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Certain Aspects of the Responses of Laboratory Rats to Exposure to (a) Nitrogen Dioxide and (b) Tobacco Smoke

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Exposure of rats to 150 ppm NO_2 for 2 hours causes death from pulmonary oedema. Continuous exposure to 25 ppm for 150 days causes gross enlargement with loss of elastic recoil and proliferative and metaplastic epithelial changes in the vicinity of the terminal bronchioles. Continuous exposure to 2 ppm results in an initial loss of cilia and focal hyperplasia of the terminal bronchiolar epithelium, but these changes, for the most part, quickly subside.

Chronic daily exposure of rats to tobacco smoke results in proliferative and metaplastic epithelial changes in the vicinity of the terminal and respiratory bronchioles. Also, both at this site and elsewhere, aggregates of golden-brown pigment-laden macrophages accumulate in the lungs. The proliferative/metaplastic changes are similar to those seen in response to NO_2, asbestos and other irritant gases and particles. The aggregates of golden-brown macrophages are seemingly a special feature of the response to tobacco smoke. Some rats exposed for over two years to tobacco smoke, develop foci of squamous metaplasia, firstly in the region of terminal bronchioles, but later at points scattered throughout the lung parenchyma. Comparable changes have not been reported in rats exposed to NO_2.

Although no strictly comparable data for NO_2 and tobacco smoke exposure are available, it is reasonable to conclude that, whereas the NO_2 in tobacco smoke may contribute to the production of cuboidal/columnar metaplasia in the vicinity of terminal bronchioles, it otherwise plays little part in the aetiology of lesions in the lungs of smoke-exposed rats.

INTRODUCTION

The nature of irritants is that they cause irritation. Moreover, they usually do this in a dose-related or concentration-related manner. In this context, nitric oxide (NO) and nitrogen dioxide (NO_2) are undeniably both irritants for the respiratory tract of man and for all animal species for which data are available. The question is: "Is there anything special or exceptional about the irritant effects of these oxides of nitrogen which distinguishes them from other respiratory tract irritants?". The present paper is concerned with this question.

RESPONSE OF RATS AND MICE TO THE INHALATION OF NITROGEN DIOXIDE

The morphological changes in the lungs of rats and other species was reviewed in a WHO Enviromental Health Criteria document published in 1977 (21).

Kleinerman and Wright (14) exposed rats to controlled concentrations of nitrogen dioxide for a period of 2 hours. All of a group of rats exposed to 150 ppm NO_2 died within 24 hours from acute pulmonary oedema. A single two-hour exposure to 75 ppm NO_2, however, was not fatal. In rats killed 24 hours after such exposure, the lungs exhibited oedema and inflammatory exudate especially in the region of the respiratory bronchioles where epithelial degeneration and regeneration were observed. By the 4th day the acute inflammation was replaced by macrophage infiltration. Repair was more or less complete by 2 weeks, except for some persistent "epithelization of alveolar spaces about respiratory bronchioles". "Epithelization of alveolar spaces" is just one of the many different names that have been given by different observers to the same

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metaplastic change that is seen in response to various irritants. In 1964 Freeman and Haydon (10) reported the first of a series of studies conducted by them of the effects of exposing rats to NO_2. Their aim was to see whether continuous exposure to lower levels of NO_2 would damage the lungs. In rats exposed to 25 ppm NO_2 for 40 days the main changes were hypertrophy and hyperplasia of the bronchial and bronchiolar epithelium with the columnar epithelial cells becoming tall and mitotic figures becoming frequent. Rats exposed for about 150 days showed remarkably voluminous lungs at necropsy. Microscopically they showed hyperplastic changes in the airways that were even more marked than those observed at 40 days. In addition, there were marked changes in the vicinity of the terminal bronchioles. These included proliferation of connective tissue and the presence of macrophages and desquamated epithelial cells within alveolar spaces. The authors considered that the changes present at 150 days, particularly the voluminosity of the lungs, to be indicative of emphysema and suggested that blockage of the small airways by desquamated cells macrophages and mucus might have been the immediate cause of the pulmonary enlargement.

