheim, H. Schreiber, D. A. Creasia, and C. B. Richter **

1. Introduction

Epidemiological investigations of the past 15 to 20 years leave little doubt that tobacco smoke is the most important single factor in the etiology of lung cancer. Nevertheless, many questions concerning the pathogenesis of this neoplastic disease — the role of predisposing host and environmental factors and the possibility of synergistic or potentiating effects from combinations of various biological, chemical, and physical agents — are still unanswered (Roe and Walters, 1965; Saffiotti, 1969). Some evidence for the interaction of etiological factors has been obtained in humans exposed to radon daughters and cigarette smoke (Saccomanno *et al.*, 1971) and to asbestos and cigarette smoke (Selikoff *et al.*, 1968).

The topic of our investigations — infectious (and inflammatory) agents as possible potentiating factors in carcinogenesis of the respiratory tract — has been actively debated for many years (for review see Spencer, 1963; Roe and Walters, 1965; McClung, 1967; Eck *et al.*, 1969). Chronic bronchitis, influenza pneumonitis, interstitial pneumonia, and tuberculosis are all thought to be associated with a high risk of lung cancer in man. However, because of the conflicting results of numerous clinical and pathological case studies (e. g. Macklin and Macklin, 1940; Raeburn and Spencer, 1957; Meyer and Liebow, 1965; Stavraky, 1971), it is difficult to come to any firm conclusion regarding the role of inflammatory processes in the development of bronchogenic carcinoma. This uncertainty is reflected in the ambiguity with which the subject is discussed in several reviews on lung cancer etiology (Roe and Walters, 1965; Eck *et al.*, 1969); and two recent epidemiological studies (McClung, 1967; Stavraky, 1971) deny completely the existence of any compelling evidence for or against an association of lung cancer with pulmonary infection (or for that matter with any other "precursor disease"). It appears to us that one of the reasons for this confusion lies in the fact that tobacco smoke, the major etiological factor in pulmonary carcinogenesis, also interferes with the pulmonary defense systems against infectious agents (e. g. Rylander, 1968, 1969), making it difficult to single out the effect of respiratory infection in the pathogenesis of human bronchogenic carcinoma.

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** From the Carcinogenesis Program, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830, U. S. A.

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That oncogenic as well as non-oncogenic viral agents can interact with chemical carcinogens to enhance neoplastic responses has been shown in several tumor systems (MARTIN *et al.*, 1961; DURAN-REYNALS, 1963), but only a few investigators have studied this phenomenon in the respiratory tract (KOTIN and WISELEY, 1963; KOTIN, 1966; LEUCHTENBERGER and LEUCHTENBERGER, 1965; HARRIS and NEGRONI, 1967; NETTESHEIM *et al.*, 1970, 1971, a, b), even though this organ system, because of its intimate contact with the environment, may receive frequent exposures to both viral and chemical agents.

In this presentation we review the main efforts currently being made in our laboratory to determine whether respiratory infections can act as co-factors in the pathogenesis of lung cancer. In discussing the various experiments, we have tried to include pertinent data from the literature. It should be mentioned at the outset that there is no single answer: the variables to be considered in these studies are simply too numerous. The type of carcinogen, carcinogen dose, type of exposure (single, repeated, or chronic), type of microbial agent, type of infection (chronic or acute), time relationship between carcinogen exposure and infection, and species differences (depending on which infectious disease model is to be tested with which lung tumor model) all are factors that influence the outcome of experiments of this type.

Rather than present a biased sample of results that might tend to support or negate a co-carcinogenic effect of respiratory infection, we discuss our current research as it relates to the topic, even though the findings are sometimes equivocal, or possibly contradictory. By taking this approach we can indicate many of the complexities facing the laboratory researcher who chooses to work in this area and, simultaneously, provide a framework of suggestive results that might give impetus to further experimentation.

We will first discuss modifications of the lung tumor response by respiratory infection and then describe our studies that are concerned with alteration of some host responses that might be important in the induction and progression of pulmonary neoplasms. A number of mechanisms by which infections might alter the respiratory tract tumor response are summarized in Table 1.

Table 1. Hypothetical co-factor effects of respiratory infection on pulmonary carcinogenesis

1. Disturbance of mucociliary clearance
2. Disturbance of alveolar clearance
3. Alteration of metabolic clearance
4. Destruction of protective cell layers
5. Disturbance of cellular steady state with increased cell proliferation (and faulty differentiation)
6. Suppression of immune surveillance mechanism

2. Effect of Respiratory Infection on the Lung Tumor Response Induced by Chemical Carcinogens

a) Influenza Pneumonitis

Between 1965 and 1970 we undertook a series of experiments designed to evaluate the carcinogenic and co-carcinogenic activity of various chemical, physical, and biological agents known or suspected to play a role in human lung cancer develop-

ment (NETTESHEIM *et al.*, 1970, 1971 a, b). Three different air contaminants (ozonized gasoline fumes, Cr_2O_3 and $CaCrO_4$ particulates), X-irradiation, and PR8 influenza virus were used in these studies and were tested singly or in combination for tumorigenicity in the respiratory tract. The data will be reviewed here only insofar as they pertain to the interaction of respiratory infection and respiratory carcinogens.

Our investigation was prompted by the reports of KOTIN *et al.* (KOTIN and WISELEY, 1963; KOTIN, 1966; KOTIN *et al.*, 1958) describing the development of squamous cell tumors in the respiratory tract of C57BL mice exposed to ozonized gasoline fumes (synthetic smog) and three different respiratory viruses. One complication in these studies was the common occurrence of "spontaneous" pneumonitis in control animals (KOTIN *et al.*, 1958). Since one of our major objectives was to determine whether influenza pneumonitis would alter the tumor response to inhaled air contaminants (and X-irradiation), we conducted our experiments with mice free of respiratory disease. The mice were raised and maintained in a rigidly controlled barrier facility and were free of internal and external parasites, pathogenic bacteria, and eight common murine viruses (SIMMONS *et al.*, 1967). At 6 weeks of age, half of all the C57BL/6 mice used in the study were infected by aerosolization of lung homogenate containing influenza virus, strain PR8-34-60 (only animals positive for antiviral antibodies were used in the inhalation experiment). Two weeks later, daily exposure to one of three air pollutants was begun and was continued for the lifespan of the animal (5.5 hours per day, 5 days per week). We found that two of the air contaminants, synthetic smog and $CaCrO_4$ particles, caused a significant increase in lung tumor incidence. The effect of influenza virus infection on the lung tumor response induced by synthetic smog and $CaCrO_4$ exposure is illustrated in Fig. 1. A significant *reduction* in cumulative lung tumor incidence as well as a delay

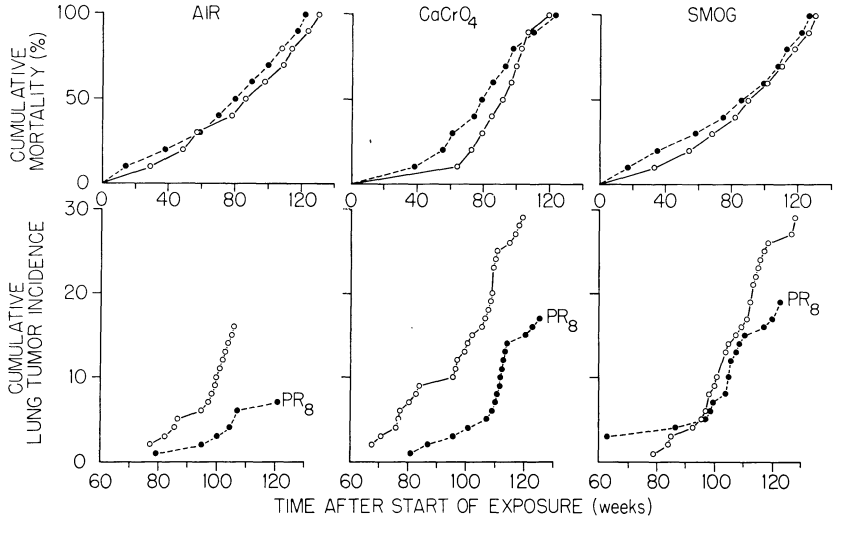


Fig. 1. Cumulative mortality and lung tumor incidence in C57BL/6 mice exposed to various air contaminants. ○, Uninfected mice; ●, mice infected with PR8 influenza virus

in the appearance of lung tumors was observed in the infected groups. Analysis of survival data showed that these differences were not the result of differences in mortality rates (NETTESHEIM *et al.*, 1970, 1971 b). Thus a true suppression of either tumor induction or tumor growth seemed to have occurred in the animals infected with influenza virus. All tumors were classified as pulmonary adenomas or adenocarcinomas. No squamous cell metaplasias or squamous cell tumors were observed.

