JEROME KLEINERMAN*
C. RICHARD COWDREY**

Department of Pathology Research and Clinical Pathology, St. Luke's Hospital, Cleveland, Ohio 44104

THE EFFECTS OF CONTINUOUS HIGH LEVEL NITROGEN DIOXIDE ON HAMSTERS

Approximately eight years ago we[1] became interested in the use of nitrogen dioxide as an agent to produce experimental lung injury. At that time our interest in this agent was prompted by reports that it was capable of producing bronchiolitis obliterans in accidental human exposures,[2,3] and because the early experimental work of Winternitz,[4] *et al.* directed our attention to the ability of certain chemical agents to injure the more distally placed airways and spare the more proximal. Our previous studies have encompassed daily two-hour exposures to low (15 to 25 ppm) and moderate (75 to 85 ppm) concentrations for periods up to two years in guinea pigs, rats and rabbits. To date, except for one irreproducible episode, we have not been successful in producing a consistent lesion which has features comparable to those seen in human emphysema that we have just now described. The purpose of the present study is to alter an experimental design of the exposures so that animals could be exposed continuously (20 to 22 hours/day) for a prolonged period to concentrations of NO_2 sufficiently high so that a moderate mortality would be produced. Since our previous work with several animal species has not been rewarding we resolved to use in this study the Syrian hamster. To date no spontaneous emphysema lesion has been reported in this species; its lung is free of lymphoid infiltrates and it did not appear to be unusually susceptible to pulmonary infections.

METHODS AND MATERIALS

Seventy-two male Syrian hamsters, 80 to 100 grams, were placed in stainless steel Gerbil cages, two to a cage and separated by a stainless steel divider. They were exposed to concentrations of NO_2 ranging from 45 to 55 ppm for 21 to 23 hours daily for ten continuous weeks. Chow pellets and water were offered continuously, and animals were removed from the exposure chamber for one to two hours daily for cage cleaning and feeding. The chamber consisted of a lucite box 30" x 24" x 24" with tapered mixing chamber. Nitrogen dioxide was introduced from a monitored tank of pure NO_2, mixed with unfiltered room air, and exhausted to the outside through filters. The chamber was under a constant negative pressure of −2 cm H_2O and the air flow through it was 200 L/min., enough to exchange the volume of the chamber

* Head, Department of Pathology Research and Clinical Pathology.
** Research Associate.

every 1½ minutes. Nitrogen dioxide concentration was monitored continuously by a Mast meter and recorded on a Honeywell-Brown 2.5 millivolt recorder. The Mast meter was calibrated by chemical analysis for NO_2 on samples obtained simultaneously from the chamber; chemical analysis was performed by the method of Saltzman.[5] Calibration in this way was performed 4 to 6 times daily. Temperature and humidity were also recorded continuously from the chamber. Control animals were kept under exactly similar conditions in a separate control chamber, where air flow was maintained at the same levels but without the addition of NO_2. Animals were weighed twice weekly.

At the completion of the experiment animals were sacrificed by light Nembutal® anesthesia and exsanguination. Complete autopsies were performed. Lungs were fixed by formalin fume at 60° C. for 24 to 48 hours at a pressure of 20 cm. of water while floating in dilute formalin solution. They were then placed in Bouin's solution overnight for hardening. After cutting, they were vacuum processed and embedded. Hematoxylin and eosin, PAS, Perl's and trichrome stains were performed on 6-micra sections; 35 to 40 micra sections were also prepared and studied. Lung volumes were measured after fixation, by water displacement. Right ventricular weights were measured by removing the auricles and fat, and separating the right ventricular wall from its septal connections. The isolated right ventricle (and the septum and left ventricle, together) were kept moist and weighed on an analytical balance to a tenth of a milligram.

Plan of experiment

Forty-eight animals were exposed to nitrogen dioxide as outlined and 24 kept as controls. After one week of continuous exposures, six experimental and four control animals were sacrificed, fixed and prepared for study. At the end of ten weeks of continuous exposure approximately ten controls and ten experimental animals were sacrificed for study; at this time postmortem lung volumes and right ventricular weights were measured. The remaining animals, approximately ten controls and ten experimentals, were allowed to survive in room air for four additional weeks. Following this period they were sacrificed and studied in the manner recorded for the previous group.