In 1968, the same group of investigators (12) described the response of rats exposed continuously to only 2 ppm NO_2. Rats so exposed developed persistent tachypnoea, but survived normally into old age without developing fatal lung disease. Resistance to airflow and dynamic compliance were normal during life, but at necropsy, after approximately 2 years exposure, lung weight were slightly (20%) increased and slight morphological changes were present in the terminal and respiratory bronchiolar epithelium. These changes included loss of cilia, but the metaplastic change described above was not seen.

Between them, these studies provide three benchmarks for the effects of NO_2 on rats: 150 ppm for 2 hours caused fatal pulmonary oedema; continuous exposure to 25 ppm for 150 days caused gross enlargement and pathological changes in the vicinity of the terminal bronchiole; but continuous exposure to only 2 ppm for 2 years had no effect on survival and little effect on lung morphology. However, Stephens *et al* (19) and Evans *et al* (7) thought it desirable to look for early responses in the lungs of rats exposed to 17 or 2 ppm NO_2. The immediate response to the higher of these two concentrations included foci of loss of cilia and sloughing of Type 1 lining cells at the level of the terminal bronchiole. During the second day of continuous exposure to 17 ppm, epithelial continuity was repaired with a low cuboidal cell type which could withstand NO_2 exposure. A wave of increased thymidine labelling was complete by 5 days. Later the non-ciliated cells developed crystalloid inclusions of unknown significance visible in the electron microscope. In response to 2 ppm NO_2, early loss of cilia, hypertrophy and focal hyperplasia of terminal bronchiolar epithelium appeared to return to normal after 21 days of continuous exposure. These latter findings suggest that, to a large extent, the rat lung can adapt to a level of 2 ppm NO_2 although, if exposure persists for up to two years, the eventual focal loss of cilia is indicative of a low level of damage. It is interesting that this tolerance evolved to cope with NO_2 could also cope with exposure to ozone (8), indicating similar mechanisms of toxicity.

Although continuous exposure to NO_2 at concentrations of less than 2 ppm does not shorten life, they are not completely without effect in the rat. Thus, Freeman *et al* (11) reported slight but persistent tachypnoea in rats continuously exposed to 0.8 ppm NO_2.

ARE CHANGES PRODUCED BY NO_2 IN THE LUNGS OF EXPERIMENTAL ANIMALS INDICATIVE OF EMPHYSEMA?

It has been suggested that generalized enlargement of alveoli, as seen in a standard section of lung, is indicative of emphysema. This interpretation should only be accepted with extreme caution, particularly if at necropsy lungs are fixed by distension with fixative. For the histological diagnosis of emphysema, one needs, I would suggest, evidence of destruction of alveolar septa and evidence of thickening and fibrosis of surviving alveolar septa. In the case of freshly killed animals, the failure of lungs to deflate spontaneously by elastic recoil after their removal from the thorax is possibly a fairly reliable sign of small airways obstruction unless it is due to oedema or consolidation.

Kleinerman and Wright (15) reported seeing centrilobular (microbullous) emphysema in

guinea-pigs, but not in rats or rabbits, following intermittent exposure to 25–100 ppm NO_2 for 15–18 months. The main histopathological feature was striking dilatation in the region of the respiratory bronchiole, unaccompanied by fibrosis. It seemed that the alveolar structures that are normally present in these bronchioles had been lost by an essentially non-inflammatory process.

In an attempt to develop a laboratory model for emphysema, Gross *et al* (13) exposed guinea pigs and hamsters to combinations of various dusts, papain and NO_2. They reported a low incidence of centrilobular emphysema in guinea pigs exposed intermittently to from 10 to 90 ppm NO_2 for 16 months and then left untreated for 8 months. The combined exposure to dusts and NO_2 did not increase the incidence of emphysema and had no effect on the incidence of subpleural bullous emphysema that was a feature in untreated hamsters of the strain used. The main effect of NO_2 in hamsters was, in the authors' own words, "metaplasia of alveolar epithelium to the cuboidal or columnar variety, thereby transforming the alveoli evaginating off alveolar ducts and respiratory bronchioles into pseudoglandular structures producing an appearance often designated as adenomatosis".