Our finding of an inhibition of the pulmonary adenoma response in influenza-infected animals, though unexpected, is not unique. The first evidence for such a suppressive effect was suggested by CAMPBELL (1940), who exposed influenza virus-infected mice to dust containing coal tar and found a lower lung tumor incidence in the infected animals. Subsequently, STEINER and LOOSLI (1950) observed that influenza pneumonitis delayed the appearance of spontaneous pulmonary adenomas in strain A mice and resulted in a lower tumor incidence (compared with that of uninfected mice) in at least one of the two reported experiments. Though the authors were uncertain about the statistical significance of these findings, they felt that a "possible inhibitory or anticarcinogenic effect of influenza virus" might exist. In KOTIN's own study (1966), such an inhibition of the lung adenoma response is apparent (Table 2), since mice exposed to "flu-viruses" and to flu-viruses plus smog had a lower incidence of pulmonary adenomas than the corresponding control mice. The nature of this seemingly inhibitory effect of the influenza pneumonitis on the pulmonary adenoma development is presently unclear. One possible explanation is that it is due to the permanent loss of rather large amounts of alveolar parenchyma from pneumonitis and subsequent extensive fibrosis with complete atelectasis, frequently of one or two entire lung lobes. This explanation might particularly apply to situations where the tumors are expected to arise many months after the infectious episode. Studies are presently underway in our laboratory to determine whether this inhibitory effect of the influenza pneumonia can also be demonstrated with the faster developing urethane-induced lung adenomas in BALB/c mice, and whether the time relationship between carcinogen exposure and viral infection is critical.

In contrast to KOTIN's findings (KOTIN and WISELEY, 1963; KOTIN *et al.*, 1958), squamous cell tumors did *not* occur in our studies. It is known that squamous metaplasias can develop in mice and rats suffering from chronic murine pneumonitis, and it has been pointed out repeatedly that lesions induced by infectious agents can, to some extent, resemble neoplastic lesions (WINTERNITZ *et al.*, 1920; LOOSLI, 1949; LEUCHTENBERGER and LEUCHTENBERGER, 1965; RICHTER, 1971). This seems to be

Table 2. Incidence of tumor-bearing animals [a]

	No. of C57BL mice	No. of Survivors	No. of tumor-bearing animals	
			Pulmonary adenoma	Squamous cell carcinoma
Control	450	330	28	0
Smog	450	231	45	0
Flu	450	270	17	0
Flu + Smog	450	328	16	33

[a] From KOTIN (1966).

particularly pronouced when exposure to toxic chemicals is superimposed on infection, as has been documented by LEUCHTENBERGER and LEUCHTENBERGER (1965), although these authors have not reported an augmentation of the lung tumor response in experiments in which tobacco smoke exposure and influenza virus infection were combined. Whatever the true nature of the lesions observed by KOTIN, we believe that the main reason for the discrepancies between his and our observations is the difference in the microbiological status of animals used.

Recently, STAEMMLER *et al.* (1970) reported induction of pulmonary carcinomas in mice 2—3 weeks after infection with PR8 influenza virus. Supposedly no other viral or chemical agent was involved that could account for this dramatic carcinogenic effect. However, in view of the extensive literature on experimental influenza virus infection, it is difficult to accept this claim until it has been convincingly demonstrated that the presumed tumors are truly invasive, metastasizing, or transplantable.

b) Chronic Murine Pneumonia

Chronic murine pneumonia (CMP) is common in many "conventional" mouse and rat colonies. The etiology and pathogenesis of this disease, as well as the problem it can create for the experimentalist whose animal colony is affected by it, have been discussed in two recent reviews (BRENNAN *et al.*, 1969; LINDSEY *et al.*, 1971). Histopathologically, the disease is characterized by bronchopneumonia, bronchiectasis, and abscess formation; metaplastic lesions in various parts of the respiratory tract are a common finding. The major etiological factor appears to be mycoplasma pulmonis, although other infectious agents may also play a role in the pathogenesis of the disease.

We decided to investigate the effect of this *chronic* respiratory infection, as a counterpart to *acute* influenza pneumonitis, on a rat lung tumor system (SCHREIBER *et al.*, 1972). The nitrosamine *N*-nitrosoheptamethyleneimine had been shown previously by LIJINSKY *et al.* (1969) to be highly carcinogenic for the respiratory tract and esophagus of rats. When it was given in the drinking water, squamous cell tumors developed in the lung within 25—30 weeks. This carcinogen-tumor system seemed to be particularly well suited to our purposes, since the nitrosamine will induce tumors in two organ systems while the infectious process involves only one of them. Thus, if the pneumonitis exerted an effect on the tumor response, we would know whether it was a systemic or a localized one.

Conventional Sprague-Dawley rats selected for clinical signs of respiratory illness were obtained from a commercial source. Shortly after their arrival in the laboratory, 29 of the rats were killed and autopsied to verify the pneumonia. Serological testing revealed serum antibodies against at least one of the respiratory viruses listed in Table 3; only 7 out of the 29 were negative for all three of the viral agents listed. *Mycoplasma pulmonis* could be isolated from 19 animals; and macroscopic lung lesions, indicative of CMP, were found in 24 of the 29 rats. Specific-pathogen-free (SPF) rats were used as controls. These animals were obtained from a colony originally derived (by Cesarian section) several years ago from the same commercial breeder stock that produced the infected animals. The diseased and SPF groups were housed in different isolation facilities but received identical treatment. The carcinogen was administered to the rats at night in the drinking water, at a concentration

Table 3. Evidence of preexisting and current respiratory infection in conventional Sprague-Dawley rats

Agent	No. positive / No. tested (%)	
Pneumonia virus of mice [a]	7/29	(24)
Sendai virus [a]	8/29	(28)
Respiratory corona virus [a]	15/29	(52)
Mycoplasma pulmonia isolates [b]	19/29	(66)
Gross lesions of chronic murine pneumonia at autopsy	24/29	(83)

[a] Serological results (Dr. JOHN C. PARKER, Microbiological Associates, Inc., Bethesda, Md.). Eighty-five rats were selected for experimentation on the basis of clinical signs of respiratory infection. Twenty-nine were killed for serological and bacteriological testing one week before the carcinogen treatment of the remaining rats was started.

[b] From upper and/or lower respiratory tract.

of 100 mg per 1000 ml of distilled water. The amount consumed was determined the following morning. The carcinogen administration was continued for 22 weeks. The calculated cumulative carcinogen dose per rat was 137—140 mg. Body weights were determined every 4—6 weeks (Fig. 2). No differences in body weights were observed between uninfected and infected animals of the same sex.