RESULTS

The exposures to 50 ppm resulted in the death of over one-third of the experimental animals within 2 or 3 days. Seven animals died on the first day of exposure and six on the second day. Six were sacrificed after one week and only two additional deaths occurred in the eight-week period following the first two weeks of exposure. The exposed animals showed rapid labored respirations in the first several weeks of the experiment; however, this became less noticeable in the latter period. After an initial period of excitement the animals rested quietly in a corner of their cage during the periods of exposure. They were not observed to eat or drink to any significant degree while in the chamber. After several weeks of exposure, the animals appeared to become increasingly irritable as evidenced by their hostile reaction during routine handling. The coats of the exposed animals

acquired a brown discoloration, undoubtedly the result of the reaction of NO_2.

In the animals sacrificed immediately following cessation of exposure, the characteristic tissue reaction was seen in the region of the terminal and respiratory bronchioles and proximal alveolar ducts. This consisted of an extensive epithelial hyperplasia and hypertrophy. The cells in this region were normally cuboidal or attenuated so that they cannot be seen by light microscopy; inflammatory cells are not present, and ciliated cells are rare. At sacrifice, following ten weeks of exposure, there were extensive focal collections of inflammatory cells and hyperplastic and hypertrophied epithelial cells in the regions of the respiratory and terminal bronchioles. The epithelial cells lining these structures were hypertrophied and tall cuboidal to columnar. Many nuclei were hyperchromatic. These hypertrophied epithelial cells extended down into the respiratory bronchiole and the proximal alveolar ducts. Many of the alveolar structures in and about the respiratory bronchioles and alveolar ducts contained abundant inflammatory cells, predominantly neutrophils and macrophages. In addition, desquamated epithelial cells were seen in various stages of disintegration. Inflammatory cells are also present in the peribronchial connective tissue. A mild degree of pulmonary edema was observed in the alveolar structures throughout the lung. The tracheal epithelium likewise showed a mild hypertrophy. No striking alteration was observed in the ciliated cell population but the number of PAS positive droplets in the epithelial cells appeared to increase. The epithelium of the medium sized and small bronchi similarly showed only a slight hypertrophy and frequently desquamated, pycnotic epithelial cells, nuclear debris and inflammatory cells lining the surface. Animals sacrificed after the first week of exposure showed similar changes but of markedly reduced intensity in the inflammatory cell infiltration and epithelial hyperplasia and hypertrophy.

While the size of the alveolar spaces appeared enlarged in the exposed animals, as compared to the controls similarly fixed, there was no evidence of destruction of alveolar septal tissue. The absence of evidence of tissue destruction in the lungs of the exposed animals is the foundation of our conviction that emphysema has not been produced in this experiment.

Several other observations are worthy of mention. While every animal which survived the ten-week exposure revealed the classical inflammatory lesion with epithelial hyperplasia, there were marked variations in the degree and extent of these lesions from animal to animal. In the same regard, in any one lung there were marked differences in the degree of involvement in the different respiratory bronchioles and alveolar duct loci even in adjacent areas.

In those animals sacrificed four weeks after the cessation of NO_2 exposures, there was a remarkable regression of the inflammatory and epithelial hyperplastic components of the lesion. A minimal degree of epithelial hypertrophy in the respiratory bronchiolar and alveolar duct regions usually existed so that one could surmise that a reaction had occurred. However, there was no evidence of pulmonary edema, the acute inflammatory cells had virtually disappeared and the bronchiolar epithelial cells did not appear as tall as those present in the lungs of the animals sacrificed immediately after cessation of NO_2 exposure. Again, no evidence of tissue destruction was observed and no emphysema could be observed.