RESPONSE OF RAT LUNG TO TOBACCO SMOKE, SMOKE CONDENSATES AND THE VAPOUR OF TOBACCO SMOKE

In a series of papers my colleagues and I (1, 2, 3, 4) reported our findings in over 2000 female rats exposed to various treatments as follows: 3,4-benzpyrene with or without carbon black by repeated intratracheal instillation; tobacco smoke condensate, or fractions derived from it, by repeated intratracheal instillation; the inhalation of the vapour phase of tobacco smoke alone or in conjunction with intratracheal instillations of smoke condensate or fractions of smoke condensate; or the inhalation of tobacco smoke with or without prior intratracheal instillation of 3,4-benzpyrene. The rats used in these studies were of a SPF grade and of a non-inbred Wistar strain. They were aged 10 weeks at the start of treatment.

During the course of the 32 month-long smoke inhalation study (4), sham-exposed controls developed certain non-specific changes indicative of a low background level of chronic respiratory disease (CRD). Thus aggregations of lymphocytes formed around main airways particularly at the points of bifurcation and, in a proportion of rats, aggregates of non-pigmented macrophages were observed usually just under the pleura but occasionally more deeply in lung tissue. In addition, a few of these sham-exposed controls exhibited isolated foci of cuboidal/columnar metaplasia (CCM) of alveolar epithelium in the vicinity of terminal bronchioles. Focal of squamous metaplasia (SqM) was a rare finding in these control animals. By comparison, rats exposed twice daily on 5 days per week in the Harrogate Smoker (5) to a 20% smoke:75% air:5% CO_2 mixture prepared from the smoke of one cigarette (eleven 25 ml puffs of 2 seconds duration at one minute intervals) showed only a slightly higher incidence and/or severity of CRD, but highly significantly increased incidences and severity of both CCM and SqM. Of 408 smoke-exposed rats, 4 developed squamous neoplasms compared with 0 out of 102 sham-exposed and 0 out of 102 untreated controls. This difference was not statistically significant. The results of this study in respect of CRD, CCM and SqM are summarized in Tables 1 and 2. The earliest change in the rats exposed to tobacco smoke was the aggregation of golden-brown pigment laden macrophages (GBM) in the vicinity of terminal bronchioles. These aggregates were evident before the development of CCM. The presence of such aggregates was an almost 100% reliable indicator of exposure to tobacco smoke. Very exceptionally isolated groups of similarly coloured haemosiderin-containing macrophages were seen in untreated or sham-exposed control rats. Figs. 1-4 illustrate the aggregation of GBM, the CCM and the SqM lesions observed in smoke-exposed rats.

Rats exposed to smoke condensate or its fractions or to 3,4-benzopyrene by intratracheal instillation (1, 2) developed the same changes (i.e. GBM, CCM and SqM) as rats exposed to cigarette smoke. However, the changes tended to be both more severe and widespread and to be complicated by much more intense chronic inflammatory reactions. Squamous neoplasms, mostly benign and highly keratinized, were seen in many of the benzopyrene-treated rats.

Of special relevance in the present context

is the fact that, compared with untreated rats, rats exposed to the vapour phase of tobacco smoke (i.e. the smoke from 10 cigarettes per week after it had passed through a Cambridge filter) gained less weight but showed no increased incidence or severity of any kind of pathological change in the lungs. (3). This negative finding confirmed that of Rylander (16).