All animals were killed at 24 weeks after the start of the experiment. At autopsy the number and size of recognizable lung tumors were determined. (*N*-nitrosoheptamethyleneimine causes multifocal tumor nodules.) Abscesses and bronchiectatic lesions could be distinguished in almost all instances from lung tumor nodules, because the latter were firm and greyish white in color. All tumors were sectioned through their longest axis and processed for histology to verify the macroscopic diagnosis and, if necessary, to correct the gross tumor count. The rest of the lung tissue was sliced

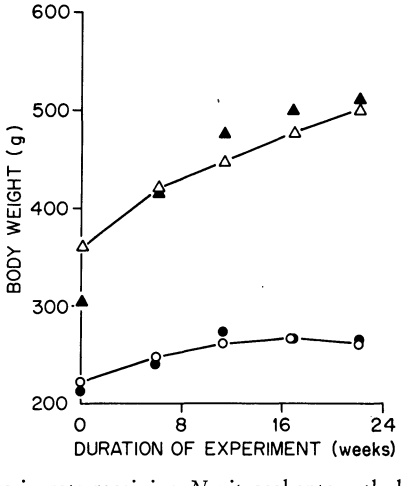


Fig. 2. Body weight changes in rats receiving *N*-nitrosoheptamethyleneimine in their drinking water. Male rats: △, SPF; ▲, with CMP. Female rats: ○, SPF; ●, with CMP. The rats were 10—12 weeks old at the start of the experiment

along the axis of the major bronchi and also processed for histology. Macroscopic and/or histologic evidence for murine pneumonia was obtained in all conventional but in none of the SPF rats treated with carcinogen. The tumor incidences in the various groups (after macroscopic and histologic evaluation) are summarized in Table 4. It can be seen that the number of animals with lung tumors is markedly higher in infected than in uninfected male rats. Similarly, the number of tumors per rat (or per tumor-bearing rat) is increased in infected males. In the female groups — which received a considerably higher carcinogen dose on a per gram body weight basis (because of the weight differences between the two sexes) — the difference between the uninfected and the infected groups ar not as striking, due to the fact that 19 out of 21 uninfected female rats had already developed tumors at the time of sacrifice. However, when the data are expressed as mean number of tumors per rat or per tumor-bearing rat, the infected females also had a significantly elevated tumor incidence. When the mean total tumor mass per rat lung was estimated, it was found to be 4 times greater in the infected than in the uninfected female rats (5200 mm^3 as compared to 1100 mm^3). No tumors were observed in infected control rats. In contrast to the lung tumor response, the esophageal tumor response was not altered by CMP. Histologically, all tumors, with the exception of a few microscopically visible adenomas (resembling the pulmonary adenomas in mice), were highly keratinizing squamous cell tumors. Neither the site of tumor development nor the morphological characteristics of the tumors appeared to be influenced by the infection. In addition to the lung tumors, massive squamous cell metaplasias were observed in the tracheobronchial tree of all carcinogen-treated animals (Fig. 3).

Our findings, though they have to be regarded as preliminary results and will need confirmation, strongly suggest that some respiratory infections may indeed significantly enhance the tumor response of the lung to chemical carcinogens. It should be pointed out that the present experiments were conducted under less than

Table 4. Lung tumor response to N-nitrosoheptamethyleneimine in specific-pathogen-free rats and rats with chronic murine pneumonia

Experimental groups	Effective no. of rats [a]	No. of rats bearing lung tumors [b]	Mean no. of tumors per rat	Mean no. of tumors per tumor-bearing rat	No. of rats with esophageal papillomas [b]
Specific-pathogen-free carcinogen-treated ♂	19 (21)	7 (37)	0.6	1.6	14 (74)
Chronic murine pneumonia carcinogen-treated ♂	29 (30)	24 (83)	2.8	3.4	22 (76)
Specific-pathogen-free carcinogen-treated ♀	21 (21)	19 (90)	2.5	2.8	17 (81)
Chronic murine pneumonia carcinogen-treated ♀	27 (30)	27 (100)	4.7	4.7	23 (85)
Chronic murine pneumonia untreated control ♀	25 (25)	0 (0)	—	—	0 (0)

[a] Only animals surviving entire carcinogen treatment were included in the study; numbers in parentheses indicate the number of rats at start of experiment.

[b] Numbers in parentheses indicate the percentage of tumor-bearing rats per group.

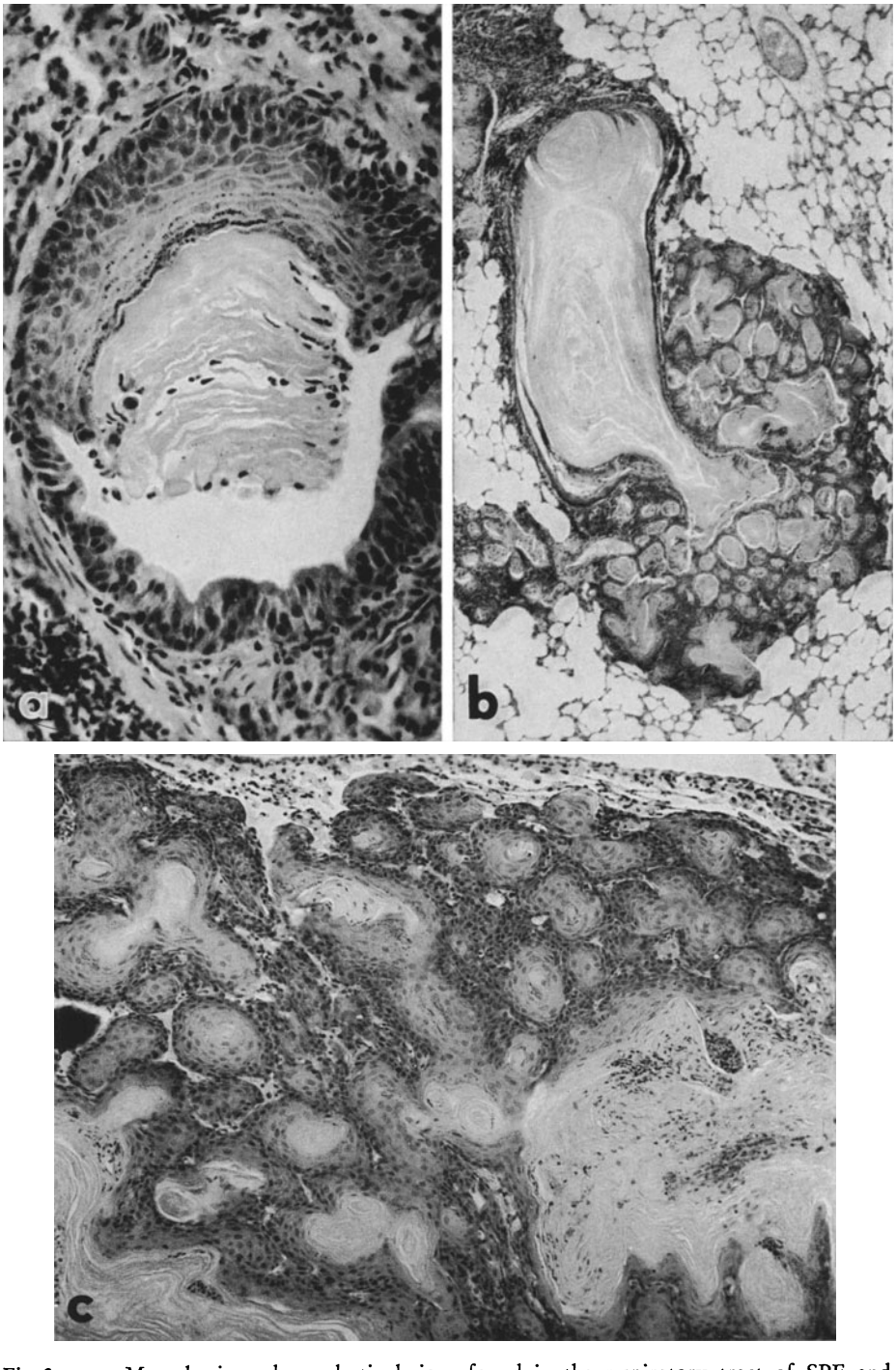


Fig. 3 a—c. Metaplastic and neoplastic lesions found in the respiratory tract of SPF and infected Sprague-Dawley rats after 22 weeks of *N*-nitrosoheptamethyleneimine administration in the drinking water. a Bronchus with keratinizing squamous metaplasia, b Keratinizing squamous cell lesion in bronchiole and peribronchiolar alveoli, c Squamous cell tumor occupying major part of a lung lobe

ideal conditions, since the carcinogen dose applied was obviously too high to allow maximal expression of the enhancement effect. The mechanism underlying this enhancement of the lung tumor response to a "systemic" carcinogen is not known, but several possibilities are worth considering: increase of susceptible cell population or accelerated expansion of malignant clones due to increased cell proliferation (WARWICK, 1971) in the infected lung tissue; alteration of carcinogen metabolism in the infected lung; suppression of immunological surveillance by the heavy antigenic burden on the lymphatic tissues during the infection (microbial antigens).