The lung volumes measured by water displacement of the fixed, inflated postmortem lungs are listed in Table 1. It can be observed that the lung volumes in the NO_2 exposed group sacrificed immediately after cessation of exposure is significantly higher than the controls. This difference holds whether the absolute lung volumes or the ratio of lung volume to body weight are compared in the control and exposed groups. However, in the groups sacrificed four weeks after cessation of exposure, the mean values of the absolute lung volumes in the exposed group have fallen by approximately 15 per cent when compared to the values in the exposed group immediately sacrificed. When the difference between the means of the absolute lung volumes in the NO_2 exposed and control animals of the delayed sacrifice group is analyzed, it still approaches the limits of statistical significance. This indicates that some overinflation of the lung in the exposed groups persists even four weeks following cessation of exposure, but that it has decreased when compared to the exposed animals sacrificed immediately. When the mean values of the lung volume/body weight ratio of exposed and control animals in the delayed sacrifice groups are compared, this difference is not significant. The latter ratio is probably a more valid basis of

TABLE 1. POSTMORTEM LUNG VOLUMES

	n	*Body wt.* (*gm.*)	*LV* (*ml.*)	$\frac{LV}{BW} \times 10^2$
		Immediately after NO_2		
Control	6	126 ± 18	4.21 ± .89	3.34 ± .11
Exp'tl	9	113 ± 13	5.48 ± .26	4.85 ± .58
P		NS	<.001	<.001
		After 4 wks normal air		
Control	8	124	4.14 ± .48	3.41 ± .55
Exp'tl	9	122	4.69 ± .55	3.81 ± .43
P		NS	<.05	NS

TABLE 2. RIGHT VENTRICULAR WEIGHTS

	RV (*mg*)	$LV + S$ (*mg*)	$\frac{RV}{BW} \times 10^4$	$\frac{RV}{LV + S}$
	Immediately after NO_2			
Control	63 ± 11	298 ± 32	5.01 ± .39	.224
Exp'tl	76 ± 10	296 ± 46	6.56 ± .88	.247
P	<.05	NS	<.01	NS
	After 4 wks normal air			
Control	72 ± 2.4	318	5.85 ± .54	.228
Exp'tl	77 ± 2.7	313	6.29 ± .42	.244
P	NS	NS	NS	NS

evaluation in this case since the body weights are practically the same and the variation of lung volume with body weight is well known.

The data concerning right ventricular weights is seen in Table 2. In the groups studied immediately following NO_2 exposure, the absolute right ventricular mean weight of the exposed animals appears significantly heavier than that of the control group. This significant difference is also observed when the mean ratio of right ventricular weight to body weight for the exposed and controlled group is compared and analyzed. When the ratios of right ventricle weight/left ventricle and septum weight for the two groups are compared, no significance is attributable statistically to the difference. This apparent discrepancy could be caused by proportionate increases in left ventricular and septum weights in several of the exposed animals which could neutralize the increase in right ventricular weight and still not be reflected as a general increase in the mean value of left ventricle and septum weight. In the analysis of right ventricular values of the groups sacrificed four weeks after cessation of NO_2 exposures, no significant difference in right ventricular weight or the ratios, right ventricle/body weight or right ventricle/left ventricle and septum, are observed between the control and exposed groups. This strongly suggests that the factors contributing to the work hypertrophy of the right heart have been diminished or eliminated.

DISCUSSION

The combined evidence from this study points strongly to the conclusion that a tissue destructive form of emphysema has not been produced. This finding concurs with that of our previous studies that nitrogen dioxide exposure in sublethal concentrations for shorter daily periods over a longer

time, in guinea pigs, rabbits and rats, does not cause emphysema. The use of continuous exposures (21 to 23 hours/day) for prolonged periods, even at concentrations that produce a mortality of approximately 35 per cent of the original group, does not appear to alter the nondestructive character of the tissue response. The hamster thus joins the other rodent groups studied by us as species that respond characteristically to NO_2 inhalation by an exudative and proliferative reaction, without evidence of tissue destruction. The character and degree of the epithelial and inflammatory response appear similar in kind and proportional in extent to the time concentration product imposed. The variability in the response from bronchiole to bronchiole in a single lung is even more disquieting than the differences in degree of reaction between animals similarly exposed. It seems to suggest that profound differences in the uniformity of distribution of the inhaled noxious agent exist and persist. These observations are consistent with physiological observation, but their fundamental basis remains unclear. The rapid and almost complete involution of the epithelial hyperplastic and inflammatory response produced by NO_2 in the animals studied 30 days after cessation of exposures is truly remarkable and once again demonstrates the extensive reparative properties of the lung. The return toward normal of the lung volumes and right ventricular weights in this group again suggests that a reversible lesion is being repaired and lends further support to the conclusion that no permanent tissue destruction has been produced by these profound experimental exposures.