THE SIGNIFICANCE OF CUBOIDAL/COLUMNAR METAPLASIA OF ALVEOLAR EPITHELIUM

It is partly from my experience with the experiments described above and partly from the descriptions by other investigators of their findings in the lungs of animals exposed to a range of different agents that I have formed the strong impression that cuboidal/columnar metaplasia and squamous metaplasia are rather non-specific responses of rat lung to a variety of irritants. Thus, a paper on the pathogenesis of asbestos-related diseases Davis *et al* (6) illustrates cuboidal/columnar metaplasia and refers to the change in the text as "– the replacement of the epithelial lining of many respiratory bronchioles, alveolar ducts and associated alveoli by epithelium of the bronchiolar type. It was not possible, however, to determine whether this was due to hyperplasia of bronchiolar lining or metaplasia of alveolar epithelial cells" Wagner *et al* (20) who illustrated the same change in asbestos-treated rats refer to it as "Thickening of the walls of the alveoli arising directly from the respiratory bronchioles with replacement of normal epithelium by type II cells." These latter authors also recorded the occurrence of focal squamous metaplasis arising in asbestotic lesions in the respiratory bronchioles and considered these foci as the points of origin of squamous carcinomas. This interpretation matches closely our interpretation of the lesions we saw in rats given repeated intratracheal instillations of 3, 4-benzpyrene or of a fraction of smoke condensate rich in polycyclic aromatic hydrocarbons (2). Earlier Falk *et al* (9) referred to "bronchiolization of the alveoli, alveolar thickening keratinization and ultimate development of squamous cancer" in C57BL mice infected with influenza virus and exposed to aerosols of ozonized gasoline. Later others who saw the same sections considered the squamous lesions to be metaplastic rather than neoplastic in nature.

In general, then, metaplasia of the epithelium of alveoli in the vicinity of terminal and respiratory bronchioles is a non-specific response to irritation. But there remains one further question, should one distinguish two different types of such metaplasia — one involving cuboidal non-ciliated cells and the other involving ciliated columnar cells? In our rats exposed to tobacco smoke we saw both kinds of metaplasia. This suggests, either that conclusions based on light microscope and e.m. studies to the effect that the change represents replacement of type 1 cells by type 2 cells (17, 18, 19) is over-simplistic; or that cuboidal (non-ciliated) metaplasia represents a more severe response to an irritant than columnar (ciliated) metaplasia.

One fact emerges clearly from the data reviewed in this paper: constituents other than NO_2 must be responsible for most of the effects of inhaled tobacco smoke on the lungs of rats.

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Table 1 Effect of treatment on survival

Group	Treatment	No. of rats in group	No. of survivors at 100 weeks	Mean survival from start of treatment (weeks)
1	10 cigarettes/week	408	103	63
2	Sham-exposed	102	67	109
3	None	102	74	113

Table 2 Effect of treatment on incidence/severity of CRD, CCM and SqM compared with untreated controls
(+ = $p<0.05$; ++ = $p<0.001$)