The significance of our findings is potentially far-reaching, particularly since a great deal of attention is being given to the nitrosamines and nitrosamine precursors as potential carcinogenic hazards for man (e. g. LIJINSKY and EPSTEIN, 1970).

The two studies on the effects of respiratory infection on lung tumor responses presented here furnished seemingly contradictory results: influenza pneumonitis *suppressed* the pulmonary adenoma response in mice induced by carcinogenic air contaminants, while chronic murine pneumonia *enhanced* the squamous cell tumor response in the lung of rats developing as a result of *N*-nitrosoheptamethyleneimine ingestion. At present we can only speculate about the possible reasons for this discrepancy. Influenza virus inoculation causes an acute pneumonitis with extensive scarring and permanent obliteration of one or two lung lobes. Since the pulmonary adenoma is a tumor of the alveolar epithelium (type II alveolar cell), it is conceivable that the reason for the suppressive effect of the influenza pneumonitis is loss and/or modification (extensive alveolar metaplasia) of alveolar epithelium, i. e. loss of target tissue for the carcinogen. The chronic murine pneumonia, on the other hand, is a protracted inflammatory process, persisting for many months and involving all parts of the respiratory tract. It causes extensive hyperplasia and metaplasia, notably of the bronchial epithelium. Since the rat lung tumors induced by *N*-nitrosoheptamethyleneimine appear to be of bronchial (or bronchiolar) origin (LIJINSKY *et al.*, 1969), it is conceivable that the enhancement of this bronchial tumor response is caused by chronic regenerative and proliferative stimulation of the bronchial mucosa exerted by the persistent inflammatory process.

3. Alteration of Host Responses by Respiratory Infection

a) Pulmonary Clearance Mechanism

Tracheobronchial and alveolar clearance are probably the most important defense systems of the respiratory tract against inhaled toxic particles. The inhalation route is generally considered to be the main point of entry of environmental carcinogens into the respiratory tract, and it is believed that carcinogens entering by this route are usually in the form of particles or are carried by particles. Thus, factors disturbing pulmonary clearance are potentially of great importance as co-factors in carcinogenesis of the respiratory tract. A review of the current literature indicates that while extensive studies have been and are being conducted on the interference of inhaled noxious gases and particles with respiratory tract clearance, no systematic investigations on the disturbance of clearance by respiratory infection exists. The influenza virus infection appeared to be a good disease model with which to conduct the initial studies on this problem, since it destroys epithelium and produces inflam-

mation both in the tracheobronchial tree and in the alveolar parenchyma (Fig. 6 a). (Thus it could be expected to interfere with the clearance mechanism on all levels of the respiratory tract.)

It also appeared advantageous that the influenza pneumonitis, in contrast to chronic murine pneumonia, is a rather acute disease, thus enabling us to study the recovery of impaired respiratory clearance as a function of time after the acute pneumonitic episode (CREASIA *et al.*, 1972 a, b).

Certain limitations are inherent in studies of respiratory clearance of radioactive particles in small rodents such as mice. Because of physical dimensions, it is difficult to distinguish with a nondestructive test between radioactivity located in various parts of the respiratory tract and in the upper digestive tract. After some initial exploratory studies using inhalation and intratracheal injection techniques with radioactive particles, we decided to use the latter method for particle deposition and whole-body counting as a means of determining the amount of radioactivity retained at various times after deposition. We recognized that this would introduce a certain artificiality into our experimental system, since intratracheally injected dust particles are mostly deposited in the deep lung, regardless of particle size. Therefore, information obtained with such a system will be largely confined to the lower respiratory tract clearance. The advantage is that whole-body counting can be used as a reliable measure of the lung burden, since alveolar clearance is so slow that no appreciable amounts of radioactivity are detectable in the intestinal tract at any given time other than the first 24—48 hours after intratracheal deposition. WATSON *et al.* (1969) recently demonstrated that the rate of particle clearance from the various pulmonary compartments is not appreciably different for inhaled or intratracheally deposited materials.

Specific-pathogen-free $BC3F_1$ mice were used in our study. Groups of mice were infected with influenza virus by intranasal inoculation. Only those animals positive for antiviral antibodies 2—3 weeks after inoculation were included in the study. Table 5 and Fig. 4 show the reproducibility of particle deposition and clearance in a large number of control mice. A total of 76 infected mice were used in the study. $^{51}Cr_2O_3$ was intratracheally administered either 2 days before, simultaneously with, or 1, 3, 5, 7, or 9 weeks after virus inoculation. In Fig. 5 the typical elimination patterns for animals with influenza infection are given; three experimental groups are included ($^{51}Cr_2O_3$ administration 2 days before, 1 week after, and 9 weeks after infection). It can be seen that the clearance of radioactive chromium was markedly

Table 5. Reproducibility of administering $^{51}Cr_2O_3$ to the lungs of mice by intratracheal injection

	Trial 1	Trial 2	Trial 3	Trial 4
No. of animals	10	10	10	10
Avg. dose [a]	279,136	274,357	254,373	255,112
Range	271,866 to 288,109	253,953 to 284,212	256,901 to 262,819	247,405 to 266,890
Standard error	± 1,545	± 2,665	± 809	± 1,594

[a] Counts/min, whole-body counting; 10.0 µg $^{51}Cr_2O_3$ approximates 2.5×10^5 counts/min.

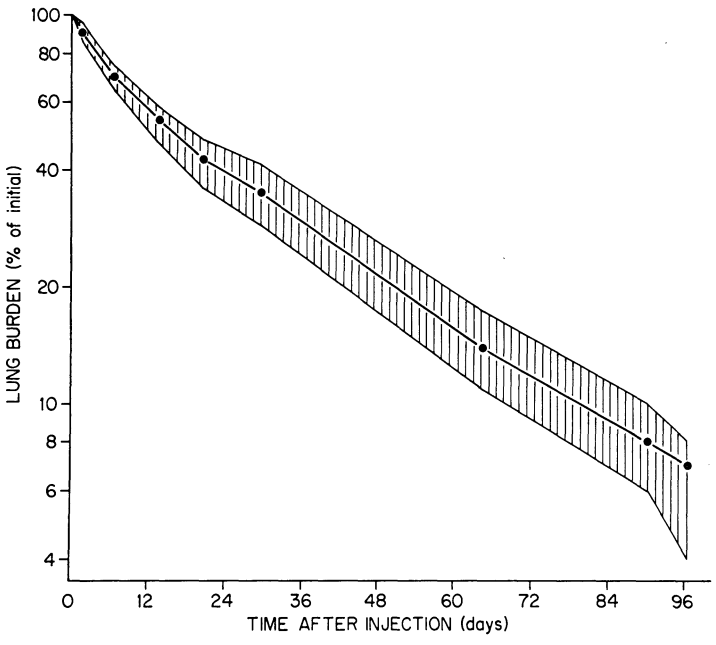


Fig. 4. Clearance of intratracheally administered $^{51}Cr_2O_3$ from the respiratory tract of uninfected mice. 10 μg of $^{51}Cr_2O_3$ (2.5×10^5 counts/min) were injected at time 0; whole-body counts were performed at various time intervals. Data are expressed as percentages of initial radioactivity remaining. Closed circles represent mean lung burden remaining (40 animals per time point); shaded area indicates range of values obtained

impaired in the majority of the animals in all three groups. At the end of the observation period, all animals were killed and were graded according to the amount of macroscopically discernible lung tissue scarring (consolidation and atelectasis). We found that all animals exhibiting impaired clearance showed massive consolidation and atelectasis of entire lobes, while animals with normal or near normal clearance had no grossly recognizable scarring of the lung tissue.