A final word is in order to attempt to explain the apparent differences in the results presented here with those of other studies[6-8] reported concerning the use of nitrogen dioxide and the production of experimental emphysema. First there arises the question of the use of the term emphysema. Most respiratory clinicians, pathologists and physiologists have come to accept the standardized definition of emphysema as proposed by the American Thoracic Society,[9] the Ciba Symposium[10] and other groups. The basis of this definition is a pathological alteration and enlargement of airspaces characterized by tissue destruction. Simple airspace enlargement based on overdistention without tissue destruction may still be called emphysema by some individuals, and may lead to confusion and inconsistency in reports. Secondly, in order to evaluate the presence of tissue destruction as a cause of airspace enlargement, the lung must be inflated and fixed by a standardized technique. The lack of proper inflation fixation and vacuum processing has contributed extensively to the confusion in reporting of experimental results regarding emphysema. Mensuration of airspace size is no panacea for the enlightened and experienced handling and interpretation of histological material in this type of experiment.

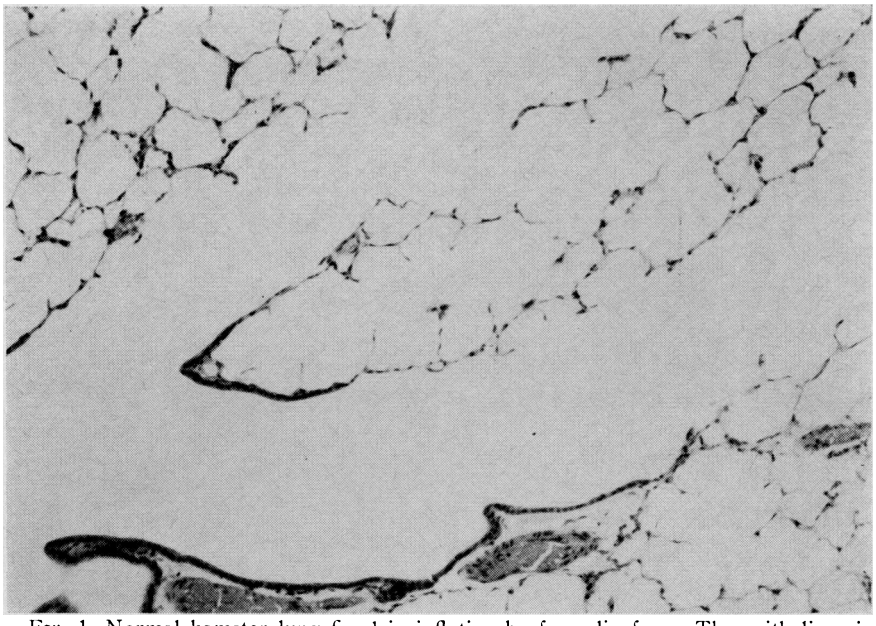

FIG. 1. Normal hamster lung fixed in inflation by formalin fume. The epithelium is single layered, thin and regular. The respiratory bronchiole is very short and the alveolar ducts are normal. (H&E x100)