Group	Treatment	CRD	CCM	Sq M
1	Smoke-exposed	+	++	++
2	Sham-exposed	0	0	0

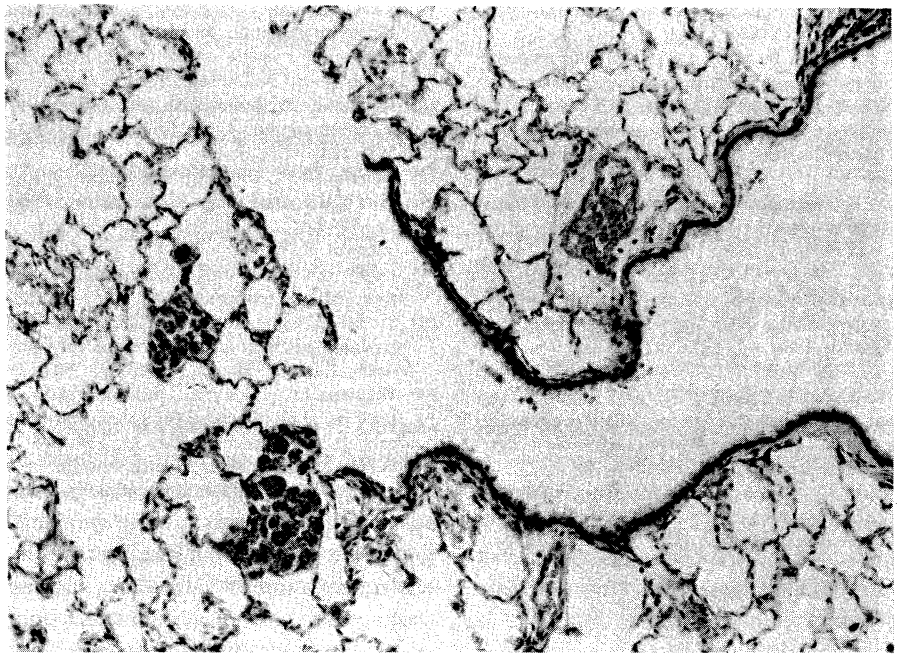

Fig. 1 Lung from rat that came to post mortem 21 weeks after the start of exposure to 10 cigarettes/week in the Harrogate Smoker.

(Rat No.367/3, Group No. 1).

The photomicrograph shows 2 clusters of golden brown pigment-laden macrophages near a terminal bronchiole. H & E, ×90

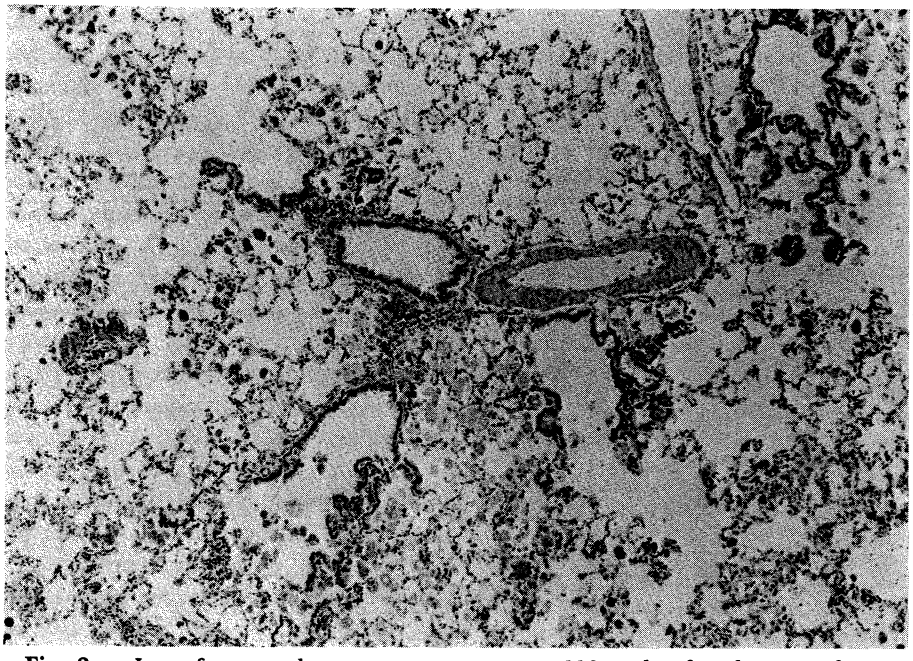

Fig. 2 Lung from rat that came to post mortem 110 weeks after the start of exposure to 10 cigarettes/week in the Harrogate Smoker.

(Rat No. 346/4, Group No. 1).