Histoautoradiograms prepared from the lungs of these mice revealed a significant difference in the localization of the radioactive dust particles dependent on the time of $^{51}Cr_2O_3$ administration relative to the time of virus inoculation. When the particles were administered before or simultaneously with virus inoculation, most of the radioactivity had aggregated in the consolidated lung lobes (Fig. 6 a—d). Presumably the radioactive chromium was deposited before the infectious process had taken hold and then was trapped in the inflamed and scarred tissues, which lose their connection to the rest of the bronchial tree. In the animals receiving $^{51}Cr_2O_3$ one or more weeks after the infection, the consolidated and heavily scarred tissue contained little radioactivity; instead, the lung lobes with only microscopic signs of a previous infection contained rather large amounts of radioactive material in thickened alveolar walls and small interstitial scars (Fig. 6 c). Thus the lung tissue only mildly involved in the infectious process sustained enough damage to exhibit a long-lasting defect of alveolar clearance.

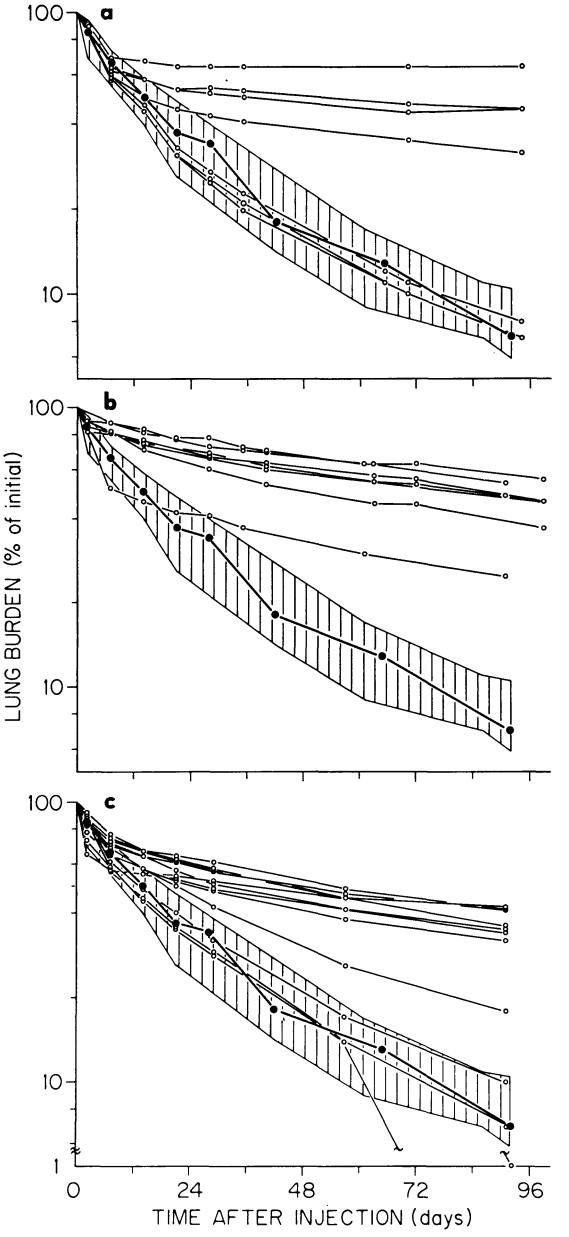
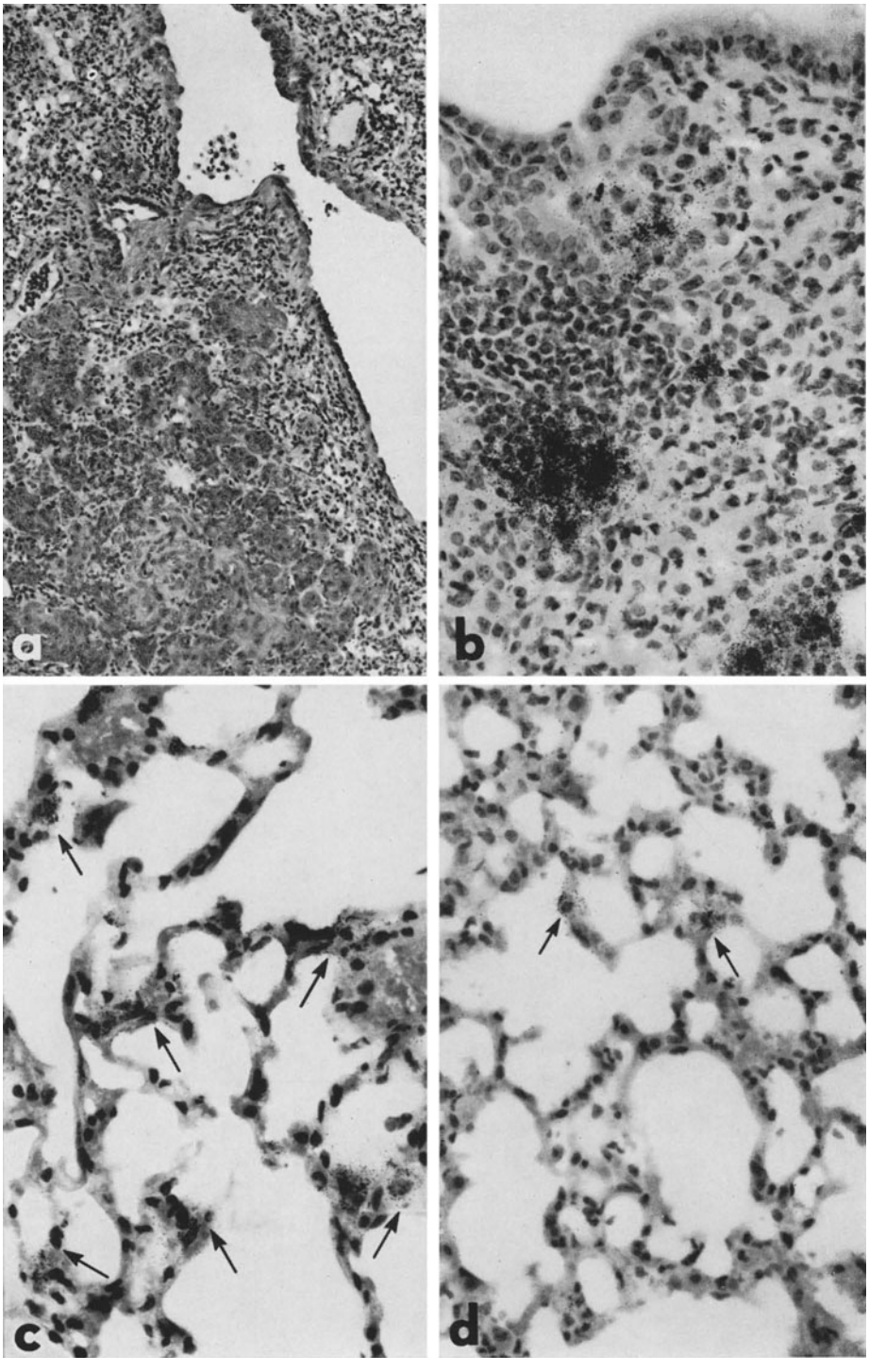


Fig. 5 a—c. Effect of influenza virus infection on $^{51}Cr_2O_3$ clearance from the respiratory tract of mice. 10 μg of $^{51}Cr_2O_3$ were administered intratracheally at different times before or after intranasal inoculation of PR8 influenza virus. Clearance in uninfected (●) and infected (○) mice was determined by whole-body counting. The control values are given as mean remaining lung burden calculated from 50 uninfected mice; shaded area indicates at various intervals. $^{51}Cr_2O_3$ was administered: a 2 days before inoculation with virus, high-low range. Data are expressed as percent of initially deposited radioactivity remaining, b 7 days after inoculation with virus, or c 9 weeks after inoculation with virus


a
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d

In summary, our experiments show that respiratory infections, particularly those that cause pneumonitis and subsequent scarring of alveolar parenchyma, can result in long-term impairment of pulmonary clearance. It does not seem unreasonable to assume that any other insult resulting in similar scarring and fibrosis of lung tissue (e. g. chemical insults, pulmonary embolism) will also cause comparable damage of the deep lung clearance.