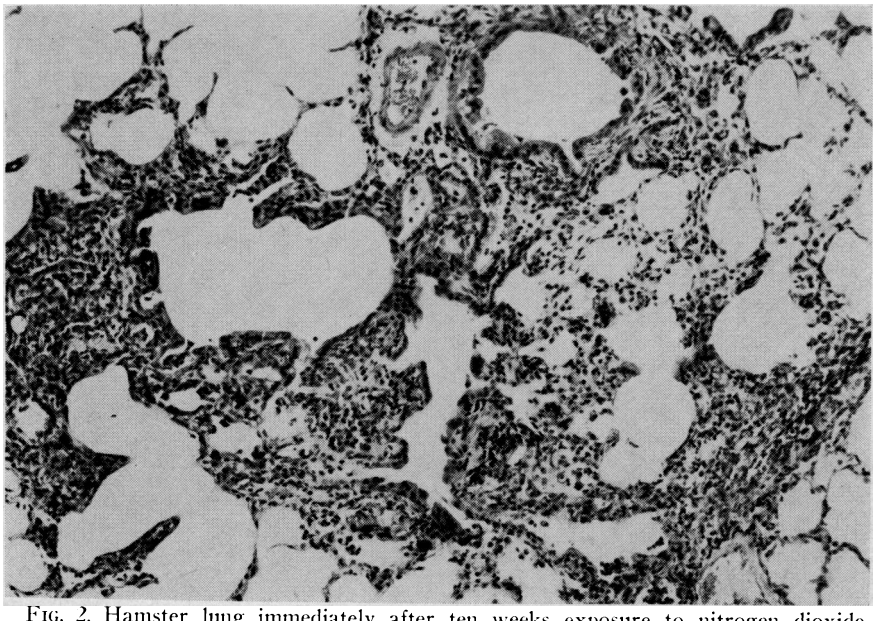

FIG. 2. Hamster lung immediately after ten weeks exposure to nitrogen dioxide. The epithelium in the terminal and respiratory bronchioles and in the proximal alveolar ducts are hypertrophied and tall cuboidal, columnar or multilayered. The interstitial tissue is edematous and thickened and infiltrated with acute and chronic inflammatory cells. The inflammatory cells are also present within alveolar spaces.

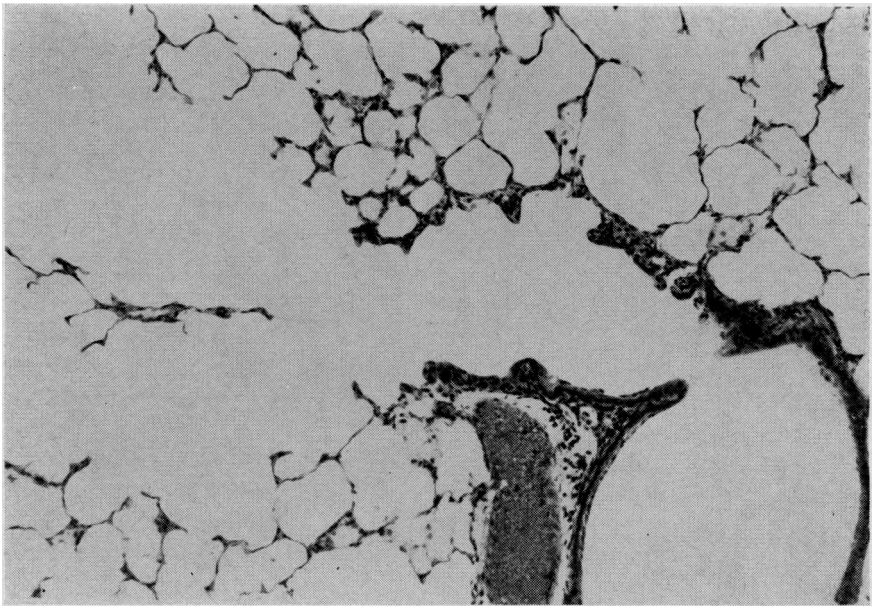

FIG. 3. Hamster lung four weeks after discontinuation of nitrogen dioxide exposure. The epithelium in the respiratory bronchiole and proximal alveolar duct shows some residual hypertrophy. Some chronic inflammatory cells persist in the peribronchiolar interstitial tissue. No evidence of tissue destruction can be observed.

The results obtained in this experiment certainly cast no light on the creation of a proper animal experimental model for the study of obstructive respiratory disease nor on the etiology of emphysema. It is hoped, however, that it may refocus interest on the enormous reparative apparatus of the lung[11] and on the need for careful preparation of pulmonary material in studies of experimental obstructive disease.

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