The photomicrograph shows widespread cuboidal/columnar metaplasia of alveolar epithelium and scattered pigment-laden macrophages. H & E, ×90

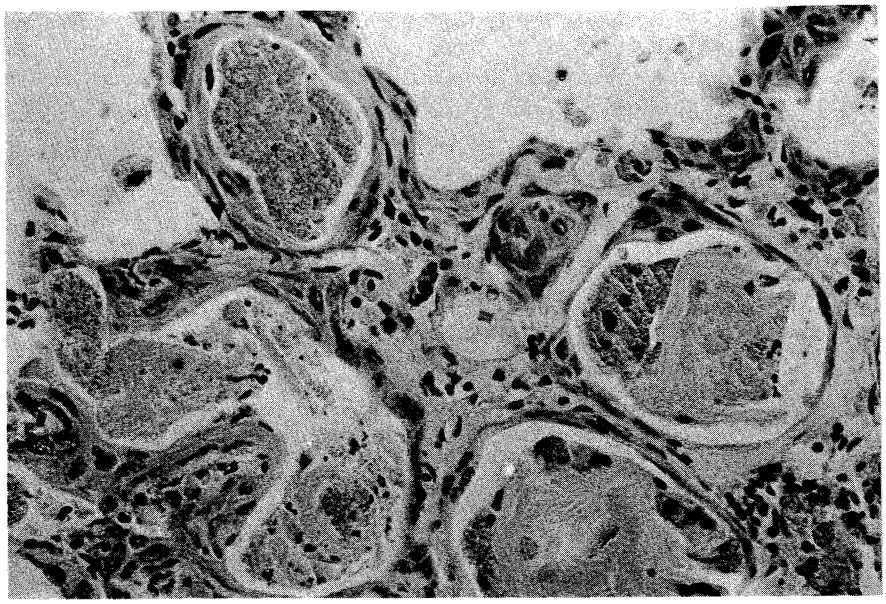

Fig. 3 Lung from rat that came to post mortem 118 weeks after the start of exposure to 10 cigarettes/week in the Harrogate Smoker.

(Rat No. 323/1, Group No. 1).

The photomicrograph shows early squamous metaplasia of the alveolar epithelium associated with the presence of golden brown pigmented macrophages. H & E, ×90

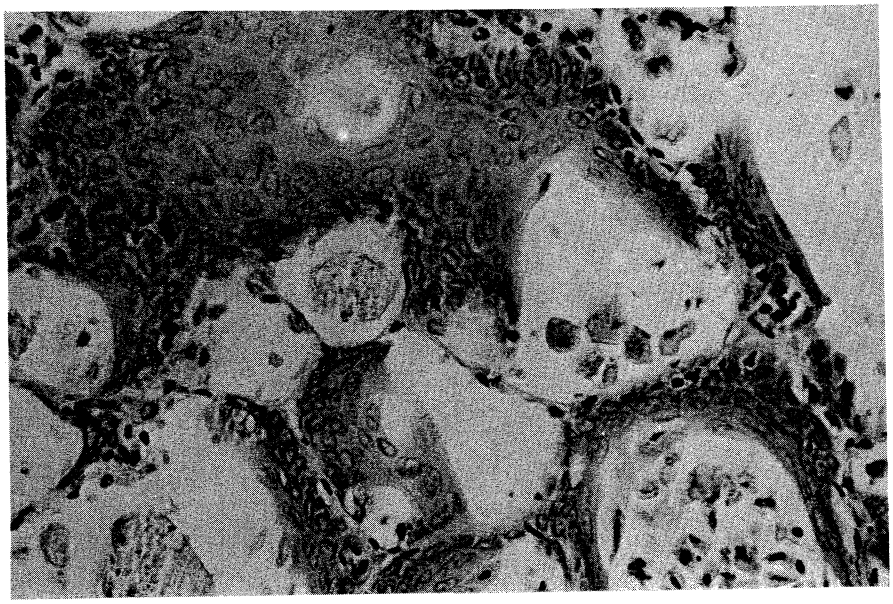

Fig. 4 Lung from rat that same to post mortem 112 weeks after the start of exposure to 10 cigarettes/week in the Harrogate Smoker.

(Rat No. 346/4, Group No. 1).

The photomicrograph shows well developed squamous metaplasia of alveolar walls with pigmented macrophages and cell debris in the alveolar spaces. H & E, ×90