Our findings may have serious implications for man, since the pulmonary residence time of radioactive particles may be considerably prolonged in damaged lung tissue, thus increasing the cumulative radiation dose. The fact that the dust particles aggregate in and near damaged lung tissue with formation of "hot spots" is probably also of significance, paraticularly in the case of radioactive particles, since the surrounding parenchyma will sustain a rather high radiation dose (see also Fig. 8 and 9 in NETTESHEIM *et al.*, 1971 a). Studies are presently underway to determine whether these results have a bearing on the pulmonary residence time of chemical carcinogens carried on dust particles.

b) Benzpyrene Hydroxylase Activity in the Lung

The capacity of tissues to metabolize chemical carcinogens to proximal carcinogens and also to less carcinogenic compounds (MILLER and MILLER, 1971), is an important factor in the overall carcinogenic process. It seems therefore important to determine whether factors such as microbial infections, which are being considered as co-carcinogens, influence the level and inducibility of enzyme systems involved in the metabolism of carcinogens (CORBETT and NETTESHEIM, 1973). Specific-pathogen-free BALB/c mice (female, 12—14 weeks old) were inoculated intranasally with a drop of a $10^{-7.0}$ dilution of a 10% lung homogenate containing PR8 influenza virus (which causes infection in 100% of the animals). In one series, mice were killed at 4, 7, and10 days after virus inoculation, and their benzpyrene hydroxylase activity was assayed according to the method described by NEBERT and GELBOIN (1968). Three to five mice were used per time point, with uninfected mice serving as controls. The results are summarized in Table 6. The enzyme activity found in infected lungs is expressed as a percentage of the enzyme activity obtained from lungs of control animals. It can be seen that the enzyme level becomes progressively lower as the

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Fig. 6 a—d. Influenza pneumonia and its effect on $^{51}Cr_2O_3$ distribution and elimination. a Pneumonia 10 days after inoculation with virus; note loss of bronchial epithelium and consolidation of lung parenchyma with squamous-like metaplasia. (Hematoxylin and eosin; × 150.), b Autoradiogram from lung of mouse intratracheally inoculated with 10 µg of $^{51}Cr_2O_3$ 2 days before inoculation with virus. The animal was killed 90 days after injection of radioactive chromium. Note large aggregates of radioactive material in scarred lung tissue (compare with d). (Hematoxylin and eosin; × 375.), c Autoradiogram from lung of mouse intratracheally inoculated with 10 µg of $^{51}Cr_2O_3$ 3 weeks after inoculation with virus. The animal was killed 90 days after intratracheal injection of radioactive chromium. Large amounts of label are present in thickened and fibrosed alveolar walls of aerated lung lobe. (Hematoxylin and eosin; × 375.), d Autoradiogram from lung of uninfected mouse intratracheally inoculated with 10 µg of $^{51}Cr_2O_8$. The animal was killed 90 days after injection of radioactive chromium. Only small amounts of label can be detected in alveolar wall cells. (Hematoxylin and eosin; × 375.)

Table 6. Effect of influenza virus infection on benzpyrene hydroxylase levels in the lung [a]

Time	per mg of protein (% of control)	per total lung (% of control)
Day 4	84	88
Day 7	61	115
Day 10	12	20

[a] Measured in fluorometric units per 20 min incubation.

influenza pneumonitis develops. (The peak of the influenza virus lesion with maximum involvement of lung tissue is between 7 and 14 days.) These data indicate that besides physical clearance (see above) the metabolic clearance (and/or activation) is an important mechanism to be considered in studies concerned with the effect of respiratory infection on the lung tumor response to a polycyclic hydrocarbon.

c) Immune Response Elicited via the Respiratory Tract

It has been amply documented that the tumor response to viral and chemical carcinogens is often enhanced in immunologically suppressed animals and that transplantation of tumor cells is facilitated by immune suppression of the host (GOOD and FINSTAD, 1969; KLEIN, 1969; BURNET, 1970). The antigenicity of the tumor and the *overall immunocompetence* of the host are major factors which determine whether an effective immune response can be mounted against the developing tumor, or whether it will enhance tumor development (see PREHN, 1971, for discussion). There is evidence that *local lymphatic tissues* near the site of tumor origin respond to the antigens released by the tumor, although the significance of these lymph nodes in the host defense against the neoplasia is uncertain (BARD *et al.*, 1969; HAMMOND and ROLLEY, 1970; FISHER and FISHER, 1971). It is therefore important to determine the ability of these lymphatic tissues to respond to antigens, particularly since it is well known that the immune response is markedly influenced qualitatively as well as quantitatively by the route of entry of the antigen.

We decided to begin our investigation with one of the test antigens commonly used to study the humoral antibody response, namely, heterologous red blood cells (NETTESHEIM and WILLIAMS, in press). We found that antigenic stimulation via the respiratory tract is a very inefficient means of inducing a primary humoral immune response in comparison to intravenous (or intraperitoneal) antigen administration (Fig. 7a). A thousand times more antigen was required with intratracheal than with intravenous antigen injection to initiate a comparable antibody response in mice. In rats the differences between intratracheal and intravenous (or intraperitoneal) antigenic stimulation did not appear to be quite as great (Fig. 7b). The difference between the two species may be related to the greater amount of lymphatic tissue present in the lung proper of SPF rats. To obtain information regarding the site of antibody production following intratracheal antigenic stimulation of mice, we determined the number of hemolysin-forming cells in cell suspensions prepared from the lung, tracheobronchial lymph nodes, and the spleen, using the direct and indirect

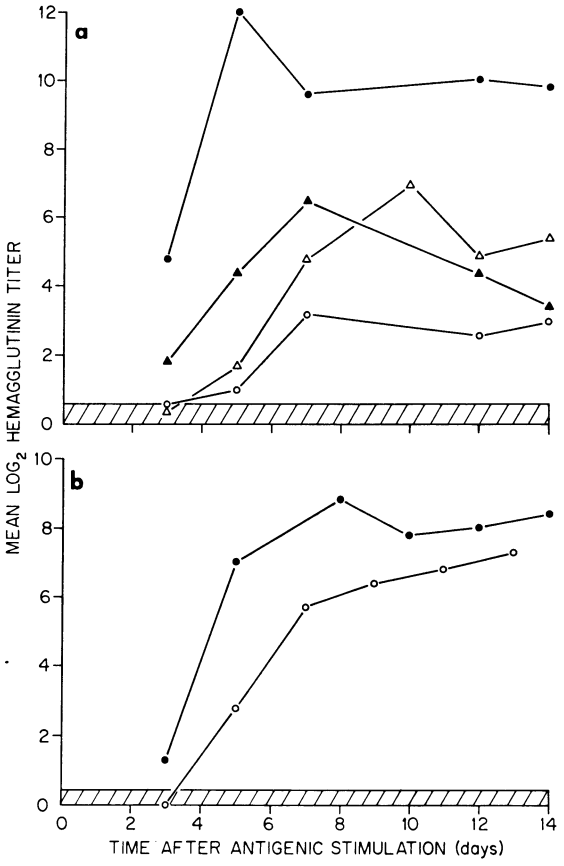

Fig. 7 a and b. Serum antibody response to sheep red blood cells (SRBC) in mice and rats. a $BC3F_1$ mice: 10^8 SRBC, intravenous (●); 10^8 SRBC, intratracheal (○); 10^9 SRBC, intratracheal (△); 10^6 SRBC, intravenous. ▲, b F-344 rats: 2×10^9 SRBC, intravenous (●); 2×10^9 SRBC, intratracheal (○). Five to ten animals per time point; shaded area indicates background titers

hemolytic plaque-forming assays as previously described (HANNA *et al.*, 1969). No more than 2—3% of the hemolysin-producing cells developing between 3—15 days in response to intratracheal injection of antigen were found in the lung and the lung-associated lymph nodes. Ninety-seven to 98% of the antibody-forming cells were found in the spleen (combined number of plaque-formers obtained from spleen, lung, and tracheal and bronchial lymph nodes were considered to be 100%). These data suggest that primary antigenic stimulation via the respiratory tract is very inefficient. At least in SPF mice and rats, the respiratory tract appears to be poorly equipped to respond to antigens. The major site of antibody production when elicited with massive antigen doses is *not* the respiratory tract and the associated lymph nodes but the spleen (we have not yet attempted to determine the gamma A antibody response after intratracheal administration of antigen).

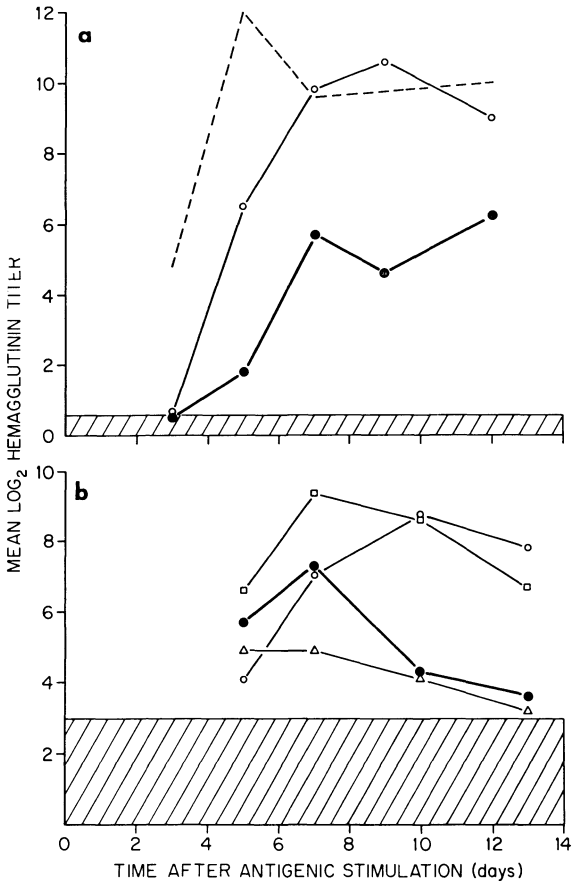

Fig. 8 a and b. Effect of *B. pertussis* vaccine and Sendai virus infection on serum antibody response to intratracheally administered sheep red blood cells (SRBC). a $BC3F_1$ mice: 5×10^8 SRBC (●); 5×10^8 SRBC given 6 days after intratracheal administration of 4×10^9 killed *B. pertussis* organisms (○). Hemagglutinin response to 10^8 intravenously administered SRBC (- - -) is given for comparison, b BALB/c mice: 10^9 SRBC (●); 10^9 SRBC given 4 days (○), 7 days (□), or 14 days (△) after intranasal inoculation with Sendai virus (10^{-3} dilution of a 10% lung homogenate containing Sendai virus). Ten to 20 animals per time point; shaded area indicates background titers

We then considered the possibility that this situation might change if the respiratory tract were exposed to bacterial or viral agents, since this often causes a severe influx of lymphoid elements into the lungs. Two studies were performed: one in which $BC3F_1$ mice were preexposed intratracheally to *Bordetella pertussis* vaccine, a potent adjuvant; and another in which BALB/c mice were preinfected with Sendai virus, a common respiratory tract pathogen in rodents. A marked enhancement of the antibody response was observed when the vaccine was given 1—14 days prior to stimulation with sheep red blood cells. Enhancement of the hemagglutinin response was maximal when the interval between vaccination and injection was 6 days (Fig. 8 a, b). The magnitude of the humoral antibody response under these conditions approximates

that seen after intravenous administration of antigen. A study of plaque-forming ability showed that the number of hemolysin-forming cells in the lung and tracheobronchial lymph nodes was only twice that of mice not preexposed to *B. pertussis*. In contrast, the peak plaque-forming response in the spleen was approximately 20-fold greater in animals preexposed to *B. pertussis* than in those receiving only an intratracheal injection of sheep red blood cells. Thus, even in the vaccinated animal, the major organ producing antibodies to sheep red blood cells (gamma M and gamma G) is the spleen. Similar observations were made by KARELSE (1970) in a very extensive study, using tetanus toxoid as antigen.

The effect of Sendai virus infection on the hemagglutinin response to sheep red blood cells in BALB/c mice is summarized in Fig. 8b. It can be seen that an enhancement of the hemagglutinin response occurred during the first week of infection, and a suppression of immunocompetence occurred after the second week of infection, i. e. when sheep red blood cells were administered 14 days after intranasal inoculation with virus. (The results are somewhat complicated by the fact that BALB/c mice had rather high background titers.)

These experiments indicate that profound changes in the capacity to respond to antigens entering the respiratory tract can occur as a result of bacterial and viral infections of the lung. Further studies will be needed to determine whether the cell-mediated immune response, particularly against tumor antigens is similarly affected.

4. Summary and Conclusions

We have discussed the various experimental approaches currently being used in our laboratory to determine whether respiratory infection plays a decisive role in the pathogenesis of lung cancer. The tumor studies performed so far indicate that there is no single answer to the question: do respiratory infections enhance the lung tumor response to chemical carcinogens, and that the outcome of studies concerned with this problem will depend on the nature of the infectious disease (acute or chronic, major target site etc.) the carcinogen-tumor model (systemic or topical, acute or chronic exposure, target site etc.) and the time relationship between infectious and carcinogenic insult. One can only expect to determine whether and under what circumstances a particular respiratory infection will alter the neoplastic response of the lung in a given carcinogen animal model.

The few studies on *lung tumor response* and respiratory infection conducted to date can be summarized as follows: 1) In chronic inhalation studies performed with mice, influenza virus infection appears to suppress the pulmonary adenoma response induced by carcinogenic inhalants (NETTESHEIM *et al.*, 1970, 1971 a, b; KOTIN, 1966; CAMPBELL, 1940). 2) Development of squamous cell carcinomas was reported by KOTIN (1966) in mice exposed to synthetic smog and influenza viruses. 3) A significantly increased squamous cell lung tumor response to a cyclic nitrosamine was observed in rats suffering from chronic murine pneumonia (SCHREIBER *et al.*, 1972).

Investigations aimed at identification of *pathophysiological mechanisms* which might render the respiratory tract more susceptible to carcinogenic insults or might facilitate tumor development have also been described. Lung clearance studies showed that respiratory infection can significantly alter the distribution of inhaled (NETTESHEIM

et al., 1971 a) and intratracheally deposited (CREASIA *et al.*, 1972 a, b) particulates and can markedly increase the pulmonary residence time of particles deposited in the peripheral segments of the respiratory tract. These investigations also showed that recovery from this defect in deep lung clearance is very slow. At present, these studies have been performed only with PR8 influenza virus. Other microbial agents with an affinity for the respiratory tract will have to be tested in the future. It also needs to be determined whether these findings apply to chemical carcinogens (possibly adsorbed to carrier particles) deposited in the respiratory tract. Studies of the arylhydroxylase levels in the respiratory tract showed a marked alteration of benzo(a)pyrene metabolism during the course of influenza pneumonitis. Since the enzyme systems involved appear to be responsible for detoxification of the carcinogen as well as for the formation of the proximal carcinogen, it is presently difficult to decide what this suppression of the benzpyrene hydroxylase during infection means in terms of tumor induction.

Studies on the immune response elicited via the respiratory tract and its alteration by respiratory infection are presently limited to investigations of the humoral antibody response. The data indicate: 1) that there are considerable strain and species differences in the ability of rodents to respond to intratracheally injected antigens; 2) that the lung and lung-associated lymph nodes respond very poorly to antigens delivered to the respiratory tract, and that the main site of antibody production is the spleen; and 3) that exposure to *B. pertussis* or respiratory infection with Sendai virus markedly alters the antibody response to sheep red blood cells: an enhancement of the humoral antibody response occurred after stimulation with *B. pertussi* and during the first week after viral inoculation; a suppression of the antibody-forming capacity developed during the second week of the viral infection. The data indicate that it might indeed be very important to investigate thoroughly the changes in immunocompetence of the lung and lung-associated lymphatic tissues occurring during respiratory tract infection, since profound changes in the immunosurveillance against malignant tumors might occur in the cours of infectious respiratory disease.

We feel that our studies, even though they are still incomplete, have at this point yielded important information and have demonstrated the need for extensive experimental efforts to help resolve the old debate over the possible role of respiratory infection in the pathogenesis of lung cancer.

References

BARD, D. S., HAMMOND, W. G., PLICH, Y. H.: Cancer Res. **29**, 1379 (1969).
BRENNAN, P. C., FRITZ, T. E., FLYNN, R. J.: Lab. Anim. Care **19**, 360 (1969).
BURNET, F. M.: Progr. exp. Tumor Res. (Basel) **13**, 1 (1970).
CAMPBELL, J. A.: Lancet **1940 II**, 487.
CORBETT, T. H., NETTESHEIM, P.: J. Nat. Cancer Inst. **50**, 779 (1973).
CREASIA, D. A., NETTESHEIM, P., HAMMONS, A. S.: Hlth Phys. **23**, 865 (1972).
CREASIA, D. A., NETTESHEIM, P., HAMMONS, A. S.: Arch. environm. Hlth **26**, 197 (1973).
DURAN-REYNALS, M. L.: Progr. exp. Tumor Res. (Basel) **3**, 149 (1963).
ECK, H., HAUPT, R., ROTHE, G.: In: Handbuch der speziellen Pathologischen Anatomie und Histologie, III/4. Berlin: Springer-Verlag 1969, p. 250.
FISHER, B., FISHER, E. R.: Cancer (Philad.) **27**, 1001 (1971).
GOOD, R. A., FINSTAD, J.: Nat. Cancer Inst. Monogr. **31**, 41 (1969).
HAMMOND, W. G., ROLLEY, R. T.: Cancer (Philad.) **25**, 368 (1970).

HANNA, M. G., JR., NETTESHEIM, P., FRANCIS, M. W.: J. exp. Med. **129**, 953 (1969).
HARRIS, R. J. C., NEGRONI, G.: Brit. med. J. **4**, 637 (1967).
KARELSE, M. P. C.: Mechanismen van Respiratoire Immunisatie. Proefschrift. Rotterdam: Dukkerij Brouder-Offset N. V. 1970.
KLEIN, G.: Fed. Proc. **28**, 1739 (1969).
KOTIN, P.: In: Canadian Cancer Conference, Vol. 6. London: Pergamon Press 1966, p. 475.
KOTIN, P., WISELEY, D. V.: Progr. exp. Tumor Res. (Basel) **3**, 186 (1963).
KOTIN, P., FALK, H. L., MCCAMMON, C. J.: Cancer (Philad.) **11**, 473 (1958).
LEUCHTENBERGER, C., LEUCHTENBERGER, R.: In: Lung Tumours in Animals. Ed.: L. SEVERI. Perugia: Division of Cancer Research 1965, p. 445.
LIJINSKY, W., EPSTEIN, S. S.: Nature (Lond.) **225**, 21 (1970).
LIJINSKY, W. L., TOMATIS, L., WENYON, C. E. M.: Proc. Soc. exp. Biol. (N. Y.) **130**, 945 (1969).
LINDSEY, J. R., BAKER, H. J., OVERCASH, R. G., CASSELL, G. H., HUNT, C. E.: Amer. J. Path. **64**, 675 (1971).
LOOSLI, C. G.: J. infect. Dis. **84**, 153 (1949).
MACKLIN, M. T., MACKLIN, C. C.: Arch. Path. **30**, 924 (1940).
MARTIN, C. M., MAGNUSSON, S. M., GOSCIENSKI, P. J., HAUSER, G. F.: Science **134**, 1985 (1961).
MCCLUNG, J. P.: J. chron. Dis. **20**, 65 (1967).
MEYER, E. C., LIEBOW, A. A.: Cancer (Philad.) **18**, 322 (1965).
MILLER, J. A., MILLER, E. C.: J. nat. Cancer Inst. **47**, 5 (1971).
NEBERT, D. W., GELBOIN, H. V.: J. biol. Chem. **243**, 6242 (1968).
NETTESHEIM, P., HANNA, M. G., JR., DOHERTY, D. G., NEWELL, R. F., HELLMAN, A.: In: Inhalation Carcinogenesis. Oak Ridge: AEC Symposium Series No. 18, 1970.
NETTESHEIM, P., HANNA, M. G., JR., DOHERTY, D. G., NEWELL, R. F., HELLMAN, A.: In: Morphology of Experimental Respiratory Carcinogenesis. Oak Ridge: AEC Symposium Series No. 21, 1971.
NETTESHEIM, P., HANNA, M. G., JR., DOHERTY, D. G., NEWELL, R. F., HELLMAN, A.: J. nat. Cancer Inst. **47**, 1129 (1971).
NETTESHEIM, P., WILLIAMS, M. L.: Ann. N. Y. Acad. Sci., in press 1973.
PREHN, R. T.: Res. J. reticuloendothel. Soc. **10**, 1 (1971).
RAEBURN, C., SPENCER, H.: Brit. J. Dis. Chest **51**, 237 (1957).
RICHTER, C. B.: In: Morphology of Experimental Respiratory Carcinogenesis. Oak Ridge: AEC Symposium Series No. 21, 1971.
ROE, F. J. C., WALTERS, M. A.: Progr. exp. Tumor Res. (Basel) **6**, 127 (1965).
RYLANDER, R.: Acta physiol. scand. (suppl.) **306**, 1 (1968).
RYLANDER, R.: Arch. environm. Hlth **18**, 551 (1969).
SACCOMANNO, G., ARCHE, AUERBACH, O., KUSCHNER, M., SAUNDERS, R., KLEIN, M. G.: Cancer (Philad.) **27**, 515 (1971).
SAFFIOTTI, U.: Progr. exp. Tumor Res. (Basel) **11**, 302 (1969).
SCHREIBER, H., NETTESHEIM, P., LIJINSKY, W., RICHTER, C. B., WALBURG, H. E., JR.: J. nat. Cancer Inst. **49**, 1107 (1972).
SELIKOFF, T. J., HAMMOND, E. C., CHURG, J.: J. Amer. med. Ass. **204**, 106 (1968).
SIMMONS, M. L., RICHTER, C. B., FRANKLIN, J. A., TENNANT, R. W.: Proc. Soc. expt. Biol. (N. Y.) **126**, 830 (1967).
SPENCER, H.: London: Pergamon Press 1963, p. 623, p. 645.
STAEMMLER, M., FOITZIK, E., HEYDENREICH, M.: Z. Krebsforsch. **74**, 238 (1970).
STAVRAKY, K. M.: J. chron. Dis. **23**, 691 (1971).
STEINER, P. E., LOOSLI, C. G.: Cancer Res. **10**, 385 (1950).
WARWICK, G. P.: Fed. Proc. **30**, 6, 1760 (1971).
WATSON, J. A., SPRITZER, A. A., AULD, J. A., GUELTHOFF, M. A.: Arch. environm. Hlth **19**, 51 (1969).
WINTERNITZ, M. C., WATSON, J. M., MCNAMARA, F. P.: The Pathology of Influenza. New Haven: Yale University Press 1920.